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**Preparation and Evaluation of Liquid
and Nanocapsule Formulations
containing Biomolecules**

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**A thesis submitted in partial fulfilment
of the requirements of the University
of Sunderland for the degree of
Doctor of Philosophy**

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Abstract of Research

The formulation, therapeutic delivery and prediction of the stability of proteins are very challenging due to their varied chemical and physical properties. A number of approaches to stabilise protein formulations, such as protein encapsulation and characterisation, using physical and chromatographic methods, were evaluated in an attempt to overcome these challenges. The aim of this project was to evaluate the impact of common formulation variables (pH, strength and composition of buffers and excipients) on liquid formulations of lysozyme and trypsin. The use of a Quality by Design (QbD) approach was adopted in liquid and nanocapsule formulations with the application of mathematical models to obtain optimised formulations in order to tailor the desired attributes.

Protein formulations were prepared according to a mathematical design of experiments by changing the pH, type of buffer and concentration, and nature of excipients. Each formulation was characterised by Differential Scanning Calorimetry (DSC) and enzymatic assay. Subsequently, each factor was optimised, and optimised formulations were prepared. These new formulations were characterised, and their stabilities investigated using the 'Size Exclusion Chromatography Method', which was developed and validated as a stability indicating assay for encapsulated lysozyme, deoxyribonuclease I (DNase I), and trypsin. Hydrophilic liquid chromatography methods were applied to measure the excipients' stability during the shelf lives of the formulations.

Polymeric nanocapsules were prepared by double emulsion methods (solid/oil/water and water/oil/water), based on the QbD experiments. Critical quality attributes were determined in order to achieve the quality target product profile. The formulations were developed by using Poly (DL-lactide-co-caprolactone) copolymers in two different molar ratios (86:14 and 40:60) for lactide and ϵ -caprolactone blocks, respectively. The nanocapsules' spherical morphology and size were investigated by Transmission Electron Microscope and Dynamic Light Scattering (DLS), respectively. In addition, protein entrapment efficiency was determined. The proteins' release profiles, in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), were assessed. The application of QbD principles reduced the length and cost of development and provided optimum protein formulations and promising results within a short time.

The formulations of lysozyme, at pH (4-5) and trypsin, at pH 3, retained their biological activity and conformational stability as illustrated, by having the highest transition temperature values. The phosphate buffer had the most stabilising effect on formulations and trehalose maintained the proteins' integrity and biological activity. Using DSC and DLS to predict long term stability produced promising results. The proteins' encapsulation efficiencies were significantly ($p < 0.05$) affected by the copolymers' compositions. Moreover, the drug release profile in SIF over 24 hours was affected by copolymer ratios, with 64% drug release in total. The release in SGF was 8%, suggesting protection of orally-delivered proteins from degradation by gastric enzymes. Adding trehalose and the encapsulation of solid proteins helped proteins to retain up to 97.4% of the original biomolecule activity.

Research activity

Abu Abed, Omar S., Elkordy, Amal, Chaw, Cheng Shu, Williams, Lee and Carlile, Mark (2016) *Development and Characterisation of Polymeric Nanocapsules Containing Macromolecule*. Molecular Therapy (Nature group), 24 (Supp 1). S237-S238. ISSN 1525-0016

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Conference presentations

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List of abbreviations

BAEE	N α -Benzoyl-L-Arginine Ethyl Ester
CMA	Critical Materials Attribute
CMC	Critical Micelles Concentration
CPP	Critical Process Parameter
CQA	Critical Quality Attribute
CV	Coefficient of Variance
DAD	Diode Array Detector
DLS	Dynamic Light Scattering
DNase I	Deoxy Ribonuclease I
DOE	Design of Experiment
DSC	Differential Scanning Calorimetry
DSF	Differential Scanning Fluorimetry
EE	Encapsulation Efficiency
ELSD	Evaporative Light Scattering Detector
EMA	European Medicine Agency
FMEA	Failure Mode Effect analysis
HILIC	Hydrophilic Interaction Liquid Chromatography

HPLC	High Performance Liquid Chromatography
HSDSC	High Sensitivity Differential SCANNING Calorimetry
ICH	International Conference of Harmonisation
KDa	Kilodalton
KFT	Karl Fisher Titration
LLOD	Lower Limit of Detection
LLOQ	Lower Limit of Quantitation
MLR	Multi Linear regression
PEG	Polyethylene Glycol
PI	Isoelectric point
PNC	Polymeric Nanocapsule
QbD	Quality by Design
QC	Quality Control
QTPP	Quality Target Product Profile
RH	Relative Humidity
RP-HPLC	Reverse phase High Performance Liquid Chromatography
RPN	Risk Priority Number
SD	Standard Deviation

SEC	Size Exclusion Chromatography
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
TEM	Transmission Electron Microscope
TFA	Trifluoroacetic Acid
Tg	Glass transition temperature
Tm	Thermal denaturation temperature (mid point)
ULLQ	Upper Limit of Quantitation
UV	Ultra Violet

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Chapter One: Introduction

1. Introduction

Proteins are large biomolecules be made of one or more long chains of 20 amino acids and play essential roles in all living biological systems, which include; mechanical or structural elements, and physiological functions such as; immune reactions, and enzymes [1].

1.1. Protein structure

The 20 Amino acids are considered the main backbone in the proteins structure. Hence, the main role of the amino acids is to be monomers condensing together to accomplish the structure of the polypeptide chain [2].

All amino acids are composed of chiral carbon (apart of Glycine) attached to carboxyl (-COOH), amine (NH₃), hydrogen, and R group, Figure 1.1. Containing a chiral carbon drives the amino acids to exist in two possible racemic forms or enantiomer configurations L- amino acid and D- amino acid, Figure 1.2.

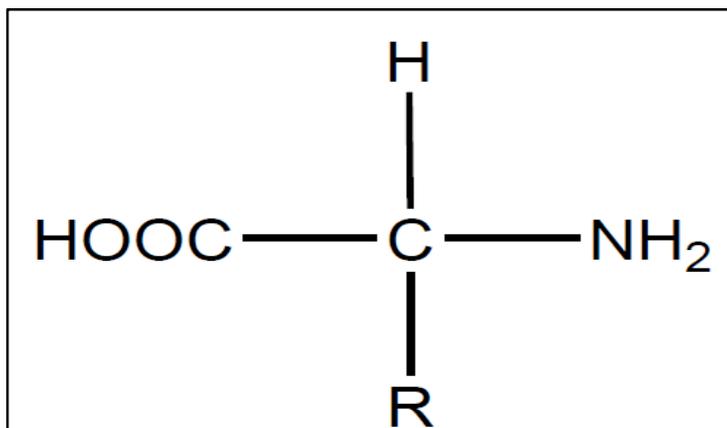


Figure 1.1: The general structure of amino acids. The structure was drawn by the author using Chem Draw®.

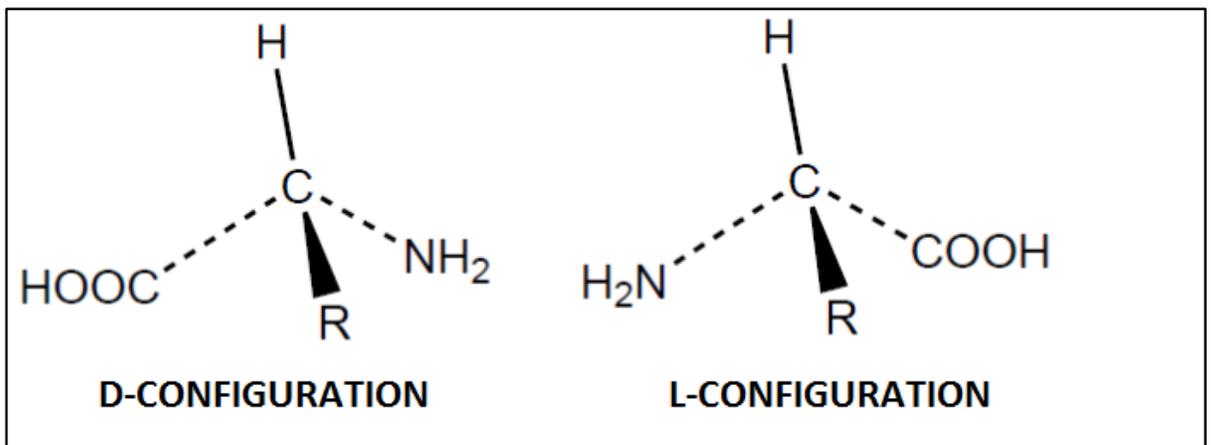


Figure 1.2: Amino acids two different enantiomers, L- configurations and D- configurations. The structures were drawn by the author using Chem Draw®.

The L- configuration amino acid is the naturally occurring form of the protein structure.

The amino acids are divided into four main different groups depending on the structure of the R group, Figure 1.3, Figure 1.4, Figure 1.5, and Figure 1.6.

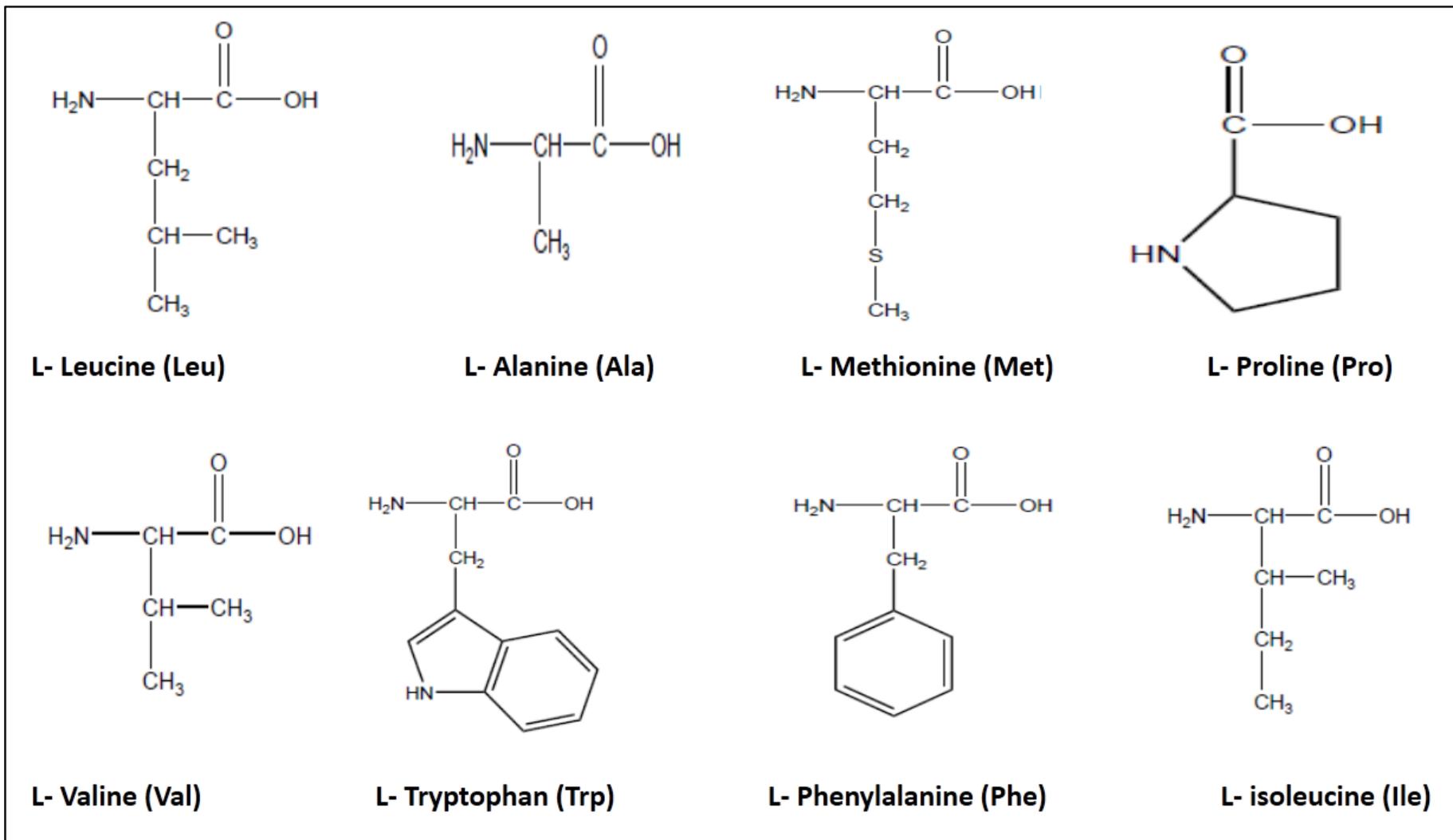


Figure 1.3: Non-polar or hydrophobic R groups containing Amino Acids. The structures were drawn by the author using Chem Draw®.

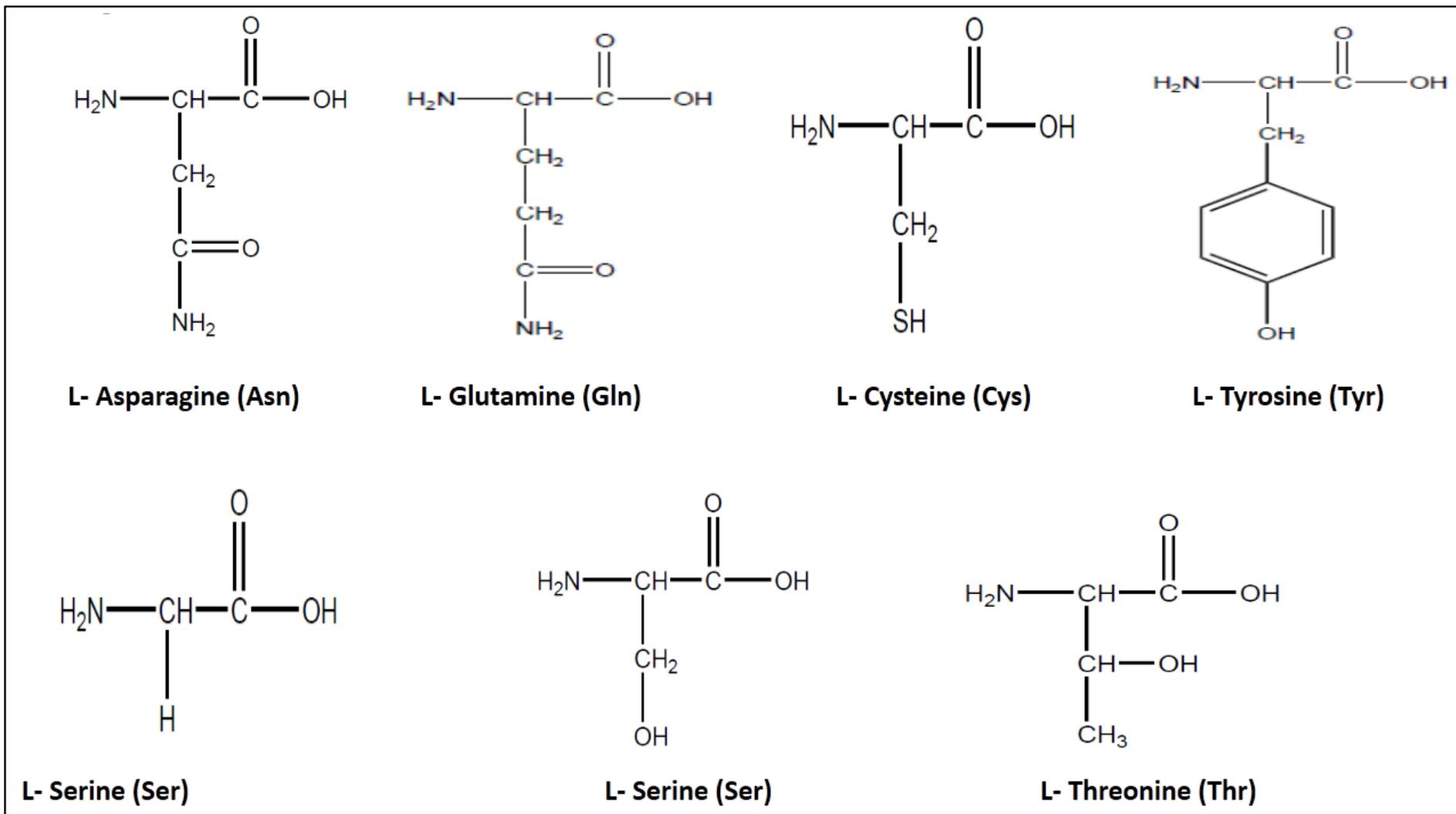
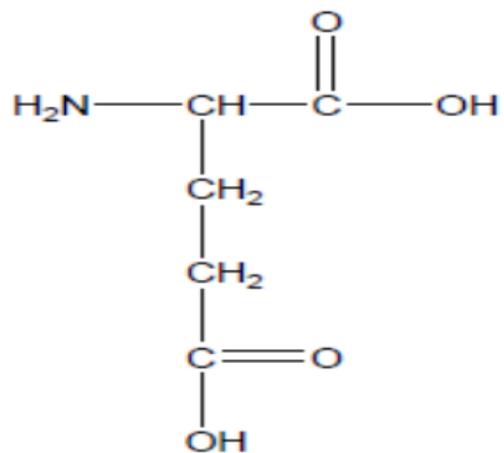
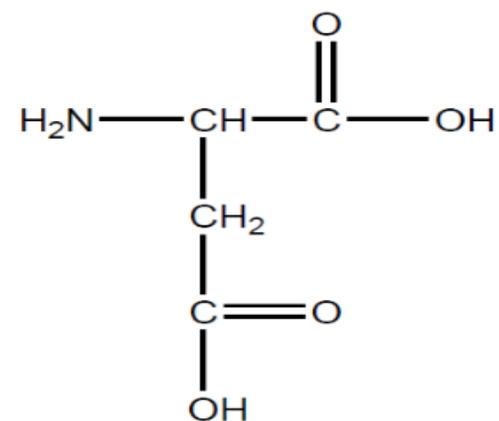


Figure 1.4: Uncharged or hydrophilic R groups containing Amino Acids. The structures were drawn by the author using Chem Draw®.

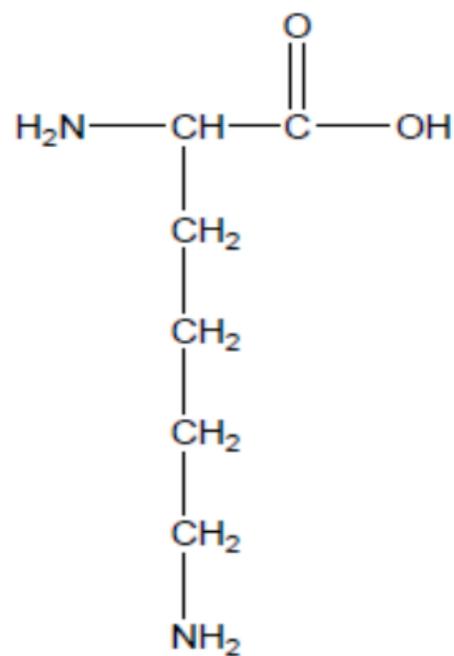


L- Glutamic acid (Glu)

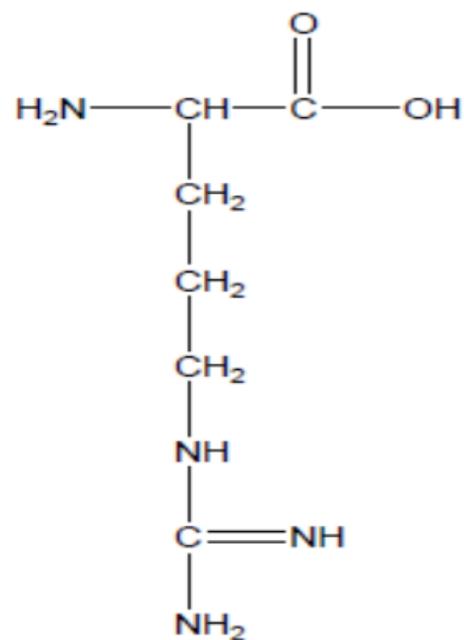


L- Aspartic acid (Asp)

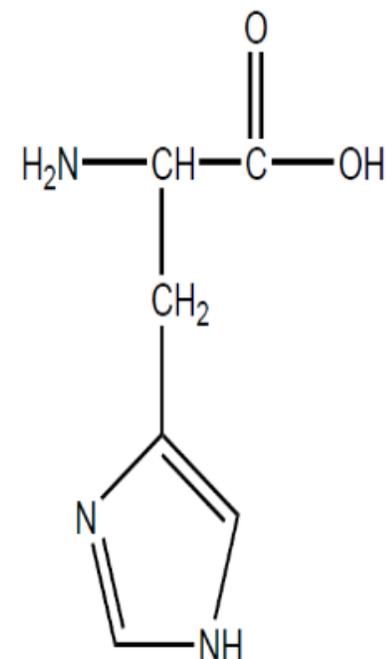
Figure 1.5: Negatively charged R groups (at pH 6-7) containing Amino Acids. The structures were drawn by the author using Chem Draw®.



L- Lysine (Lys)



L- Arginine (Arg)



L- Histidine (His)

Figure 1.6: Positively charged R groups (at pH 6-7) containing Amino Acids. The structures were drawn by the author using Chem Draw®.

As illustrated in Figure 1.3, Figure 1.4., Figure 1.5, and Figure 1.6, R group may be non-polar or hydrophobic as in for example; Methionine, negatively charged at pH 6-7 such as; Glutamic acid, positively charged at pH 6-7 such as; Lysine, or neutral or hydrophilic state e.g. Glutamine.

The amino acids link together via covalent amide or peptide bonds to form a sequence of amino acids or the primary structure of the protein, Figure 1.7.

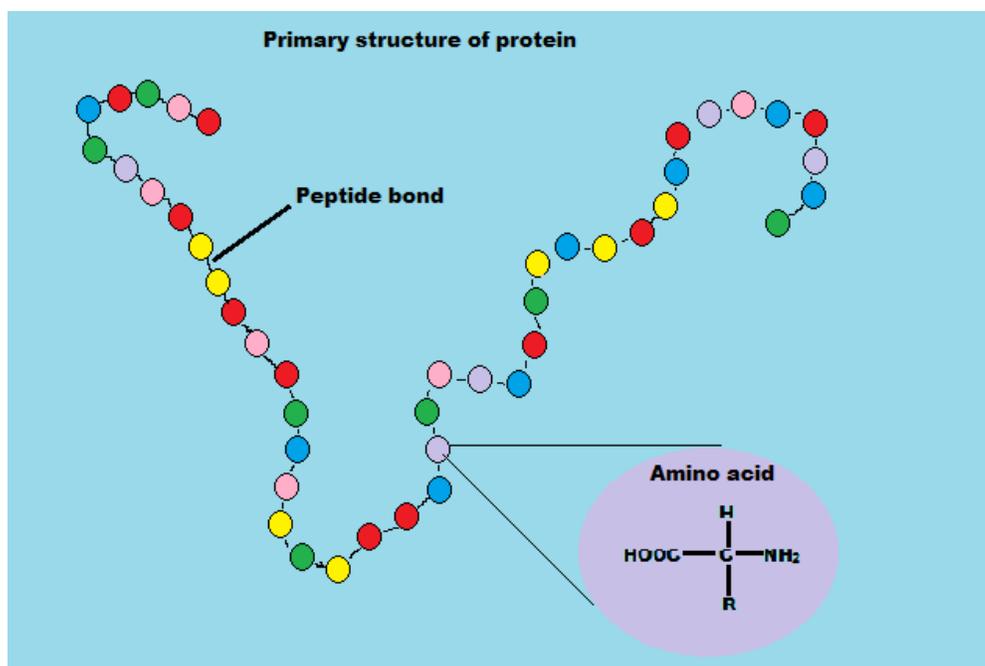


Figure 1.7: The primary structure of proteins. Figure drawn by the author using Chem Draw®.

The primary structure of the protein is defined as the polypeptide chain and consists of a certain number of amino acids connected via peptide bonds in a predefined sequence. As defined by *Campbell et al. 2005*, the peptide bond is a rigid structure chemical bond, which is formed by the interaction between the $-NH_3$ and $-COOH$ terminals of two adjacent amino acids by the elimination of one water molecule [3].

In 1951, *Linus Pauling* and *Robert Corey* discovered the secondary structure of the protein based on the core chemistry principle, their experimental observations, and the former scientists' explanations. Pauling and Corey have drawn the secondary structure and identified the two most important protein conformations. They concluded that; the close range of amino acid residues on primary sequence interact to each other via hydrogen bonds to form certain conformations; α -helix, β -sheet, and random conformation, to create the secondary conformational structure of the protein [4], Figure 1.8.

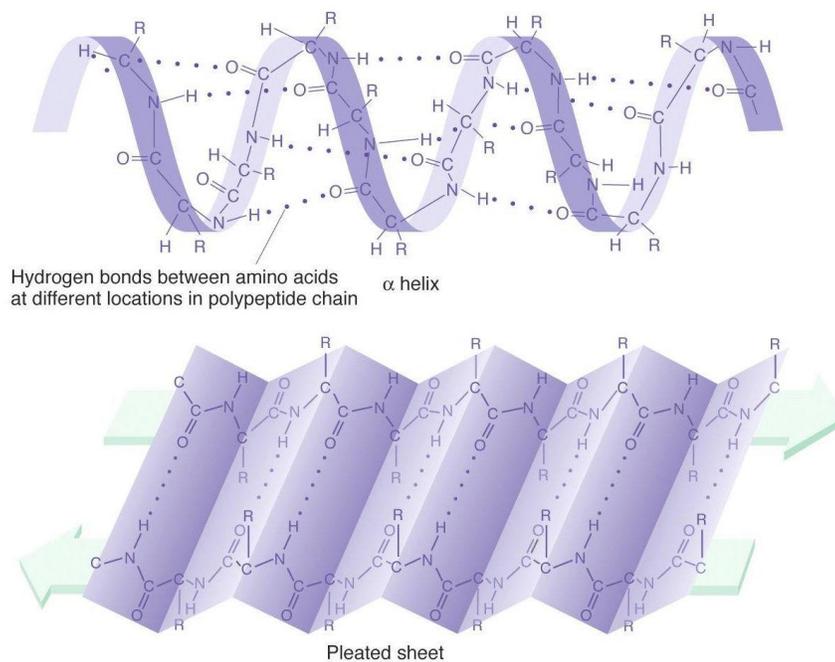


Figure 1.8: Alpha-Helix and beta- sheets secondary structure of the proteins. The figure was obtained from [5].

Figure 1.8 demonstrates the α - helix and β - sheet structures of the proteins, which comprises the secondary structure of most of the proteins. The secondary structure

shapes some of the physicochemical, chemical, and physiological characteristics of the protein. In α - helix, the maximum number of hydrogen bonds exists within the peptide bonds. However, no intramolecular hydrogen bond is present in β - sheet conformation, and all hydrogen bonds are formed between different chains [3].

After that, the polyamide folds further by forming hydrogen bonds, van der Waals, hydrophobic interaction, and disulphide bonds to shape the three-dimensional structure of the proteins which is called the tertiary structure of the protein. Some proteins have a higher level (quaternary structure) which is the interaction of two or more subunits of polypeptides such as haemoglobin and keratin, Figure 1.9.

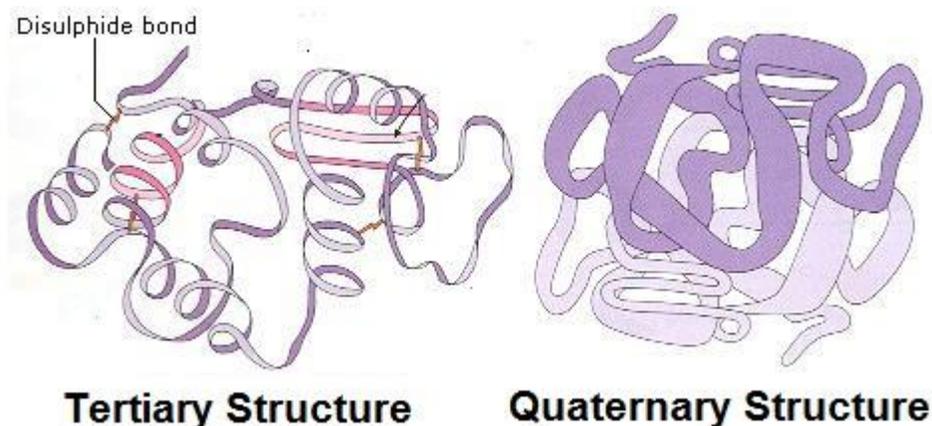


Figure 1.9: The tertiary and quaternary structures of the protein. The figure was obtained from [6].

Rangwala. H. et al. 2005, [7] classified the proteins based on their higher levels of the structure into two different main groups: a) fibrous proteins and b) globular proteins. Fibrous proteins are described that their polypeptide chains are arranged in long strands or sheets, while, the polypeptide chains in globular proteins are folded into a spherical or globular shape. The way that the polypeptides arrange in is the main

determinant of their functions and roles in the biological systems and organisms as will be detailed later in this project.

1.1.1. Isoelectric point (IP) of proteins

Like all molecules, proteins at certain pH can be either charged or neutral. Proteins' charge depends entirely on their primary structures which reflect the sequence of amino acids. The R groups connected to the chiral carbons in the amino acids determine the charge of the protein at the surrounding pH. Therefore, the "Isoelectric point" term came to identify the pH value at which the protein presents in a neutral state or with zero net charge [8]. Identifying the isoelectric point of proteins helps in determination the optimal solubility and activity conditions.

1.1.2. Folding and unfolding

In the default native state, proteins exist in a folded and three-dimensional structure. The proper folding of the proteins is an essential constraint to be biologically active. After the genetic codes transcription and translation into a polypeptide chain, the chain impulsively folds to shape the three-dimensional protein structure. Two different hypothesis usually explain the polypeptides folding, the thermodynamics and kinetics models. Thermodynamic folding and unfolding explain the changes in the free energy of the protein in both states [9]. Theoretically, proteins in their native folded state are the most thermodynamically stable and have the lowest energy [10]. Proteins unfolded

state may result due to physical, chemical disruption, external factors such as; temperature or pH, or more than one of them [11]. The majority of proteins except very small proteins fold to their native state or vice versa by forming one or more partially intermediates [12].

Some kinetic models explain folding and unfolding of the proteins, where some of them assume that proteins fold and unfold in only two states either fully folded or fully unfolded with no intermediates in between [13].

1.2. Biological functions and therapeutic proteins

1.2.1. Biological functions

The biological roles of proteins are determined by their structures. Hence, the proteins in their native three-dimensional structures are active. Therefore, the basic protein structure must be in a native state in order to achieve its desired functions.

Proteins play essential structural and physiological roles in the living organisms. Protein groups determine their physiological functions in the biological systems. Hence, fibrous proteins (large and insoluble proteins) e.g. collagen have mechanical support functions. Fibrous protein roles are usually comprised of structural and mechanical functions rather than physiological ones. For example, collagen is the most abundant protein in the animal species as the main component of the connective tissues and considered the major source of gelatine [14].

Proteins in the Animal bodies present in different forms and perform several Physiological functions other than the structural role. Proteins physiological functions are concluded in the following: a) enzyme to control the body reactions such as acetylcholine esterase b) hormones to control growth and differentiation such as; insulin c) transport and storage e.g. serum albumin d) motion coordinator e.g. actin and myosin e) antibodies for immune responses e.g. IgG f) neurotransmitters and their receptors for signalling such as Substrate P.

1.2.2. Therapeutic proteins

The genetic revolution and DNA-technology made the protein synthesis easier than before and generated the recombinant proteins in specific host cells e.g. bacteria, yeast, or mammalian. The recombinant proteins engineered in the lab for pharmaceutical uses are called therapeutic proteins, amongst these proteins; Insulin was the first one to be introduced [15]. The therapeutic proteins are being clinically used to treat broad range serious diseases, cure of many of them was a dream or even scientific fiction just a few decades ago.

Protein pharmaceutical formulations have emerged as promising therapeutic agents in recent years. *Walsh 2010* addressed that the therapeutic proteins are almost 50% of the new drugs approved recently by the United States Food and Drug Administration (FDA) [16]. The proteins in these formulations should be in their native conformation throughout the pharmaceutical process in order to be biologically active [17]. However, finding stable formulations and their delivery to the target site are a challenge due to

physical and chemical instabilities of proteins, including the most stable refrigerated ones, even during storage, as stated by [18].

Recombinant proteins are forming the majority of the therapeutic market proteins, and more hundreds are still in clinical trials phase and intended to treating cancers, immune disorders, infections, and other diseases [19]. Dimitrov 2012 reviewed the types of the therapeutic proteins and classified them according to their pharmacological activity into five different groups. The first group is the proteins used to replace a deficiency or abnormalities of the endogenous proteins e.g. Insulin in Diabetes mellitus Type I. The second group encompasses the proteins augment an existing physiological pathway such as Erythropoietin in anaemia caused by renal failure [20]. In addition, therapeutic proteins may provide a novel function or activity as in the case of Botulinum Toxin Type A when it is used as a drug of choice for patients suffering from muscle dystonia [21]. Moreover, some proteins are given to the patients targeted for a particular activity by interfering with a molecule or organisms such as; monoclonal antibodies to treat immunity disorders. Finally, some proteins are being used as delivering agents for other medications or proteins, e.g. Gemtuzumab is used as a conjugate treatment of acute myeloid leukaemia in elderly patients [20].

1.3. Pathways of proteins degradation

Proteins such as; therapeutic proteins and enzymes play vital roles in the biological systems. Thus, therapeutic proteins manufacturing is being increased every day. However, the production of proteins is quite restricted for different reasons. Noteworthy

amongst the reasons is the complex synthesis and purification processes, and poor long-term stability. Moreover, the proteins are prone to chemical and physical instabilities, which limits the choices of dosage form [22].

The biological activity and potency of the proteins are highly affected by the physical and chemical structure of the proteins. Therefore, the chemical and physical degradation pathways were extensively investigated by the researchers [23, 24].

According to *Wang 1999*, [11], a number of mechanisms could destabilise the proteins chemically or physically. Chemical degradations include deamidation, oxidation, proteolytic, β -Elimination, Condensation, and Asp isomerization. Whilst, physical degradation processes were concluded in Adsorption, Aggregation, Denaturation, and Precipitation, Figure 1.10.

However, Usually, both chemical and physical degradation pathways are coming together and synergistic (e.g., chemical degradations usually trigger physical ones and vice versa).

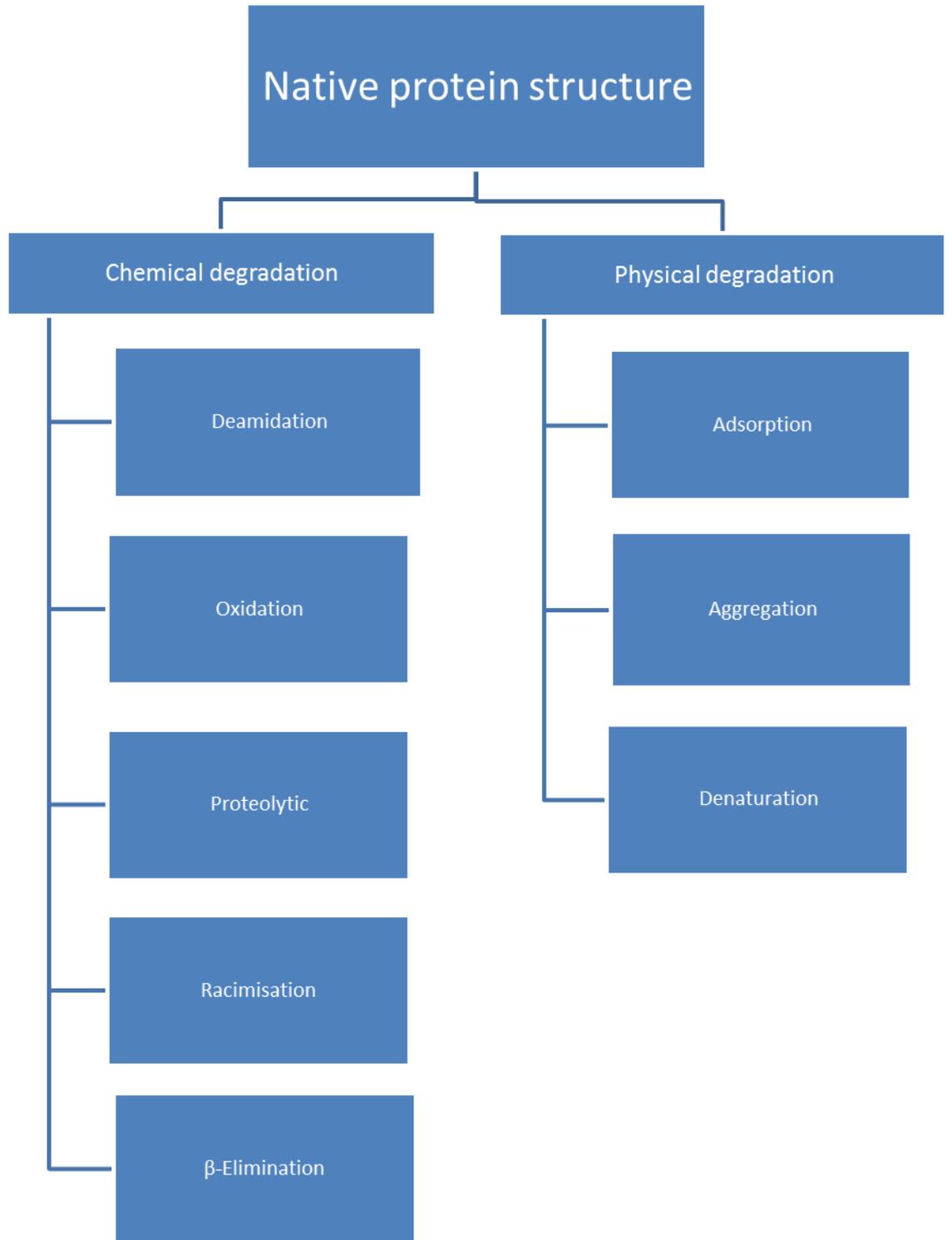


Figure 1.10: A schematic diagram illustrating both of the chemical and physical pathways of protein degradation.

1.3.1. Proteins degradation by chemical pathways

Chemical degradation of a protein refers to several chemical reactions which may change the chemical nature of proteins by the formation or destruction of covalent bonds within the structure of protein molecules (e.g., deamidation), and caused by changing the primary structure of the protein [25].

This type of degradation contributes to the changing in the amino acids residue in protein (primary structure) and thus disturbing the higher level of protein structure. The chemical degradation of proteins structure is caused by the different reactions. The following sections detail the main reactions causing the chemical instabilities, their effect on the protein structures, the precursor conditions of these reactions, and general tactics to decrease the chance of happening of the main degradation mechanisms. Some understandings of the impact of these chemical reactions on the protein physical stability are also provided.

1.3.1.1. Deamidation

The Deamidation reaction can be considered as the most common hydrolytic chemical degradation mechanism in proteins [26]. Deamidation reaction often happens in the Asparagine (Asn) side chain. Asparagine under deamidation is converted into

Aspartate (Asp) and/or isoaspartate (isoAsp), Figure 1.11. Hence, deamidation of the protein active site modifies the primary structure of the protein, and consequently, it changes the secondary and tertiary structure, which eventually, ends up with the loss of activity in some proteins. Moreover, converting Asparagine into Aspartate side changes the net charge of the protein. In addition to altering the amino acid sequence, converting Asparagine (Asn) into isoaspartate (isoAsp) changes the peptide backbone by adding extra methylene group [25].

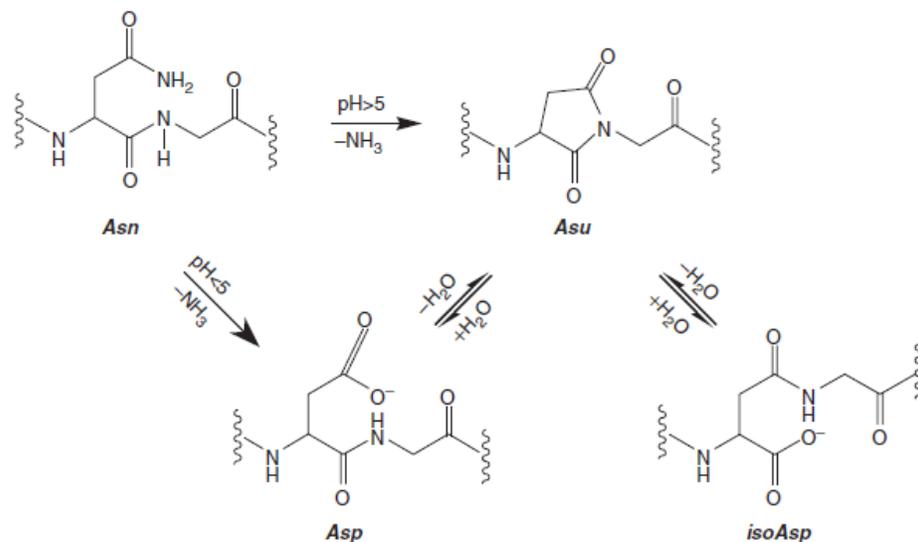


Figure 1.11: Asparagine (Asn) deamidation reaction and the isomerization aspartate (Asp), this figure was adapted from [25].

The impact of deamidation differs between the proteins. Therefore, some proteins get deactivated by deamidation, such as lysozyme, while other proteins potency is not affected by the deamidation reaction [27]. The reason behind the variation of the proteins activity towards the deamidation degradation is; that deamidation occurs mainly at asparagine (Asn) and glutamine (Gln) residues. Despite glutamine propensity to deamidation, its deamidation reaction is not as such observed as in Asparagine [25].

Thus, lysozyme activity is diminished when it is prone to deamidation; since Asparagine is one of the key amino acid residues in its active site [28]. The rate of deamidation reaction on Asn site is triggered by several factors e.g. protein primary structure, the surrounding pH, and Temperature.

There is a close link between the deamidation and the physical stability of the protein. Deamidation prompts the protein towards the aggregation and the connection between deamidation of some proteins and their aggregation is observed by several researchers [29, 30].

1.3.1.2. Oxidation

Oxidation is a common reaction which alters the protein chemical structure. According to Parkins et al., oxidation often happens at certain amino acid residues e.g. Methionine, Histidine, Cysteine, Tryptophan, and Tyrosine due to the propensity of their chemical structure to be oxidised [31]. It is a pH-dependent reaction and changes the primary sequence of proteins and forms aggregates [32]. The oxidation reaction can be concluded into three major mechanisms: free radical oxidation, metal catalysed oxidation, and photooxidation [33], Figure 1.12.

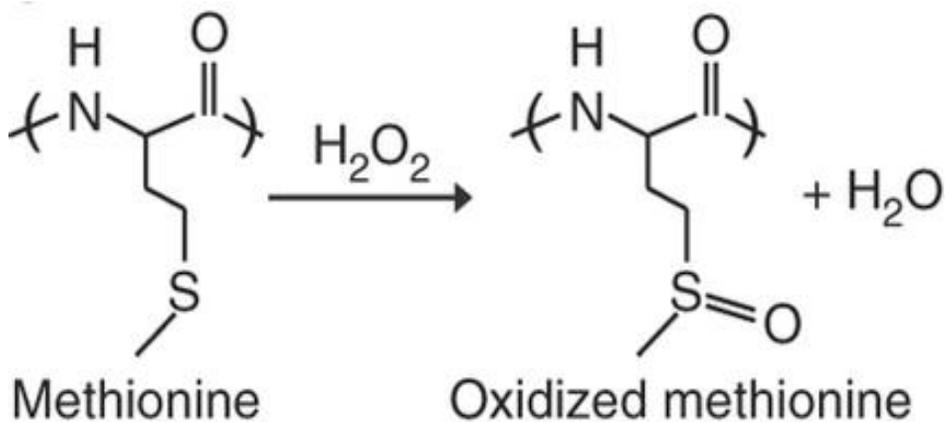


Figure 1.12: Oxidation reaction of methionine amino acid by the free radical pathway.

Some excipients can protect the proteins from degradation caused by oxidation. Adding excipients with high susceptibility to oxidation can safeguard the proteins from oxidation via sacrificial mechanisms such as antioxidant e.g. ascorbic acid, and methionine [34, 35]. Oxidation reaction in protein formulations should be reduced or even avoided due to its consequences on the physical stability of the proteins. Although the insight of effect of oxidation on protein aggregation rate is still poor, several researchers proved the link between oxidation and aggregations either by reducing the hydrophobicity [36] or changing the conformation of the proteins [37].

1.3.1.3. Racemization

As mentioned former about amino acid structure, all amino acid contain chiral carbon except Glycine. At high alkaline media, the hydrogen connecting to the carbon could be subtracted leaving the α - carbon in an ionic racemised form. The racemization of carbon atom can create non-metabolised amino acid in D- configuration, or enhance the creation of an irreversible peptide bond which can not be broken [38].

1.3.1.4. β-Elimination

A strong link can be noticed between the mechanism of β-Elimination and racemization. As in racemization reaction, β-Elimination is promoted at high temperature and the alkaline pH environment. β-Elimination occurs at Cysteine residue by the elimination of disulphide bond which eventually results in deactivation of the protein [27].

1.3.1.5. Proteolytic and fragmentation

As all chemical degradation pathway, proteolytic reaction affects mainly the sequence of amino acid. Protein proteolytic reaction is the fragmentation process which breaks the peptide bonds connecting the amino acids at the site of the link between the carbonyl and amide functional groups to convert it into small peptide chains [39], Figure 1.13. Proteolytic mainly occurs at Aspartic acid residue irreversible and may cause serious pathogenic conditions when it happens to the endogenous proteins [40].

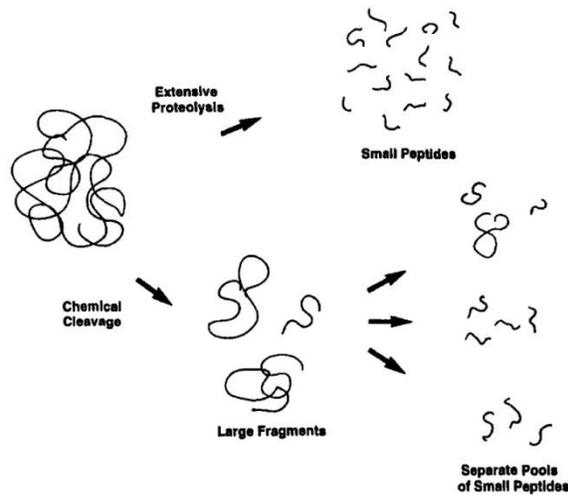


Figure 1.13: Fragmentation of proteins into smaller peptides.

1.3.2. Proteins degradation by physical pathways

Unlike the chemical degradation of proteins, the physical deterioration of the protein usually disrupts the secondary and tertiary structures, which may result due to changes in non-covalent bonds (H, van der Waals, hydrophobic interaction, and electrostatic). The major physical degradation pathways of the proteins are aggregation, denaturation or unfolding and adsorption. In addition to some minor pathways e.g. dissociation, and precipitation.

1.3.2.1. Aggregation

Protein aggregation is the common type of physical degradation; the aggregation may be driven by chemical changes, which is called chemical aggregation, e.g. the aggregation caused by changes in covalent bonds. It could be soluble or insoluble, reversible or irreversible, which will eventually, leads to the loss of protein activity [41].

1.3.2.2. Unfolding or denaturation

As explained earlier in this project, proteins are natively folded. The unfolding of the protein is the main denatured form, and it may lead to further degradations e.g. aggregation. The unfolding may be reversible or irreversible. It may also be caused by the formulation compositions e.g. pH, or the solvent nature, or by external causes such as temperature, or pressure.

1.3.2.3. Adsorption

Protein structure may be degraded due to the adsorption on many interfaces; e.g. air-water interface, solid-water interface, hydrophobic surface water interface, and container surface. Several hypotheses can explain the degradation mechanisms by adsorption, for example; protein molecules may be reoriented and rearranged at the interface which may enhance the unfolding of the protein [42]. Moreover, the instability of protein may be caused by the interaction between the hydrophobic amino acid residues (which must be impeded inside the folded protein) with the hydrophobic surface and may change the three-dimensional protein structure [38, 43]. According to *Burke et al., 1992*, no clear relation was observed between the protein size or isoelectric point and the severity of the adsorption [44].

1.4. Toolbox for protein analysis

Protein therapy may end up with an undesirable side effect for example; anti-drug antibody [45]. The undesired effect could be raised up by the complex physicochemical characteristics of the proteins as large susceptible molecules to the chemical and physical degradation, which eventually leads to unwanted immunogenicity, that affects the products efficacy and safety [46, 47]. Therefore, the identification and prediction of the degradation pathways of the proteins have been forming a challenge for the researchers for decades [48].

Advances in analytical chemistry made the identification of degradation or stabilisation mechanism possible. Several techniques have emerged as analytical tools to assess the stability of proteins. However, selecting the right technique is one of the most important steps in protein formulations development. A broad range of the analytical tools is available to assess the proteins degradation. Hence, the selection of the analytical tools must be well studied in advance to suit the desired purpose of analysis and the characteristics of the protein under investigation. According to Filipe et al. 2013, the available analytical tools are divided into four main classes based on the pathway of degradation which they can detect. These classes are analytical methods to assess the proteins a) conformational changes e.g. Fourier Transmitter Infrared (FTIR), and Circular Dichroism spectroscopy (CD), b) physical instabilities e.g. turbidimetry, c) chemical degradation e.g. reverse phase high performance liquid chromatography (RP-HPLC), and d) biological activity and potency [49],

Table 1.1.

Table 1.1: Frequently used analytical methods for the assessment of proteins degradation.
Table adapted from den Engelsman et al., 2011, [50].

Conformational stability	Uses	Chemical degradation	Uses	Physical instabilities	Uses
UV Spectroscopy	Tertiary structure	RP-HPLC	QC, hydrophobicity	SEC	QC, Protein size
Raman spectroscopy	Secondary structure	LC-MS	Molecular weight change	Turbidimetry	QC, non-soluble, aggregates
FTIR	Secondary structure	MS	Molecular weight change	Light Obscuration	QC, size
Fluorescence	Tertiary/ Quaternary structure	IEX Chromatography	QC, Charge variant	DLS	Size
CD spectroscopy	Secondary/ Tertiary/ Quaternary structure	cIEF	QC, Charge variant	Native MS	Fragment and aggregate
DSC	Unfolding onset temperature, Thermal variable			Optical microscopy	Morphology
DSF	Melting temperature, Thermal variable			SDS- PAGE	QC, Molecular weight

Abbreviations: UV: Ultraviolet; FTIR: Fourier Transmitter Infrared; CD: Circular Dichroism, DSC: Differential Scanning Calorimetry; DSF: Differential Scanning Fluorimetry; RP-HPLC: Reverse Phase High Performance Liquid Chromatography; LC-MS: Liquid Chromatography Mass Spectrometry; MS: Mass spectrometry; IEX chromatography: Ion Exchange Chromatography; cIEF: capillary Iso-Electric Focusing; SEC: Size Exclusion Chromatography; DLS: Dynamic Light Scattering; SDS-PAGE: Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis.

1.4.1. Analytical methods to assess the conformational changes within protein structure

In order to study the conformational stability; the changes in the three-dimensional structure of the protein should be detected. Many options are being used for this purpose; which can be concluded, based on the detection mechanisms, by two main categories; spectroscopic and thermal analytical tools. Spectroscopic instruments are the analytical methods designed to investigate the production or emission of spectra as a result of the interaction of electromagnetic radiation with matters via for example; electronic excitations (UV) or molecular vibration (FTIR) [51, 52]. In protein science, spectroscopy is used to evaluate the conformational changes in the protein structure include; Fourier Transmitter Infra-Red (FTIR) Spectroscopy, Ultraviolet (UV) Spectroscopy, Fluorescence spectroscopy, Raman Spectroscopy, in addition to Circular Dichroism (CD) Spectroscopy [50].

Moreover, the thermal analytical methods include two main common methods: a) Differential Scanning Calorimetry (DSC) b) Differential Scanning Fluorimetry (DSF) [53].

The selection of the analysis option depends on the desired structure detection, either secondary or tertiary structure, purpose of the study (QC, or screening), the size of the samples, or whether to use high, medium, or low throughput.

Amongst the spectroscopic analytical methods, FTIR is the most common used for secondary structure changes detection. FTIR has been intensively investigated, and

the relevant fingerprints are well-defined in the literature [54]. The tertiary structure of a protein can be assessed by several precise tools. UV and fluorescence spectroscopy are the most common tools used for this purpose [55].

Recently, thermal analytical methods (calorimetric, and fluorimetric) have been emerged to assess the conformational stability of proteins, by measuring different parameters during thermal treatment [56, 57]. Differential scanning calorimetry (DSC), microcal VP-DSC, and differential scanning fluorimetry are the main examples of the used thermal techniques. DSF is a thermal high throughput method and often used to evaluate protein conformational stability by determining the melting temperature (T_m) and the onset of unfolding. DSF mechanism is primarily based on the measuring of fluorescence intensity of hydrophobic dyes upon binding to unfolded parts (hydrophobic part) of protein during under a thermal treatment [58].

Calorimetric methods, DSC and VP-DSC, are sensitive, are high throughput, and user-friendly techniques and usually used to determine the calorimetric parameters associated with conformational changes of the protein under heating effect, in a simple analytical approach.

Microcal VP-DSC or so-called High sensitivity differential scanning calorimetry (HSDSC) provides data about the unfolding temperature, (T_m), the energy of unfolding in addition to unfolding reversibility, which can be measured by comparing the energy of unfolding of two consecutive runs, all calorimetric parameters can be determined by determination of onset, rate, extend and thermodynamic of heat-induced protein

unfolding processes. The thermodynamic properties of a protein depend on its conformational state and solution conditions [59, 60]. More stable proteins have a high value of T_m thereby, needing very high thermal energy to unfold [60]. The resolution of VP-DSC is greater than DSC, and it is used for analysing liquid formulations. This method has been used by researchers as a predictive tool for the long-term stability of different formulations [61].

1.4.2. Analytical methods to assess physical degradation of proteins

Protein aggregation is the main concern in therapeutic protein delivery, because of the generated immunogenicity. The aggregation should be defined, detected, and controlled before delivering the protein to the patient, even if the protein is fully active. Different analytical techniques were emerged to evaluate the proteins evaluation. However, not all of them can be used as Quality Control (QC). The pros and cons of each method are described and investigated in the literature. Therefore, the selection of the method of analysis depends on the aim of the study. For example, for stability indicating studies require using sensitive, QC, high throughput methods.

Table 1.1 lists the commonly used analytical methods along with their detections and observations.

Amongst the listed methods, size exclusion chromatography, turbidimetry, light obscuration, and Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) can be used for QC purposes. Size exclusion chromatography (SEC) is a high throughput and an accurate method usually used for large molecules analysis.

SEC mechanism is concluded in chromatographic separation of large molecules based on their sizes [62]. SEC is able to quantify the native proteins, aggregations and their fragments. However, SEC, like all the chromatographic methods, is only able to analyse and quantify the soluble moieties. Hence, using SEC for detection of aggregation over the stability study period must be accompanied by an analytical method able to detect the non-soluble aggregates. Turbidimetry is often used for detection of non-soluble protein aggregates by determination the changes of intensity of transmitted light through the sample. Turbidimetry is a quite popular because it is simple, user-friendly, and high throughput. However, the data obtained from turbidity measurement can only be used for comparison purposes [63]. SDS-PAGE was a popular method for protein aggregation detection. However, it has recently been replaced by other methods because of its inaccuracy of quantification of aggregations [64].

1.4.3. Analytical methods to assess chemical degradation of proteins

Chemical structure of a protein can be degraded by several pathways as explained earlier. In order to detect the chemical instabilities, different analytical methods are assigned for that purpose. The main assays are chromatographic or electrophoretic methods e.g. High performance liquid chromatography, ion exchange chromatography, and capillary Iso-Electric Focusing. Amongst the chromatographic methods, Reverse Phase- HPLC is the most commonly used to assess the changes in the protein hydrophobicity [65]. Ion exchange chromatography (IEX) is applied to investigate the

changes in protein charge. The chromatographic analytical methods are very sensitive, high throughput, and able to provide valuable quantitative and qualitative information about the protein stability [66]. Also, electrophoresis based analytical methods e.g. capillary isoelectric focusing (cIEF) are often used to assess the chemical instabilities of protein.

Moreover, Mass spectroscopy (MS) and liquid chromatography-mass spectroscopy (LC-MS) are also used to detect the chemical changes in the protein structure. However, the complexity of samples preparation and the time consuming have restricted the applications of MS in the identification of the chemical degradation of the protein. LC-MS is the main method being used to assess the chemical changes. However, LC-MS is not being used as a QC technique and its uses usually restricted in identification the products of chemical changes of the proteins rather than a QC tool to track the chemical changes in protein or quantification of the remaining intact protein [49].

Usually, the chemical degradation is accompanied by physical instabilities, potency decrease, or both e.g. Lysozyme deamidation affect its activity significantly. Considering that, the assessment of physical stability and biological activity of the protein formulations over the period of storage may give a strong indicator of the chemical degradation. Therefore, it makes the need for the chemical structure assessment is not necessary.

1.4.4. Potency analytical assays to assess the protein's activity

Potency is the main vital quality of the therapeutic protein, potency is a good indicator of the biological activity of the protein and should be high in order to achieve the key purpose of the protein formulations.

Biological activity of the protein can be assessed by several potency assays, and there is no one assay suitable for all proteins. Potency assay selection is based on the principal function of the protein. The biological activity of the protein can be evaluated by using cells, tissues, organs, and animal. Sometimes, due to the high cost of the biological assays, the potency tests are performed after ensuring that the protein is physicochemically stable. Enzymatic reactions based assays are often used to evaluate the biological activity as a cost effective test can be carried out in the lab e.g. measuring the biological activity of lysozyme as an efficient enzyme to hydrolyse the cell wall of bacteria. Moreover, enzyme-linked immunosorbent assays (ELISA) is used to assess the biological activity of certain proteins.

1.5. Protein formulations

1.5.1. Liquid formulations

Designing the pharmaceutical protein in the desired dosage form is not always possible due to various restrictions related to stability issues. Therefore, the most common

therapeutic protein dosage form is the injection or parenteral form. Liquid formulations popularity came from their relatively reduced manufacturing cost, and ease of handling. However, proteins face stability issues in the liquid formulation; due to the nature of protein – water interaction [67]. In order to attain the desired protein activity, the protein surface hydration should be well maintained to protect the native folded state. However, the ratio of protein water should be wisely adjusted to avoid the plasticisation of water towards the proteins which therefore will increase the proteins mobility and ends up with denaturation or aggregation [68].

All the formulation factors should be will studied, investigated in advance and optimised, in order to enhance protein stability and obtaining persistent formulations. Thus, a critical evaluation of the impact of each factor on the stability of these formulations should be carried out, to reduce a chemical and physical withstand loss due to the different forms of instabilities [11]. These factors include pH, buffer types, buffer concentrations, protein concentrations, the used excipients, container and the external conditions, such as; temperature, shaking, and relative humidity [11].

The additives and excipients are needed to keep the adequate hydration around the protein molecules and to avoid the denaturation at the same time e.g. using some sugars or so-called extromolytes, such as trehalose, in protein formulations protected the proteins by balancing the water content surrounding the protein by preferential hydration/ exclusion mechanism [69]. Moreover, the additives may protect the protein molecules from the various chemical and physical degradations which may be triggered by the aqueous media, as discussed before.

1.5.2. Solid protein formulations

Although liquid protein formulations are the most common amongst the therapeutic protein formulations, solid protein formulations are more stable.

The solid state places the protein in a rigid, and inert matrix. It, also, separates the protein molecules and limits their mobility, which, minimises bimolecular interaction. Accordingly, it slows down unfolding and other chemical degradation due to their presence in a strongly coupled protein with rigid matrix [70].

Different techniques have been applied to develop solid protein formulations to enhance protein stability. Amongst these approaches; protein drying by either freeze-drying or spray drying [71], and crystallisation [72].

According to *Elkordy et al. 2002*, [72], protein drying is the process of removing water from liquid protein formulations to convert them into solid powder in order to prolong their storage stability.

1.6. Quality by Design

Product and process development and even product manufacturing were traditionally based on experienced and fixed procedures in the pharmaceutical industry. The

situation has been influenced by the rigid regulatory environment which controlled the pharmaceutical industries business and consequently limited the improvement in the manufacturing technology. These restrictions led to economic problems, e.g. 5% - 10% of the produced medications were being discarded because of manufacturing shortcomings as shown in a survey of the Wall Street Journal, which contributed to increase the manufacturing expenses [73]. Considering this economic background and its technical consequences, the FDA launched the Quality by Design (QbD) initiative in 2004, in order to produce high-quality products without extensive regulatory oversight [74]. The QbD approach is defined as a “systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control based on sound science and quality risk management” [75].

The rationale behind the QbD approach is to build the quality into the product from the beginning of the design, through understanding the relations of product quality and parameters affecting it, instead of testing it [76]. Accordingly, QbD can promote faster and more consistent product and process development. Thus, to increase flexibility in manufacturing in order to reduce production cost. Adapting the QbD is important in biologics manufacturing, due to the high cost of the biological materials such as proteins, antibodies, cells and genes. The regulatory agencies (e.g. International Conference on Harmonization ICH) released several documents defining the key steps and innovative tools of QbD implementation, which prompted the manufacturers to adopt systematic science-based tools, including mathematical modelling tools, Figure 1.14.



*FMEA: Failure Mode and Effects Analysis.

**DOE: Design of Experiment.

Figure 1.14: A schematic diagram comprises the QbD approach as stated by ICH Q8, Q9, and Q10.

The QbD approach starts with the identification of the critical Quality Target Product Profile (QTPP) to form a clear aim for the product development and to design the rest of the pharmaceutical development process to suit the pre-defined targets. Accordingly, the Critical Quality Attributes are determined in relation to the product characteristics. Then, the risk assessment including risk identification, risk analysis, and risk evaluation takes place before the manufacturing as hazard determination processes as recommended by [75]. As a part of the QbD, a product lifecycle management plan should be drawn, and the quality should be continuous monitoring made over time to update the process [76].

Appropriate mathematical modelling tools have been made available to enhance the pharmaceutical products development and manufacturing. The design of experiment (DOE) is considered the most common statistical example of these mathematical tools [77]. DOE is defined as a planned statistical approach which is able to figure out the relationship between the causes (inputs) and responses (outputs). DOE can effectively analyse the vast amounts of data generated from the measurement systems to collect the maximum amount of information with minimum expenditure time and resources, [78]. The main purpose of DOE development is to reveal the relationships between the variables and responses, especially when the multivariate product is available, in addition to analysing several model responses variables [79].

In comparison to the conventional way of experimental design (one factor a time), applying the design of experiment approach consumes 10% of the time required to collect the same data by the conventional empirical approach [78, 80]. Therefore, when designing an experiment by applying the empirical approach, many samples have to be prepared in order to obtain the desirable results which pursue the purpose, that ends up with high raw material consumption and longer time with less accurate results. Moreover, the conventional way can only investigate the effect of one factor a time, which means the rest of the independent variables are held fixed, which means valuable information e.g. the effect of factors interaction are missed [79].

All the mentioned benefits made applying of DOE approach appealing to the researchers and manufacturers. The researchers' insight towards the results analysis has been developed, and the awareness of products development have increased due to the provided research explanations and the optimisation processes.

DOE approach has been used in the literature in different disciplines e.g. chemical manufacturing, engineering, and pharmaceuticals due to the previously detailed benefits.

1.7. Shelf stability

Protein stability during manufacturing, shipping, shelf storage, and even in patient's body is a vital consideration during the biopharmaceutical development. Therefore, the formulation conditions should be investigated, well studied, and wisely selected.

In addition to the nature of proteins themselves, as inherently sensitive diverse large molecules and their tendency to aggregate, there are numerous external factors affecting proteins stability and have either positive or negative significant impact. For example, temperature, pH, the type of solvent or buffer, protein concentration, and excipients. The factors surrounding the protein and forming the environment of the formulation should be controlled to avoid the unfolding state or aggregation, which eventually leads to loss of native protein activity.

The regulatory agencies have established the requirements for the pharmaceutical products to have a shelf life clearly labelled on the products container [81]. The shelf life is "The time period during which a drug product is expected to remain within the approved shelf life specification, provided that it is stored under the conditions defined on the container label.". The above definition is adapted from The International

Conference on Harmonisation (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use guidance document Q1A.

ICH Q1A R2 has placed that there are three different approaches to studying the pharmaceutical products based on the duration of the study and the conditions of storage: accelerated stability study, intermediate stability study, and long-term stability study [75].

The accelerated stability study is the shortest approach and it encompassed by storing the pharmaceutical products under harsh conditions e.g. temperature reaches 40 °C with up to 75% relative humidity (RH) for at least six months. In the intermediate approach the drug products are recommended to be kept at either 25 °C or 30 °C (based on the original conditions of storage for the long term) and 60% RH for at least six months. However, the storage for long-term stability should be under conditions suitable for the active pharmaceutical ingredients (API), for example; highly sensitive API like the biologics should be stored under 5 °C or – 20 °C [75].

All new active pharmaceutical ingredients (API), require long-term stability studies in order to be registered. Stress conditions are usually employed in formulations to establish degradation pathways, and then validate the stability by using indicative power analytical procedures. Physical and chemical stability of protein should frequently be tested during the long-term and accelerated stability study.

Therefore, too many efforts were applied, and various analytical methodologies were employed to predict instability events well before commencing long-term stability studies, in order to reduce the materials, operation, labour costs, and time.

In protein formulations, the instability events, physical and chemical changes are not predictable and do not obey linear behaviour. That means the prediction of protein stability requires intensive investigation to find suitable techniques to predict the long-term stability, which is still challenging until now [82].

Accelerated stability studies showed success in some protein formulations. Moreover, high-throughput formulations screening methods have been employed by the researchers to predict long-term stability [83].

The combination of different analytical techniques is required for characterization of protein formulations as a complex duty. However, determination of the biological activity of the protein, measured by potency assays, over the period of storage is the most important and can tell if the protein can fulfil its main purpose or not.

1.8. Polymeric Nanoparticle carriers' applications for oral and controlled release protein delivery systems

1.8.1. Oral protein formulations

Oral drug delivery systems are the most common and acceptable route of administration. However, it is too difficult and challenging to deliver the therapeutic proteins by the oral route, due to different hurdles, for example; extreme stomach

acidity, enzymatic degradation, physiological (permeability) barriers, and physicochemical instabilities of proteins [84, 85]. In order to design oral protein formulations, the previous hurdles should be overcome. Therefore, many researchers developed different approaches to enhance oral route bioavailability such as; protease inhibitors to inactivate the gastrointestinal enzymes (which denature the therapeutic proteins) e.g. chymotrypsin and trypsin [86]. Therapeutic proteins degradation rate in the digestive tract by the digestive enzymes was diminished by the enzyme inhibitors, which consequently led to increasing in protein availability and improved the absorption through intestinal wall in rats in a study performed using insulin [87]. However, the daily need of insulin in diabetic patients requires the long-term intake of these enzyme inhibitors, but their safety still not well established and some potential hazards may be developed [85]. In addition to the previous approaches, absorption enhancers have been used. Absorption enhancers can improve the therapeutic proteins oral bioavailability by increasing gastrointestinal tract epithelium permeability through targeting the epithelial cells lipid bilayers, for example, bile salts and fatty acids [88]. However, pathogens and toxins can cross the highly permeable epithelium into the blood circulation [89]. Likewise, different protein chemical structure modifications e.g. PEGylation, and different protein carriers' techniques, for instance, nanocapsules and liposomes have been employed to overcome the low protein bioavailability after oral administration [85].

Nanocarriers are drug delivery systems of a nanoscale particle size. Nanocarriers are usually used in protein therapy to deliver the therapeutic proteins and improve their pharmacokinetics properties either by encapsulation or bioconjugation [90]. According to different researchers, nanoparticle systems show promises as drug carriers due to their efficiency in drug release [91], their highly intracellular uptake than the larger

particle systems due to their subcellular sizes [92], and the ability to protect the encapsulated drugs and improving their stability [93].

Therefore, nanoparticulate carriers such as polymeric nanoparticles and micelles are employed for the oral delivery of insulin. These nanocarriers protect insulin from degradation and facilitate insulin uptake via a transcellular and/or paracellular pathway. Various nanoparticle systems like polymeric nanocapsules (PNCs), solid lipid nanoparticles (SLN), liposomes, and metallic nanoparticles, were employed to deliver protein nanomedicines, to enhance their stability, and to control the drug release [94].

1.8.2. Applications of polymeric nanoparticles in protein delivery systems

Polymeric nanoparticles drug delivery systems have been developed and widely investigated by researchers in the past couple of decades [95, 96]. According to *Soppimath et al. 2001*, [95], polymeric nanoparticles are carrier drug delivery systems have particle diameters up to 1000 nm with a structure composed mainly of biodegradable polymers. Polymeric nanoparticle systems are classified based on their structure into two main forms: polymeric nanocapsules and polymeric nanospheres, Figure 1.15.

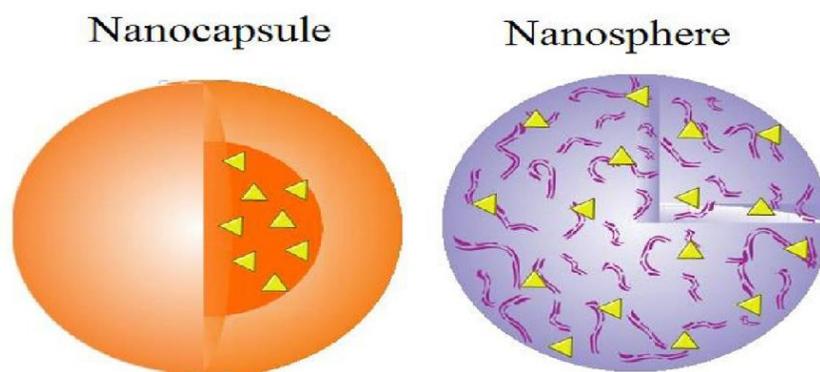


Figure 1.15: Illustration of the difference between polymeric nanocapsule and a polymeric nanosphere. The source of the image is from [97].

As illustrated in Figure 1.15, Polymeric nanocapsules (PNCs) system is simply defined as a reservoir-shell vascular system, where the active substances are confined in a cavity or core and surrounded by a polymeric shell [98, 99]. While polymeric nanosphere system is a carrier composition presenting matricial organisation of the polymeric structure [100].

Different polymer categories are used in the synthesis of polymeric nanoparticle carriers. The commonly used polymers are usually characterised by being biodegradable which can be degraded and eliminated from the body by the normal body physiological degradation pathways. Moreover, the polymers should be biocompatible which are non-toxic with no antigenicity due to their structures which are compatible and adaptable with the body [101]. Two major kinds of biodegradable polymers are being used in the nanoparticles preparation based on their origin: natural

or synthetic. There are around 20 common natural polymers include; gelatine, alginate, starch, chitosan, and hyaluronic acid. Recently, the polymer synthesis revolution resulted in too many synthetic biodegradable polymers which have several applications in the biomedical field, drug delivery systems, tissue engineering, and regenerative medicine e.g. polycaprolactone, polylactide, poly ethyl oxide, poly glycol, blend of more than one polymer, and copolymer composed of different monomer blocks [102].

Using polymers to formulate core- shell nanocapsules shows advantages over other nanoparticle systems like matrix nanospheres system, as the system needs less amount of polymer contents to protect the vulnerable drugs e.g. protein against harsh conditions, such as stomach pH [103]. Moreover, the polymeric nanocapsule systems have the higher efficiency to encapsulate the drugs due to enhancing drugs solubility in the nanocapsules cavity. Another advantage of nanocapsule systems is that; using biodegradable polymers to synthesise the polymeric nanocapsule systems enhances the system's capacity to be biocompatible with tissues and cells, hence, improves the particles distribution and metabolism [104]. Furthermore, polymeric nanocapsules can act as a controlled release system; hence, it reduces the systemic toxicity of the drug [93].

Different methods were applied in the literature to prepare polymeric nanocapsule systems. Nanoprecipitation, emulsion diffusion, double emulsification, emulsion coacervation, polymer coating, and layer by layer are the main common six methods for polymeric nanocapsules preparation [105]. As asserted by different authors, each preparation method has its advantages and disadvantages; therefore, no methodology is ideal to prepare polymeric nanocapsules. Various factors affect the process for

selecting of specific preparation methods; like; physicochemical properties of the drug, its solubility, the therapeutic objective of nanocapsule. The solubility and physiochemical nature of the drug play a crucial role in selecting the suitable preparation method. Nevertheless, all preparation methods are only able to encapsulate lipophilic drugs apart of double emulsion method, which can entrap hydrophilic drugs, like, proteins. Therefore, double emulsion method is usually used to prepare protein containing polymeric nanocapsules.

1.9. Model and therapeutic proteins and the essential excipients used in this project

Two proteins, lysozyme and trypsin, were selected as model proteins in this study. Lysozyme and trypsin were investigated by several researchers [28, 57]. Moreover, Deoxy ribonuclease I (DNase I) as a therapeutic protein has been selected in this study to apply the results obtained from the model proteins.

Lysozyme as a model protein is considered a good candidate for studying the influences of several formulation factors and processes on its stability and integrity due to its well-known structure and characteristics. Lysozyme consists of 129 amino acids single polypeptide chain with a molecular weight of 14.3 KDa and isoelectric point around 10.7. Lysozyme secondary structure is compromised in dominantly α -helix shape, and its conformation is stabilised by four disulphide bridges formed between cysteine amino acid residues, Figure 1.16. The active centre of lysozyme contains several AAs (Ala, Asn, Asp, Glu, and Trp), and they are involved in the enzyme-

substrate interaction. This interaction demonstrates the enzyme activity by assessing its ability to control of predisposed bacteria. The structure of lysozyme is easy to be investigated by spectroscopic analysis, due to the presence of aromatic amino acids tyrosine and tryptophan in its amino acid backbone [106].

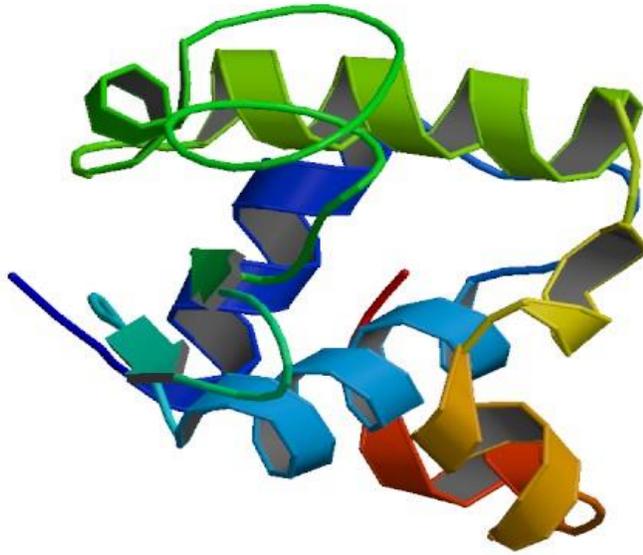


Figure 1.16: Ribbon representation of lysozyme. The structure was adapted from Protein Data Bank (PDB), file 5E4P.

Trypsin as a model protein, with characteristics different than the one in lysozyme, has been chosen for study in order to obtain insight into the effect of the formulation material and process attributes on its structure. Trypsin is a serine protease consists of 223 amino acids polypeptide chain, with a molecular weight of 23.3 KDa, with an isoelectric point between 11.0 and 11.4, and dominant β -sheet secondary structure [107], Figure 1.17.

Enzymatic- substrate reaction demonstrates the enzymatic activity by measuring its ability to convert N α -Benzoyl-L-Arginine Ethyl Ester to N α -Benzoyl-L-Arginine. Trypsin is used in biotechnological processes [108].

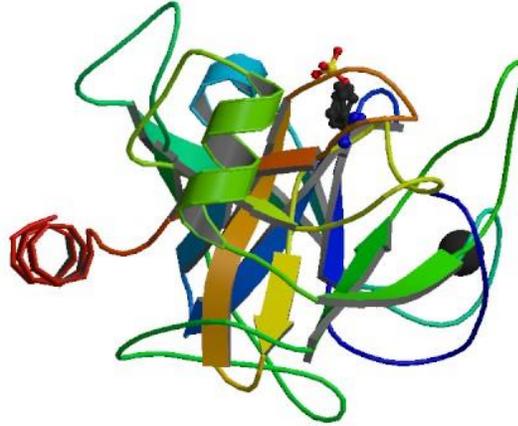


Figure 1.17: Ribbon representation of trypsin. The structure was adapted from Protein Data Bank (PDB), file 5F6M.

Deoxy ribonuclease I (DNase I) is an enzyme enhancing the cleavage of phosphodiester bonds between the nucleotides in DNA backbone. DNase I consists of a single polypeptide amino acids sequence containing disulphide bridge with a molecular weight around 30 KDa and isoelectric points 5.1 ± 1 [109], Figure 1.18. Recently, DNase I emerged as a drug of choice for treatment of cystic fibrosis. In cystic fibrosis, the patients suffer from high sputum viscosity which results in difficult breathing. The reason behind the high viscous gel sputum is the high DNA content in the patients' sputum. Therefore, providing the patients with DNase I can reduce the viscosity of the sputum by DNA hydrolysis [110].

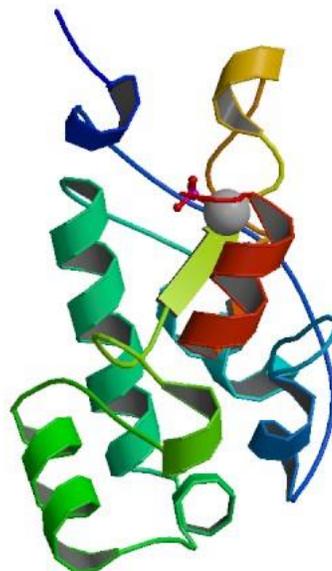


Figure 1.18: Ribbon representation of Deoxy Ribonuclease I. The structure was adapted from Protein Data Bank (PDB), file 1FSJ.

Pluronic F127 is a non-ionic surfactant, and bifunctional triblock copolymer, with a molecular weight of approximately 12.5 KDa; it is a type of general class of copolymers known as poloxamers. It consists of a central hydrophobic block polypropylene glycol connected to two hydrophilic polyethene glycol (PEG). These amphiphilic properties, 100% activity, and safety profile make the compound suitable to be used in many pharmaceutical applications, as a solubility enhancer of water –insoluble materials in physiological media [111], Figure 1.19.

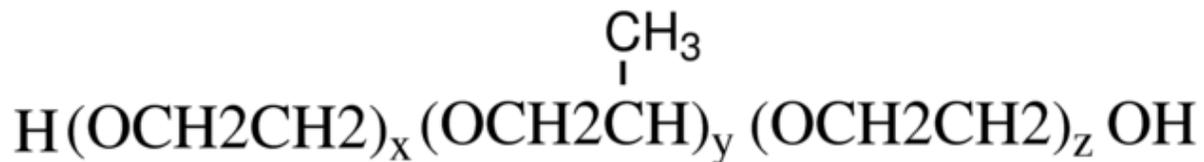


Figure 1.19: Chemical structure of Pluronic F 127. The figure was adapted from [112].

Sodium ascorbate is a mineral salt of ascorbic acid (vitamin C), as a reducing agent, it helps reduce oxidative stress. It could be used in a broad range of application, as an anti-oxidant and acidity regulator. Figure 1.20.

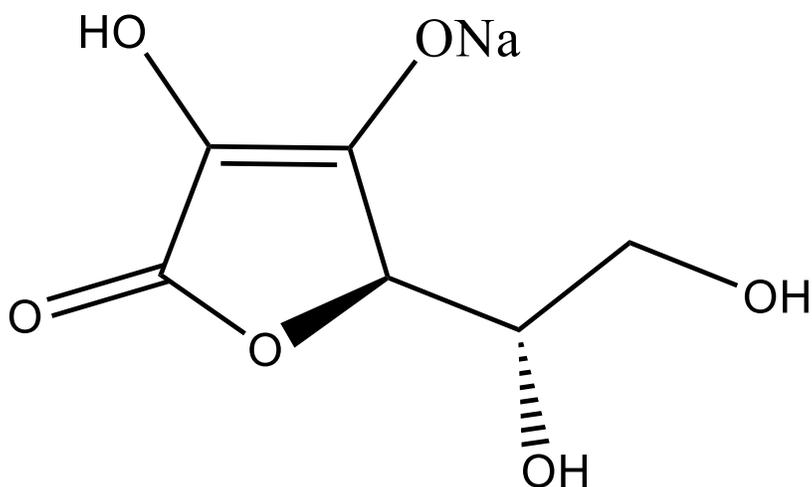


Figure 1.20: Chemical structure of (+)-Sodium L-ascorbate. The chemical structure of sodium ascorbate was drawn by the author by using Chem Draw®.

Trehalose dihydrate is sugar, belongs to a general group called extromolyte; and considered one of the most widely used extremolyte in protein formulations. It is disaccharide consists of two glucose units. Trehalose is found in extremophilic

microorganisms and protects them from extreme environmental stresses [113]. Depending on this natural background in addition to its safety profile, trehalose has been used by researchers as an excipient in protein formulations due to its well-known stabilising effect on the conformational stability of proteins. Its impact on conformational stability has been explained, as it increases the hydration of proteins, which makes proteins unfolding less favourable [114], Figure 1.21.

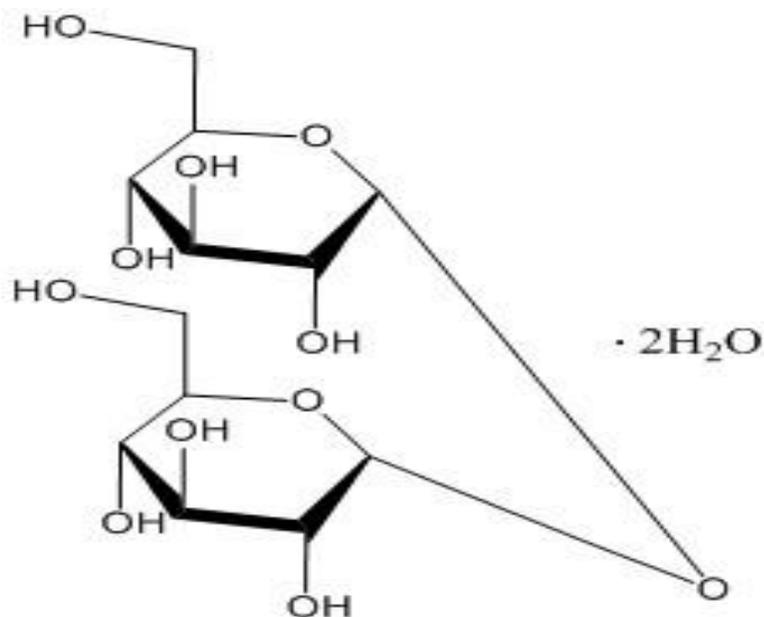


Figure 1.21: Chemical structure of Trehalose Dihydrate. The chemical structure of trehalose dihydrate was drawn by the author by using Chem Draw®.

1.10. Aims and Objectives

- I. To investigate the effect of buffer conditions (Type and concentration of buffers), pH, and excipients on the conformational stability, by using high sensitivity differential scanning calorimetry, and biological activity of model

proteins (lysozyme and trypsin) before and after storage for the accelerated period under the Quality by Design (QbD) approach.

- II. To develop and validate precise, accurate, and robust analytical methods (size exclusion chromatography and hydrophilic interaction liquid chromatography) as stability indicating and quality control (QC) assays for lysozyme and trypsin liquid formulations, in addition to the accompanying excipients.
- III. To adopt the Quality by Design concept to developing a strategic approach to preparing oral polymeric nanocapsules containing stable and active macromolecules with reduced processing cost and development time.
- IV. To prepare polymeric nanocapsule formulations, intended for oral delivery, containing model proteins (lysozyme and trypsin) by applying S/O/W and W1/O/W2 methods combined with the design of experiments, in addition to characterising the formulations to pursue the desired quality attributes.
- V. To evaluate the effect of formulation conditions, such as buffers, excipients, pH, temperature, and relative humidity, on the biological activity and physical stability of trypsin and lysozyme in liquid formulations by applying three different approaches: conservative, intermediate, and aggressive.

The thesis is constructed as follows:

Chapter One: Introduction.

Chapter Two: Materials and Methods.

Chapter Three: Quality by Design (QbD) based preparation of liquid lysozyme and trypsin formulations.

Chapter Four: Analytical methods development and validation.

Chapter Five: Development of a strategic approach for preparation of oral polymeric nanocapsules containing biomolecules.

Chapter Six: Preparation and characterisation of polymeric nanocapsules containing lysozyme and trypsin: intended for oral route delivery.

Chapter Seven: Liquid formulations containing lysozyme and trypsin stability study.

Chapter Eight: General Conclusion.

Chapter Nine: Bibliography.

Chapter Two: Materials and Methods

2. Materials and Methods

The objective of this chapter is to provide briefs about the chemicals, reagents, and methodologies used in this study. All the materials details are presented as provided by the suppliers. Methods and instrumentations are presented in a general approach in this chapter.

2.1. Materials

2.1.1. Proteins used in this project

Lysozyme (Mucopolysaccharide N-acetylmuramyl hydrolase, Muramidase, lyophilized powder, $\geq 40,000$ units/mg protein) obtained from chicken egg white, was provided by Sigma-Aldrich.

Trypsin (Serine protease, lyophilized powder, 13,000-20,000 BAEE units/mg protein) obtained from porcine pancreas, was supplied by Sigma-Aldrich.

Deoxy Ribonuclease I (DNase I lyophilized powder, ≥ 400 Kunitz units/mg protein) obtained from bovine pancreas, was provided by Sigma-Aldrich.

Lysozyme and trypsin as model proteins were used in the entire project. DNase I as a therapeutic protein was incorporated into polymeric nanocapsules as an application of the developed strategy on a therapeutic protein.

2.1.2. Other Materials and Reagents

Nano pure water ($>\Omega$ 18, Milli-Q) was used all the time throughout this project. All chemical and reagents used along with their suppliers are presented in Table 2.1.

Table 2.1: A list of the chemicals utilised in this project along with their suppliers.

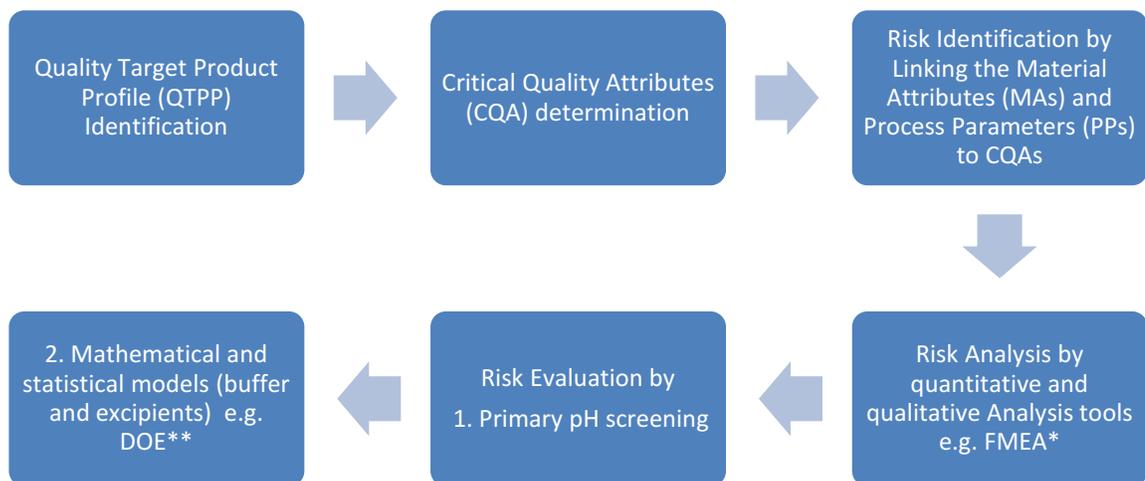
(+)-Sodium L-ascorbate	Sigma Aldrich
Acetonitrile	Fisher Scientific
Ammonium acetate	Sigma Aldrich
Deoxy ribonucleic Acid (DNA)	Sigma Aldrich
Disodium hydrogen orthophosphate	Fisher Scientific
Ethyl Acetate	Sigma Aldrich
Hydranal Dry methanol	Fisher Scientific
Hydrochloric acid (HCl)	Fisher Scientific
Micrococcus Lysodeikticus	Sigma Aldrich
N α -Benzoyl-L-arginine ethyl ester hydrochloride (BAEE)	Sigma Aldrich
Pepsin	Sigma Aldrich
Pluronic F-127®	Sigma Aldrich

Poly (DL-Lactide -co- Caprolactone) (40:60)	Sigma Aldrich
Poly (DL-Lactide -co- Caprolactone) (86:14)	Sigma Aldrich
Polyvinyl alcohol PVA	Sigma Aldrich
Potassium dihydrogen orthophosphate	Sigma Aldrich
Reagent	Supplier
Sodium acetate	Sigma Aldrich
Sodium citrate	Fisher Scientific
Sodium hydroxide	Sigma Aldrich
Sodium phosphate dihydrate	Fisher Scientific
Sodium silicotungstate	Fisher Scientific
Span ⁶⁰	Sigma Aldrich
Trehalose dihydrate	VWR
Trifluoroacetic acid (TFA)	Sigma Aldrich

2.2. Quality by Design based preparation of liquid lysozyme and trypsin formulations (Chapter Three Methodologies)

2.2.1. Quality by Design implementation

In this study, Quality by Design (QbD) was implemented by determining the Quality Target Product Profiles (QTPPs) and Critical Quality Attributes (CQAs) accordingly. Thereafter, the risk assessment has also been carried out. All the QbD approach process was performed according to ICH guidelines Q8, Q9, and Q10, [115] (Figure 2.1).



*FMEA: Failure Mode and Effects Analysis.

**DOE: Design of Experiment.

Figure 2.1: A schematic diagram comprises the Quality by Design (QbD) approach as stated by ICH Q8, Q9, and Q10.

2.2.1.1. Quality Target Product Profiles (QTPPs) and Critical Quality Attributes (CQAs) identification

QTPPs were identified as the first step of the QbD process implementation, based on the relevant literature and guidelines such as; ICH Q8 guideline, [116]. The CQAs were determined as the next step after QTPPs determination, based on QTPPs and prior knowledge.

2.2.1.2. Risk assessment

QbD approach has been implanted to scan the influencing factors and to assess the potential risks on the formulations. QbD was applied by risk assessment which is concluded by risk identification when the potential risk factors were identified, and listed in fishbone diagram. Thereafter, the risk was analysed, according to ICH Q9 guidelines, by applying Failure Mode Effect Analysis (FMEA). Hence, Risk Priority Number (RPN) for each factor was determined. Critical and rigorous risk analysis procedures were performed based on the relevant literature and the preliminary study. And as a final step in risk assessment, Potential hazards have been evaluated by; first, pH screening, and then by building mathematical design of experiments DOEs, in order to screen the buffer conditions effect and excipients, and consequently, optimise these factors [75]. For more details about the risk assessment accomplishment, see 2.4.1.3

2.2.2. Preliminary screening of the effect of pH changing on the conformational stability and biological activity of lysozyme and trypsin

Based on factors identification and the potential risk analysis, pH was identified as a high potential risk factor, and since the pH scale is limited, it can be screened and controlled before risk evaluation and design of the experiment. pH screening has been performed by preparing of 4 mg/ml of trypsin and lysozyme solutions at pH values 3-10, to tighten and select the optimum pH ranges. Biological activity and thermal integrity of protein formulations at different pH were investigated.

2.2.2.1. Preliminary screening of the effect of pH changing on the conformational stability of lysozyme and trypsin by using High Sensitivity Differential Scanning Calorimetry (HSDSC)

Proteins integrity was obtained by measuring T_m by using High Sensitivity Differential Scanning Calorimetry (synonym VP-DSC) (Microcal Inc., MA, USA) scanning microcalorimeter. Therefore, all formulations were scanned relative to the reference (contains the entire sample except the protein) in triplicate. The samples and corresponding references were freshly prepared, just before the runs, and were degassed for 5 minutes before the injection, by a Thermo Vacuum Pump (Microcal Inc., MA, USA). Both sample and reference were injected into μ DSC cells. All scanning was run under high pressure to prevent the boiling of samples during heating over a

temperature range from 30 C° to 90C°, at heating rate 90C°/hr. A baseline run was scanned as a corresponding reference versus itself and subtracted from transition scans before analysis of protein denaturation curve [68]. For data analysis purposes and curves fitting, Origin DSC software was used; then T_m for each sample was determined.

In addition to conformational stability investigations, lysozyme and trypsin biological activities were measured by applying the enzymatic potency assays established in the literature, as explained in details in the following sections.

2.2.2.2. Measurement of the biological activity of lysozyme by enzymatic assay

The biological activity of lysozyme can be obtained by applying the previously established enzymatic potency assay [117]. Lysozyme enzymatic assay is accomplished by measuring the ability of lysozyme to lysis the bacterial cell wall by breaking the b-1, 4-glycosidic linkage between N- acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM). The biological activity test for lysozyme formulations was conducted as the following: preparing a 0.01% (w/v) *Micrococcus Lysodeikticus*, lyophilized cells suspension in Potassium phosphate buffer (66 mM and pH 6.24). The A₄₅₀ of this suspension must be between 0.6–0.7 versus a Buffer blank. When necessary, the absorbance was adjusted using the appropriate amount of buffer or *Micrococcus Lysodeikticus* cells. Then enzyme solutions (lysozyme), immediately before use, were prepared to contain 400 units/ml of lysozyme (as mentioned above each 1 mg contains 40,000 units) in cold (2–8 °C) buffer, which equals 0.01 mg, that means the concentration was 0.001% w/v. To start the biological reaction, 0.1 ml of the

enzyme in the same buffer at different pH was added to 2.5ml of the bacterial suspension, and the same steps carried out by adding 0.1 ml of buffer without enzyme as a blank. Because pH values used were different compared to the documented pH (6.24) for running the biological activity, blank effects were taken into consideration, for example at pH 3 two reactions have been performed; the first reaction was (the buffer (pH 3) was added to the bacterial suspension), and A_{450} has been recorded as A_{450} of blank and subtracted from A_{450} for lysozyme at pH 3, the same steps have been repeated for all pH levels. The systems were mixed by inversion, and the decrease in A_{450} was recorded for 5 minutes. The unit activity of lysozyme is defined as the amount of the protein that reduces the absorption rate of the system (protein and bacteria) at A_{450} nm by 0.001 min^{-1} at 25 C° . The equipment used was M501 Single beam Scanning UV/Visible spectrophotometer Camspec (Biochrom, UK) and lysozyme activity determined using the following equation and:

$$\text{Units/ml enzyme} = (\Delta A_{450}/\text{min Test} - \Delta A_{450}/\text{min Blank}) (\text{df}) / (0.001) (0.1) \dots\dots\dots \text{Equation 1}$$

df = dilution factor.

0.001 = ΔA_{450} as per the Unit Definition.

0.1 = Volume (in millilitres) of Enzyme Solution.

The biological activity of enzyme formulations was expressed as a percentage relative to lysozyme at pH 6.24. The biological activity of pH 6.24 lysozyme was 100%.

2.2.2.3. Measurement of the biological activity of trypsin by enzymatic assay

The biological activity of trypsin can be determined by measuring the rate of ester link cleavage in *N*-benzoyl-L-arginine ethyl ester (BAEE), as described by [118, 119] . Trypsin samples at pH 2, 3, 4, 6, 8, and 10, were assayed for activity at 25 C°, the temperature was controlled in a water bath (unstirred water bath, Clifton, UK). The enzymatic assay of trypsin formulations was conducted by preparing (0.25mM *N*-benzoyl-L-arginine ethyl ester (BAEE) solution in 67 mM Sodium phosphate buffer at pH 7.6). Enzyme solutions, immediately before use, has been prepared containing 500 units/ml of trypsin (as mentioned above each 1 mg trypsin contains up to 20,000 units, which means 500 units equal 0.025 mg, consequently, the concentration was 0.0025% w/v) in cold (2–8 °C) diluted and concentrated HCl for pH 2 and 3, respectively. To start the enzymatic reaction, 0.2 ml of blank solution has been added to 3 ml of substrate solution as a first measurement to measure the effect of blank on the substrate to be subtracted from the last result, after that three consecutive tests were performed by, adding 0.1 ml blank and 0.1 ml enzyme sample, 0.05 ml blank and 0.15 ml enzyme sample, and 0.2 ml sample to three different substrate solutions, as first, second, and third test, respectively. The blank has been carried out to substrate the effect of pH out of the effect of the enzyme itself. Each system has been mixed by inversion, and the increase in A253 has been recorded for 5 minutes. The unit activity of trypsin is defined as the amount of the protein that increases the absorption rate of the system (protein and substrate) at A253 nm by 0.001/ min at 25C° in 3.2 ml.

The equipment used was M501 Single Beam Scanning UV/Visible spectrophotometer Camspec (Biochrom, UK). Trypsin activity determined using the following equation:

Units/ml= ($\Delta A_{253nm}/\text{min Test} - \Delta A_{253nm}/\text{min Blank}$) (df)/ (0.001) (VE) Equation 2

df = Dilution Factor

0.001= The change in A_{253nm}/minute per unit of Trypsin at pH 7.6 at 25°C in a 3.2 ml reaction

mix.

V_E = Volume (in millilitres) enzyme used in step.

The biological activity trypsin was expressed as a percentage relative to trypsin at pH

3. The biological activity of pH 3lysozyme was 100%.

2.2.3. Screening of the effect of buffer conditions on the conformational stability of lysozyme and trypsin

Buffers are usually used to control pH and salt content; hence controlling charge repulsion, and accordingly, optimising protein stability and integrity [120]. This makes the selection of buffer type a crucial decision in formulations development. DOE as a mathematical tool was applied in this study as a part of risk evaluation, in order to examine the initial formulation conditions by using the proper analytical tools. Acetate, citrate, and phosphate buffers are commonly used in parenteral formulations [121], [122], and they cover a wide range of pH values 3-10, [123]. Based on that and on the results obtained from pH screening, an initial buffer screening was performed using VP-DSC, as explained above by applying DOE at pH (4.0, 4.5, and 5.0) and (3) for both lysozyme and trypsin, respectively, and at buffer concentrations 10, 50, and 100 mM. Full factorial experimental design with every factor varied at three levels in interaction mode was created, to uncover the relevant factors and their appropriate changes regardless of linear or non-linear dependencies in addition to two factors interactions.

All these factors at different levels have been examined by VP-DSC, to screen their effect on thermal stability of both proteins, and then, optimise according to those results.

2.2.4. Screening of the effect of excipients on the conformational stability of lysozyme and trypsin

Based on the primary buffer screening and determination of optimum buffer conditions, a set of 3 different excipients (Pluronic F127, trehalose, and sodium ascorbate) out of three different chemical groups have been selected for formulations optimisation. One full factorial design was built for each protein to examine the impact of excipients on unfolding temperature, in addition to interactive effects, thus optimising the models, in order to obtain stable formulations. Three variables were included in every protein DOE, two quantitative (buffer concentration, and concentration of excipient) and one qualitative (the type of excipient). Two full factorial designs, T_m values, and statistical parameters are shown in

Table 2.2.

Table 2.2: A total of 12 Lysozyme and 12 trypsin formulations included in two different designs of experiments.

Protein	Phosphate concentration(mM)	Excipient	Excipient concentration
Lysozyme	10	Trehalose	10
	50	Trehalose	10
	10	Ascorbate	10
	50	Ascorbate	10
	10	Trehalose	50
	50	Trehalose	50
	10	Ascorbate	50
	50	Ascorbate	50
	10	Trehalose	100
	50	Trehalose	100
	10	Ascorbate	100
	50	Ascorbate	100
Trypsin	65	Pluronic	0.02
	100	Pluronic	0.02
	65	Trehalose	10
	100	Trehalose	10
	65	Pluronic	0.1
	100	Pluronic	0.1
	65	Trehalose	50
	100	Trehalose	50
	65	Pluronic	0.2
	100	Pluronic	0.2
	65	Trehalose	100
	100	Trehalose	100

2.2.5. Preparation and characterisation of liquid lysozyme and trypsin liquid formulations prepared after the factors optimisation

The optimum formulations for both proteins were selected, and the thermal stability of each formulation was performed in order to find the denaturation temperature T_m . T_m values for the optimised formulations were recorded and then compared to the predicted values. A t-test was carried out in order to determine the significances in T_m difference between the observed and predicted values. Further to proteins integrity determination by DSC, the optimised formulations biological activity was measured by applying the enzymatic assay methodologies described in [117] and [119]. Biological activity was determined for freshly prepared lysozyme and trypsin samples, and after 6 month's storage at 5 °C and 25 °C.

2.3. Analytical methods validation (Chapter Four Methodologies)

2.3.1. Development and validation of Proteins stability indicating assay by Size Exclusion Chromatography (SEC)

Proteins physical stability detection is a big challenge as protein degradation/aggregation behaviours are unpredictable. Size exclusion chromatography is considered a suitable and accurate choice to study protein physical stability if a well-developed and validated method is used.

In this study, the analysis was carried out on an integrated Agilent 1100 HPLC with an infinity UV-diode array detector (DAD) at 214 nm (Agilent technologies, Delaware, USA), using a size exclusion column (Agilent SEC-5,100A, 7.8x150mm). The column temperature was internally controlled at 25 °C. Isocratic separation system was used with a mobile phase constituent of 150 mM Sodium phosphate buffer pH 7 at a flow rate 1 ml/minute for a total chromatographic run time 10 minutes. Peak areas and retention times were obtained by utilising Agilent Chemstation software.

Whatman filter 0.2 um nylon membrane vials (General Electric, USA) containing proteins sample were injected (1 µl) by the autosampler. The method was validated for specificity, linearity, accuracy, precision, and robustness according to ICH and EMA guidelines [124, 125].

2.3.1.1. Specificity

Specificity of the bioanalytical procedure is the ability to elute the protein in a separate peak in the presence of all potential sample components such as the protein, inactive ingredients in the formulated products, degradation and aggregation products. In this study, the specificity was examined by its ability to separate the trypsin and lysozyme from their related degradants/ aggregates and other expected components like excipients. Specificity was demonstrated by analysing the samples containing trehalose, sodium ascorbate, and/or Pluronic F-127 mixed with the protein of interest. Both proteins were exposed to stress conditions to obtain their degradation products. The degradation products are usually obtained by applying stress conditions sufficient to degrade the analyte, e.g. heat, light, acid, or alkaline media. Heating is a suitable option to degrade the protein; however, the proteins may refold into their native state

after cooling. Therefore, the degradation products were generated by heating the samples in alkaline media, while the aggregation was promoted by forming concentrated proteins solutions (50mg/ml) in urea and storing it at room temperature for one week to enhance the aggregation process.

2.3.1.2. Linear range

Linearity is the proportional relationship between the response of the instruments and analyte concentration within a certain range, this relation between the response and concentration is called calibration curve. The standard calibration curve was prepared at different concentrations from 0.05 to 8 mg/mL (to 200%). Three replicate injections of each concentration were analysed for this study. The linear regression and correlation coefficient were calculated from the graph between peak area and concentration.

2.3.1.3. Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

The lower limit of detection LLOD is the lowest concentration of an analyte in a sample which could be detected qualitatively while for the lowest analyte concentration which could be detected and quantified is called the lower limit of quantification LLOQ. LLOQ and LLOD can be determined by several approaches according to (ICH Q2 R1) [125], based on visual evaluation which suitable for non-instrumental methods, signal-to-noise (a good rule of thumbs) the most conventional one, or based on standard deviation of the response and slope. ICH guidelines define the determination of LLOQ and LLOD based on Signal-to-Noise approach, the concentration that has a signal

response height equals to at least 10 or 2 folds of baseline noise, respectively. The limit of detection and limit of quantification were determined based on Signal-to-Noise method.

2.3.1.4. Accuracy

The term accuracy is defined as the closeness of concentration value of a quality control (QC) to a sample with known amounts of analyte (reference sample). The accuracy of developed method was determined for within-run accuracy, and between-run accuracy by analysing quality control samples (QC), containing the standard protein in the range of LLOQ, within four times of LLOQ (low), around 50% of calibration curve range (medium), and around 75% of the upper calibration curve range (high), for six samples per level on three different days. Accuracy was calculated by determining the obtained values of their averages, in addition to each single value. The accurate biological samples should show recovery value within 15% of nominal value except for the LLOQ, when up to 20% of nominal value is accepted.

2.3.1.5. Precision

Precision term describes how repeated measurements of the analyte under unchanged conditions are close to each other. The precision normally covers repeatability, intermediate precision, and reproducibility according to ICH guidelines. Repeatability (within-run precision), and intermediate precision (between-run) were determined by calculating the coefficient of variation (CV) of analysed QC samples at four different

levels: LLOQ, low, medium, and high. QC samples were six samples per level except for medium level since six samples were prepared per day for different six days. The coefficient of variation was calculated according to the following formula: $CV = \text{Standard deviation (SD)} / \text{the average}$ and expressed in a percentage.

2.3.1.6. Robustness

The robustness of an analytical method is its ability to remain unaffected significantly by changing the analytical conditions within a small reasonable range e.g. buffer pH, flow rate, column temperature, or mobile phase composition. In the present study, mobile phase pH, mobile phase concentration, and flow rate, were changed within 5% interval and combined into the fractional design of experiment (DOE). The fractional design QC samples, peak areas and retention times are shown in Table 2.3.

Table 2.3: A total of 10 different analytical conditions for analysing 4 mg/ml lysozyme and trypsin formulations included in one fractional design of experiments.

Concentration (mM)	Mobile pH	Flow rate (ml/min)
142.5	6.65	0.95
142.5	7.0	1.0
142.5	7.35	1.05
150	6.65	1.0
150	7.0	1.05
150	7.35	0.95
157.5	6.65	1.05
157.5	7.0	0.95
157.5	7.35	1.0
150	7.0	1.0

2.3.2. Validation of Proteins analytical assay for characterisation of polymeric nanocapsules containing protein by Size Exclusion Chromatography (SEC)

In the current study, polymeric nanocapsule formulations containing lysozyme, trypsin, and deoxy ribonuclease I (DNase I) were developed. Hence, the characterisation of the prepared formulations requires quantification of the encapsulated proteins in order to

determine the attributes of the formulations such as; encapsulation efficiency and protein release. Therefore, the developed SEC method is adapted for the characterisation purpose. However, the aims of both studies are different, and the range of concentrations of the protein samples obtained from nanocapsules is lower than the range of concentrations of liquid formulations. Thus, the analytical method parameters should be changed to suit the characterisation purpose. The SEC assay for polymeric nanocapsules characterisation had the same previous analytical conditions, but not the injection volume. The injection volume in this method was raised up to 10 μ l which will consequently change the response of the instrument towards the sample, for example; when the same sample are analysed by the same method but with different injection volume, the obtained peak area will be different. The rationale behind increasing the injection volume is to decrease the lower limit of detection (LLOD) and lower limit of quantitation (LLOQ). As the increase of injection volume increase the sensitivity of the method towards the analytes.

2.3.2.1. Partial validation of Size Exclusion method to characterise polymeric nanocapsules containing lysozyme and trypsin

According to EMA 2011, there is no need for the full validation procedures for the already validated analytical method when small changes are applied to the method, depending on the made changes. Partial validation may be conducted by doing only one requirement such as accuracy or by performing nearly the full validation. In the current study, only two changes were made: the injection volume, the analyte concentration scale, and the expected sample components. Therefore, the specificity, linearity, LLOQ, and LLOD of the method were restudied.

The specificity test was carried out by analysing a sample of obtained from the dissolution media of lysozyme and trypsin nanocapsules. The samples are expected to contain all the potential component.

Moreover, the LLOD and LLOQ were determined by signal-to-noise ratio method. The linearity of the method was studied by preparing six of different concentration samples for each of lysozyme and trypsin. The range was from LLOQ up to 200 µg/ ml which is the concentration equals the 200% of the maximum expected concentration.

2.3.2.2. Validation of Size Exclusion method to characterise polymeric nanocapsules containing Deoxy ribonuclease I (DNase I)

The previously developed and validated SEC methods were for lysozyme and trypsin. However, DNase I was also incorporated into polymeric nanocapsules, which requires an analytical method for the characterisation purposes. Therefore, lysozyme and trypsin SEC assay were adapted to characterise the DNase I nanocapsules. Full validation of the method was accomplished following the same validation procedure (specificity, accuracy, precision, linear range, LLOQ, LLOD, and robustness) mentioned in Section 2.3.1 taking into consideration that the injection volume was changed. Therefore, the linearity was at the same scale discussed in Section 2.3.2.1.

2.3.3. Development and validation of excipients stability indicating and Quality

Control assays

In order to confirm the quality into the pharmaceutical formulations, excipients should be analysed to ensure the safety of the formulations and control the harmful byproducts.

Developing an analytical method to separate structurally related compounds with similar physicochemical characteristics is not easy and needs controlling of the analytical parameters and wisely selection of the stationary phase.

In this study, the stabilities of three excipients, trehalose, sodium ascorbate, and Pluronic F 127, are under investigation. Therefore, the aim was to adapt an analytical method able to separate and quantify their concentrations in the formulations.

Initially, a gradient RP-HPLC method was selected to perform the analysis by reverse phase Jupiter 300 °A C18 (5 µm, 250 x 4.60 mm) (Phenomenex Incorporation, UK) with an infinity UV-diode array detector (DAD) at 214 nm and 1260 infinity Evaporative Light Scattering Detector (Agilent Incorporation, Delaware, USA) at 60 °C evaporative temperature and gas flow rate 2 SLM (standard litter per minutes) and PMT gain 1/40 Hz. The rationale behind using ELSD is that; trehalose as sugar does not have a chromophore which makes it unable to absorb UV radiation and therefore cannot be detected by UV detectors.

The gradient system of mobile phase A (0.1% TFA in 90:10 water: acetonitrile) and mobile phase B (0.1% TFA 90:10 acetonitrile: water) was running over 50 minutes as total chromatographic run time with a flow rate 1 ml/min and injection volume 20 μ l. The run started with 90% mobile phase A for 5 minutes and then the gradient started by mixing A and B to reach 90% mobile phase B at 30 minutes of the total run time and maintained at the same percentage for 10 minutes. Then the ratio between A and B started to increase again to reach 90% mobile phase A after 5 minutes. The system kept running at 90% A until the run time was finished. After failure to obtain acceptable detection and separation of the excipients, a gradient reverse-reverse phase HPLC method was selected to perform the analysis by Hydrophilic Interaction Liquid Chromatography HILIC with an infinity UV-DAD at 254 nm and connected to Evaporative Light Scattering Detector (Agilent Incorporation, Delaware, USA) at 60 °C evaporative temperature and gas flow rate 2 SLM (standard liter per minutes) and PMT gain 1/40 Hz. LUNA 3 μ HILIC (silica) 200 Å (4.6 X 150 mm, 3 microns) column was utilised, and the internal temperature was controlled at 30 °C, (Phenomenex Incorporation, UK). Ammonium acetate 100mM pH 5.8 was used as the hydrophilic buffer, while acetonitrile was used as the organic phase with a flow rate 1 ml/minute for a total chromatographic run time 28 minutes. The run started with 100% mobile phase A (92:8 acetonitrile: buffer) for two minute and then the mixing between mobile phase A and mobile phase B (50:50 acetonitrile: buffer) stated and reached 70:30 mobile phase A: mobile phase B at 20 minutes, and kept constantly running at this ratio for three minutes, then the ratio started to decrease to reach 100% mobile phase A at 25

minutes from the total run time, the system kept operating at this ratio before the next run for three minutes, (Table 2.4 and Figure 2.2).

Table 2.4: The gradient run and the time of mixing mobile phase A and mobile phase B. Mobile phase A composition is 92:8 acetonitrile: Ammonium acetate buffer 100 mM and pH 5.8), while mobile phase B composition is 50:50 acetonitrile: Ammonium acetate buffer 100 mM and pH 5.8)

Time (minute)	Mobile phase A: Mobile phase B
0	100:0
2	100:0
20	70:30
23	70:30
25	100:0
28	100:0

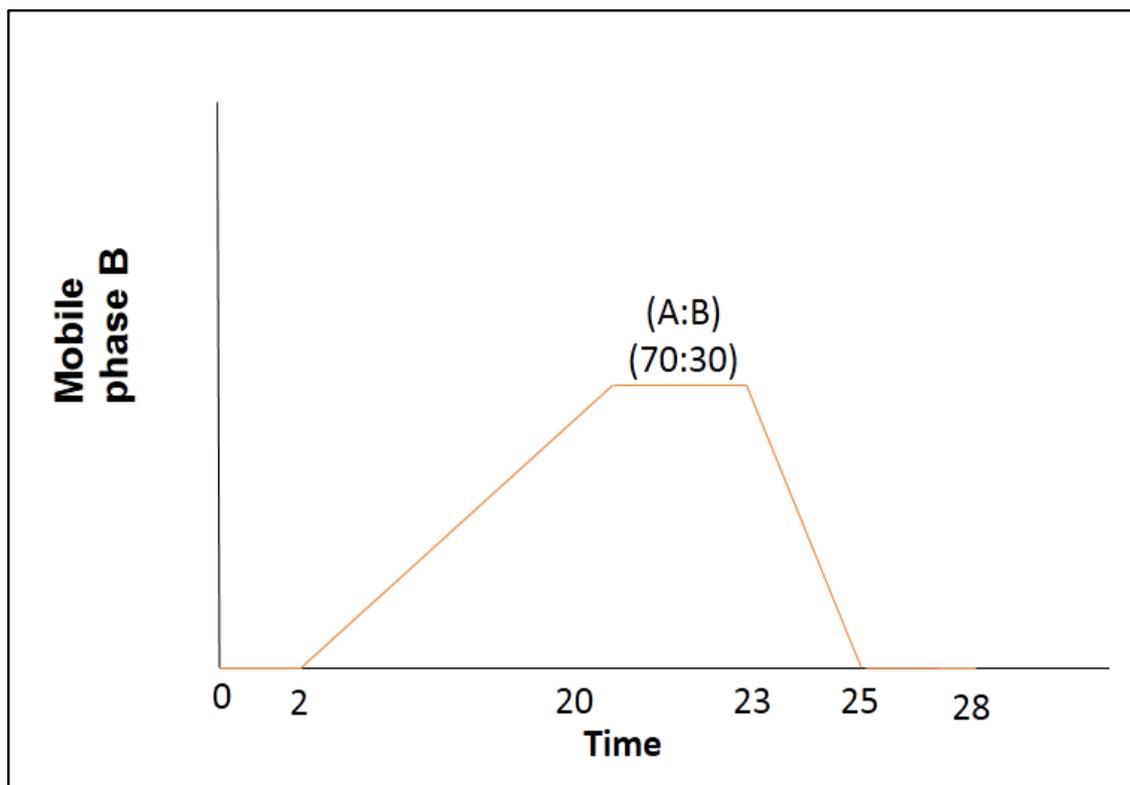


Figure 2.2: Illustrating diagram for the gradient run and the time of mixing mobile phase A and mobile phase B. Mobile phase A composition is 92:8 acetonitrile: Ammonium acetate buffer 100 mM and pH 5.8), while mobile phase B composition is 50:50 acetonitrile: Ammonium acetate buffer 100 mM and pH 5.8).

The analytical parameters were selected, monitored, controlled and adjusted by try and error method and based on the previous knowledge about the chromatographic separation, as will be explained later.

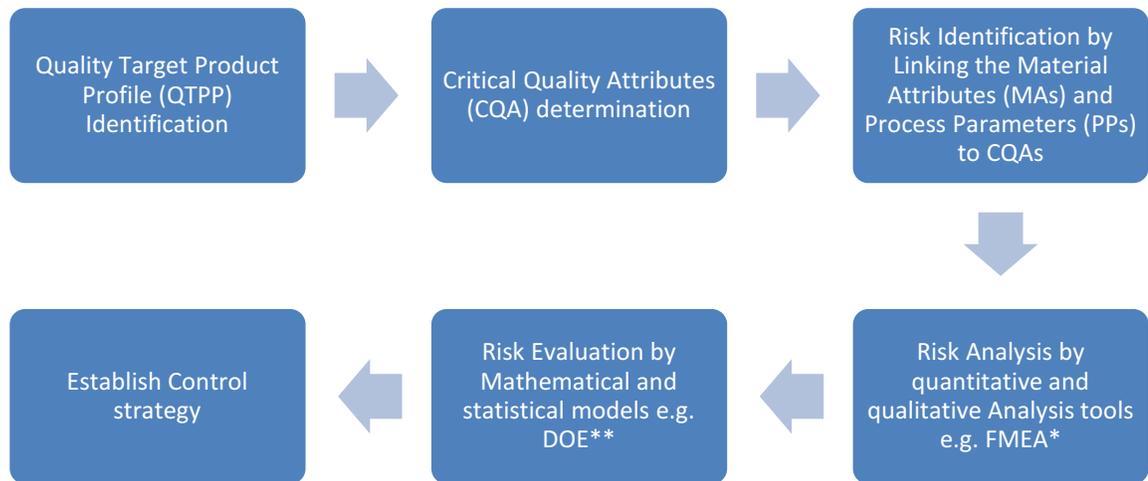
Whatman filter 0.2 um nylon membrane vials (General Electric, USA) containing the formulation sample were injected (10 μ l for trehalose and ascorbate, and 20 μ l for Pluronic) by the autosampler. Peak areas and retention times were obtained by utilising Agilent Chemstation software. The method was validated for specificity, linearity, accuracy, precision, and robustness.

2.4. Development of a strategic approach to preparation of oral polymeric nanocapsules containing biomolecules (Chapter Five Methodologies)

2.4.1. Quality by Design (QbD) implementation

Quality by Design (QbD) is a scientific, regulatory approach which designs the product and the process properties to build the quality of the product from the early stages of the product development [126].

QbD implementation has been well detailed by the regulatory agencies e.g. FDA and ICH in their released guidelines e.g. ICH Q8. In this study, the QbD approach was implemented in the early stage of the preparation of the polymeric nanocapsules containing proteins, according to the ICH guidelines. The initial steps of identification of Quality Target Product Profiles (QTPPs), Critical Quality Attributes (CQAs), Critical Process Parameters (CPPs), and Critical Material Attributes (CMAs) were performed according to ICH Q8, whilst, the risk management and risk assessment processes were performed as detailed in ICH Q9 guideline[75], Figure 2.3.



*FMEA: Failure Mode and Effects Analysis.

**DOE: Design of Experiment.

Figure 2.3: A schematic diagram comprises the QbD approach as stated by ICH Q8, Q9, and Q10.

2.4.1.1. Quality Target Product Profiles (QTTPs) and Critical Quality Attributes (CQAs) identification

The QTPP is an abbreviation for Quality Target Product Profile, which forms the first step in the Quality by Design implementing during pharmaceutical product development. The QTTPs are the professional patient relevant characteristics of the product e.g. the route of administration and the dosage strength [127]. In order to achieve the QTPP, different critical quality attributes (CQAs) were assigned. Selection of these CQAs depends on the QTPP and/or prior knowledge, as CQAs are the controlled attributes which can influence the quality of the final product [128]. Taking

the QTPPs into consideration, the CQAs in this study were determined based on the prior knowledge and relevant literature.

2.4.1.2. Identification of Critical Process Parameters (CPPs) and Critical Material Attributes (CMAs)

Preparation methods and the conditions, under which the pharmaceutical products are also prepared have influences on the overall quality and affect the quality attributes.

The process parameters, which significantly influence the quality, are considered critical process parameters (CPPs). Those parameters are usually selected and identified based on the prior knowledge and relevant literature.

The materials which they affect the overall quality are called critical material attributes (CMAs).

In this study, the double emulsion method was selected to prepare the biodegradable polymeric nanocapsules (PNCs) based on the data obtained from relevant literature [129].

2.4.1.3. Risk Assessment

The QbD is a science-based process and should follow the released guidelines by agencies, to be well implemented. Risk assessment or so-called Quality Risk Management is a crucial step in the QbD implementation to identify and control the hazard which may affect the quality of the product [130].

The risk assessment in the current study commenced with predefined risk questions as recommended by ICH Q9. The three risk questions were:

- 1- What might go wrong?
- 2- What is the probability it will go wrong?
- 3- What are the consequences?

The risk assessment was performed by the following three steps: risk identification, risk analysis, and risk evaluation, as explained below. The output of the risk assessment was qualitative and quantitative.

2.4.1.3.1. Risk identification

Identification of the potential hazards is “a systematic use of information to identify hazards referring to the risk question or problem description” (ICH Q9). This step is being performed as a crucial stage in risk assessment process to answer “what might go wrong?” question, thus, addressing the potential concerns.

In risk identification, the historical data and the theoretical analysis are used to determine the possible hazards by applying different basic risk management facilitation methodologies e.g. flowcharts, check sheets, process mapping or cause and effect diagrams.

In the current study, risk identification step is the first step in risk assessment process and forms the precursor for the following procedures in the process. Risk factors were listed and identified in a fishbone, in order to answer the risk question and avoid any

expected harmful consequences. Therefore, the “cause and effect diagram” was used and represented specifically by the fishbone diagram or so-called Ishikawa diagram.

2.4.1.3.2. Risk analysis

After the potential hazards have been identified, the risk analysis step has been performed to predict the risk related with each hazard. The risk analysis step is the linking of the likelihood of occurrence, detectability and severity of accompanying harms or hazards by a quantitative or qualitative method. The risk analysis is being addressed to answer the “What is the likelihood (probability) it will go wrong?” question.

As by ICH Q9, the initial risk assessment and analysis could be performed by different systemic tools and procedures i.e. Failure Mode Effects Analysis (FMEA); Failure Mode, Effects and Criticality Analysis (FMECA). Moreover, Fault Tree Analysis (FTA); Hazard Analysis and Critical Control Points (HACCP); Hazard Operability Analysis (HAZOP); Preliminary Hazard Analysis (PHA) were also used as tools for risk assessment.

FMEA is being applied to reduce and eliminate the potential failures. FMEA concludes the critical failure modes, the precursor causes, and the probability of occurrence of the failure, by simplifying the analysis process.

In the current study, Failure Mode Effect Analysis (FMEA) has been adopted as a tool to evaluate the effect of the process parameters, material attributes and the selected

elements which were listed in the fishbone diagram on the desired CQAs and the overall product quality.

As stated by McDermott et al. 1996 [131], and as recommended by IEC 60812 [132], the estimated risk can be expressed by a qualitative (high, medium, or low) or a semi-quantitative way. The semi-quantitative analysis has been performed by determination of Risk Priority Numbers (RPN). RPN is a number obtained by calculating the product of multiplying the numbers assigned or the severity of the element, its occurrence probability, and the detection probability, as the following equation:

$$\text{RPN} = S * P * D \dots\dots\dots \text{Equation 3}$$

S= severity.

P= probability.

D=detectability.

The severity is defined as how much the failure consequences on the final product quality is serious. Moreover, the probability is the frequency of the failure or how the failure is likelihood to happen. While the detection is how obvious the failure is to be detected e.g. if the failure is obvious and can be assigned without further investigations or any advance detection methodologies, the detectability would be given a low value, and it is considered a safer factor.

Each investigated factor was given a certain RPN value, and all the RPNs were ranked. The high RPN value factors were selected for the risk correction to monitor the hazard

and reduce the associated risk. Some factors were screened in the lab only to reduce their effects on the quality of the products e.g. the used organic solvent. While other factors were avoided and were not included in the design as their presence in the formulations is a hazard e.g. heating or high temperature. The third group of the high resultant RPN was treated by monitoring and correction during the formulation process e.g. reducing the analytical errors by analytical method validations. The last group of elements is when the factors have several levels, and in order to monitor the levels; the factors were combined in a statistical design of experiment to evaluate the factors effect and optimise their values. The last group provided the basis for the risk evaluation as is going to be explained in the next section (2.4.1.3.3).

2.4.1.3.3. Risk evaluation

The risk evaluation process is an employment of qualitative and quantitative methods to match the previously identified and analysed risk with the given risk criteria which may affect the product quality attributes. The risk evaluation is considered a crucial stage in the risk management process due to its role in answering the three assigned quality questions, mentioned in 2.4.1.3, by determination the significance of the risk.

The obtained factors from the risk analysis process have been classified into several categories, and the factors which they have influences on the risk and quality within a range of levels have been taken and studied further in a risk evaluation process.

The analysed risk is being estimated by the assessment of the relationship between the factors and the quality of the final product by applying one or more of the suggested methodologies by the ICH. The risk analysis methodologies include supporting

statistical tools i.e. Control Charts, Design of Experiments (DOE); Histograms; Pareto Charts; and Process Capability Analysis. The previous methodologies were assigned by the ICH Q9 guideline due to their abilities to support and simplify the risk assessment, by identifying the significant factors through precise data analysis, hence, obtaining reliable decisions.

The design of experiment (DOE) as a popular organised mathematical and statistical method was adapted in this study to evaluating the risk. The design of experiment was selected to connect the overall quantitative data in the experiment by a lucid way. The DOE approach was discussed and accredited as a reliable tool for the manufacturing in different fields e.g. food manufacturing, pharmaceuticals, and automotive industries [133]. As stated by [133], the benefits of applying the DOE are; it can provide more useful and precise information in fewer experiments, its ability to evaluate the results against the variability, and facilitating the decision making by generating contour plots.

Based on what was obtained from the risk analysis, both lysozyme and trypsin share the same risk factors. Thus, the same variables were selected to build the DOEs. Three factors have been designated as factors need further investigations and optimisation by DOE models, including the ratio between the blocks of the used copolymers, the physical state of the encapsulated proteins (solid, or liquid), and encapsulation of trehalose in the polymeric nanocapsules (PNCs) cores. Therefore, full experimental designs were generated for each protein including the assigned factors at different levels by using MODDE 10.1 (Umetrics AB, Umea, Sweden), Table 2.5.

Table 2.5: The Design of experiments combining different factors at various levels to prepare eight different samples for each protein (lysozyme and trypsin).

Formulation	ϵ - Caprolactone block %*	Core physical state	Trehalose (mM)
L1	14%	Liquid	0
L2	60%	Liquid	0
L3	14%	Solid	0
L4	60%	Solid	0
L5	14%	Liquid	10
L6	60%	Liquid	10
L7	14%	Solid	10
L8	60%	Solid	10
T1	14%	Liquid	0
T2	60%	Liquid	0
T3	14%	Solid	0
T4	60%	Solid	0
T5	14%	Liquid	10
T6	60%	Liquid	10
T7	14%	Solid	10
T8	60%	Solid	10

*The percentage of ϵ - Caprolactone block in the copolymer.

L indicates for lysozyme formulations.

T indicates for trypsin formulations.

As shown in Table 2.5, eight different possibilities to formulating the PNCs were suggested by the model for each protein. MODDE software generated the full experimental design based on (2^3) role, as three different factors were included at two different levels for each factor. In the experimental design above, the effect of the factors on the desired criteria (which they are CQAs) was assessed by the preparation of the all suggested formulations in triplicate and then characterise them in the light of the suggested qualities to screen the significance of the factors and to optimise them.

2.4.2. Polymeric Nanocapsules (PNCs) preparation

The double emulsion method is a method for the preparation of polymeric nanoparticles i.e. nanocapsules and nanospheres. The polymeric nanocapsules in the current study were prepared by the double emulsion method, as described earlier by [134, 135]. The double emulsion method was adapted based on the performed risk analysis, with some modifications to suit the necessary quality. This method has been employed to prepare the PNCs containing proteins to avoid the risk of formulations failure which may be caused by the other preparation methods. The risk of failure includes low encapsulation efficiency reported when the PNCs are containing hydrophilic drugs, e.g. protein, was prepared by other preparation methods [136]. The failure of the formulations to achieve the desired encapsulation efficiency will reduce the overall formulation quality and consequently, increase the cost and the bulk size of the dosage form, which means failure to achieve the assigned CQAs and QTPPs.

As stated by *Garti et al. 1997* and *Grigoriev et al. 2009*, [134, 135], in order to prepare a successful double emulsion, a hydrophobic surfactant should be added to the oily phase to avoid the separation of the emulsion phases. In the current study, span⁶⁰ was used as a surfactant based on the initial risk assessment, when the CMAs were identified. Polyvinyl alcohol was selected as a stabiliser for the external aqueous phase, while ethyl acetate was used as the organic solvent of choice with minimum effect on the protein's structure as was explained in the risk analysis process.

Protein-loaded nanocapsules were prepared in triplicate at 0.625% w/v nominal drug loading by a W1/O/W2 double emulsion solvent evaporation method or by S/O/W method as described previously in the literature, Figure 2.4.

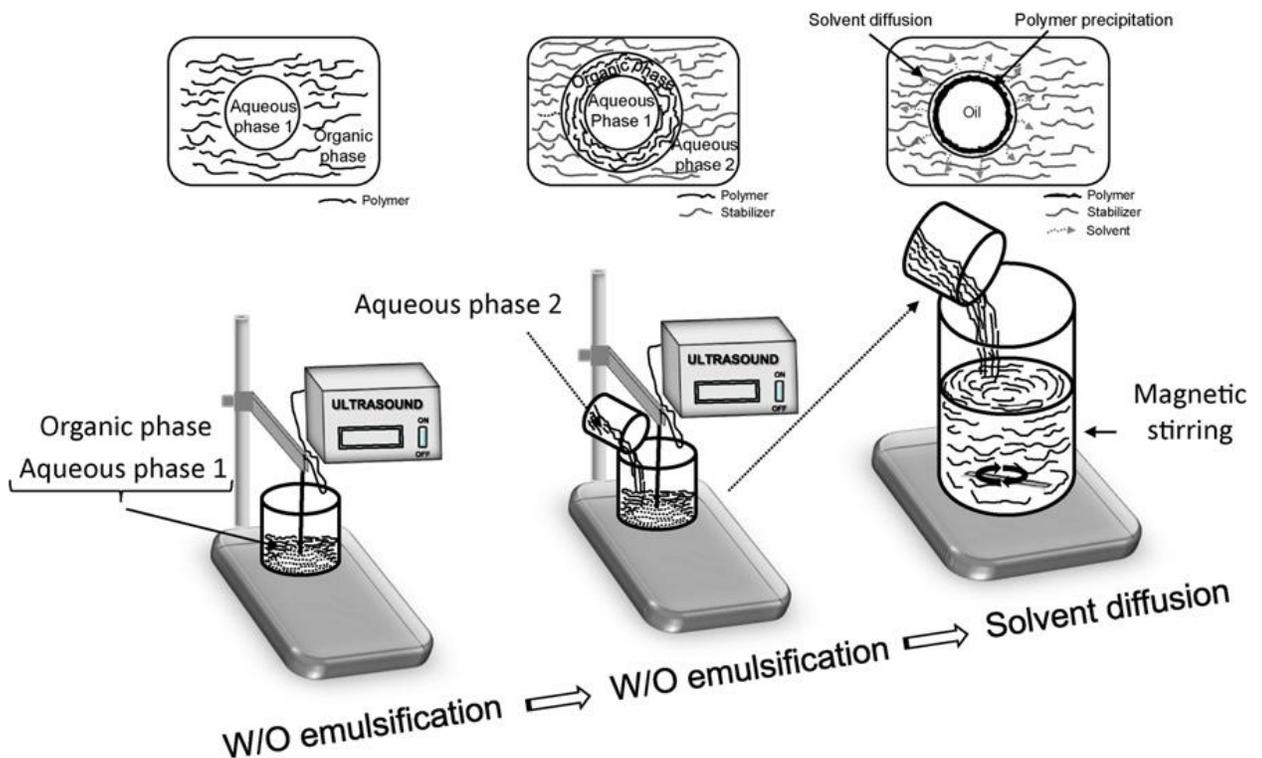


Figure 2.4: A diagram showing the double emulsion method procedure used to prepare PNCs. Adapted from [129].

2.4.2.1. Preparation of polymeric nanocapsules (PNCs) by W1/O/W2

Briefly, for the formulations prepared by W1/O/W2, 1.6 ml protein dissolved in Nano pure water ($>\Omega$ 18, Milli-Q) to form protein solution (the internal aqueous phase) was first poured into 15 ml of a 1.67% w/v copolymer dissolved in the organic solvent (ethyl acetate) and containing 6% span⁶⁰. Afterwards, the two phases were sonicated by using a probe sonicator for 10 seconds at 65 watts.

AS shown in Table 2.5, the formulations were prepared according to the design of the experiment. Therefore, their compositions were varied. Trehalose was presented in the core of some formulations at concentration 10 mM. In these relevant formulations, the trehalose was dissolved in the internal aqueous phase with the protein.

After the first W1/O emulsion had been obtained, it was added to the 50 ml external aqueous phase, which contains 3% w/v polyvinyl alcohol (PVA), to prepare the secondary aqueous emulsion via sonication for 15 seconds at 65 watts. After the sonication has taken place, a double emulsion of three phases was formed W1/O/W2.

2.4.2.2. Preparation of polymeric nanocapsules (PNCs) by S/O/W

For the S/O/W emulsions, the proteins with/without trehalose were directly suspended in the organic phase and sonicated to prepare the finely dispersed s/o suspension. Then, all following steps of PNCs preparation were as the mentioned earlier for the double emulsion method, Section 2.4.2.1.

The final emulsion (Prepared by S/O/W) and double emulsion (developed by W1/O/W2) systems were then magnetically stirred for fifteen hours, at room temperature (22 ± 2 °C), until all the organic solvent was completely evaporated. Afterwards, suspensions of the solid nanoparticles in aqueous media were obtained and centrifuged and washed for four times for 30 minutes at 15 000 rpm at a chilled temperature (almost 4 °C). The centrifuging has been performed to remove the residues of the organic solvent, polyvinyl alcohol, and the free protein from the external phase of the formulation. Moreover, as the last step in solid polymeric nanocapsule preparation, the PNC formulations suspension were frozen overnight at - 80 °C and then freeze-dried by VirTis Benchtop Freeze Dryer (Biopharma) under 25-36 m Torr and at a freezing temperature of - 105 °C for 48 hours, until the formulations have been fully lyophilised. Table 2.6 shows the composition of all phases of all PNC formulations.

Table 2.6: The raw materials and their concentrations used for the preparation of nanocapsules by the double emulsification method—aqueous and solid core.

Formulation #	Internal phase	Organic phase	External aqueous phase
F1	10 mg protein,	250 polymer ^a	50 ml Water
	1.6 ml Water	15 ml ethyl acetate 0.9 SPAN ⁶⁰	1.5 g PVA
F2	10 mg protein,	250 polymer ^b	50 ml Water
	1.6 ml Water	15 ml ethyl acetate 0.9 SPAN ⁶⁰	1.5 g PVA
F3	10mg protein	250 polymer ^a	50 ml Water
		15 ml ethyl acetate 0.9 SPAN ⁶⁰	1.5 g PVA
F4	10 mg protein	250 polymer ^b	50 ml Water
		15 ml ethyl acetate 0.9 SPAN ⁶⁰	1.5 g PVA
F5	10 mg protein, 6.05 mg trehalose	250 polymer ^a	50 ml Water
		15 ml ethyl acetate 0.9 SPAN ⁶⁰	1.5 g PVA
F6	10 mg protein, 6.05 mg trehalose, 1.6 ml Water	250 polymer ^a	50 ml Water
		15 ml ethyl acetate 0.9 SPAN ⁶⁰	1.5 g PVA
F7	10 mg protein, 6.05 mg trehalose	250 polymer ^b	50 ml Water
		15 ml ethyl acetate 0.9 SPAN ⁶⁰	1.5 g PVA
F8	10 mg protein, 6.05 mg trehalose, 1.6 ml Water	250 polymer ^b	50 ml Water
		15 ml ethyl acetate 0.9 SPAN ⁶⁰	1.5 g PVA

* Copolymer^a: 86:14 Poly (DL-Lactide -co- Caprolactone).

* Copolymer^b: 40:60 Poly (DL-Lactide -co- Caprolactone).

* F: formulation containing either lysozyme (L) or trypsin (T).

* PVA: Poly vinyl alcohol.

2.4.3. Characterisation methods

After the preparation of the formulations, the characterisation i.e. particle size, encapsulation efficiency, drug release, the biological activity of the encapsulate proteins, and the accelerated stability of the formulations, has taken place. All the characterisation methodologies were adapted from the literature with some modifications to suit the desired quality and to avoid any denaturation that may occur during the characterisation processes. Ethyl acetate was chosen to break the polymeric shell in order to collect the encapsulated proteins for characterisation. Moreover, the quantification of the proteins was performed by size exclusion chromatography. The significance of the factors on the product quality and the factors-response relationship was obtained by multi-linear regression analysis of the models via applying the following equation:

$$y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_{12}x_1x_2 + \dots + \varepsilon \dots\dots\dots \text{Equation 4}$$

The details of the characterisations will be discussed in details later on the current project, Section 2.5 its subsections.

2.4.4. Prediction and optimisation

The formulation factors were optimised to obtain set points combining the best formulation conditions which can attain the desired characteristics and predict these characteristics. The optimisation has been performed by the MODDE 10.1 software

taking into consideration all the factors at the same time and linking them to the obtained responses by performing the experiments in the lab.

After having the suggested set points, the two optimal formulations for each protein were prepared by the double emulsion method at the same preparation conditions and process parameters which have been applied earlier to prepare the PNCs, Section 2.4.2.

Then, all the prepared formulations were characterised against the encapsulation efficiency, drug release, and the biological activity. The obtained and predicted characteristics were compared and paired T-test has been carried out to record any significant difference between them. Hence, evaluate the ability of the design of experiments to predict the optimal conditions based on the provided results.

2.4.5. Validation and applying of the developed strategy

The strategy of developing polymeric nanocapsules containing macromolecule intended for oral delivery has emerged by preparation of PNCs containing lysozyme and trypsin. After the optimal PNC formulations had been prepared and characterised, a therapeutic protein (Deoxy Ribonuclease I) was selected to be encapsulated inside PNCs at the same optimal conditions to validate the developed strategy. Therefore, Deoxyribonuclease I (DNase I) enzyme, which is used as a drug of choice to treat cystic fibrosis [137], was selected to prepare PNCs at the optimal conditions. DNase I containing PNCs were developed by the double emulsion method at the same

preparation conditions and process attributes used for lysozyme and trypsin. The prepared PNCs was investigated by TEM for morphology as described in Section 2.5.4.

Moreover, their encapsulation efficiency, drug release, and the biological activity of DNase I were also studied. Encapsulation efficiency and drug release experiments were carried out by polymeric shell breaking down by using ethyl acetate and then quantified by SEC, as described for lysozyme and trypsin. However, the biological activity of the encapsulated DNase I was measured by collecting the DNase I after the shell breaking down and performing the DNase I enzymatic assay against DNA, as described by [138].

2.4.5.1. Biological activity of deoxyribonuclease

The biological activity of DNase I in this study was detected by applying the enzymatic assay procedures explained by the supplier [139], based on the method established by *Kunitz 1950* [138]. The rate of the cleaving of phosphodiester linkage of DNA is considered as a function of DNase I activity [140].

In this method solution of DNA was used at a concentration of 0.033% that was achieved by keeping the DNA solution on cold ice for 30 minutes until DNA was completely dissolved. DNA was further diluted for assay purpose with the diluting solution composed of 5 ml of 1 molar at pH, 2.5 ml of 100 mM MgSO₄, 6 ml of freshly prepared 0.033% w/v DNA. Further water was added to make up the volume of 50 ml, and the concentration of DNA achieved was 0.004% w/v.

However, standard DNase I solution was prepared by dissolving in 0.85% NaCl and further diluted up to 400-500 units per ml prior to measuring the activity.

In order to measure the activity of encapsulated DNase I the shell was disrupted by ethyl acetate previously described in Section 2.5.2. Then, a solution made of the encapsulated DNase I, after shell breaking, was prepared at a concentration equal to the standard solution concentration.

The biological activity was identified for the encapsulated DNase I and fresh DNase I. For assay the kinetic reaction was carried out and increase in absorbance was measured as a function of enzymatic activity. The final volume of reaction mixture was 3 ml with 2.5 ml of DNA and 0.5ml of the enzyme. The temperature of reaction mixture was maintained at 25 °C by using a static water bath. The kinetic reaction was carried out by measuring the changing in the absorbance at wavelength 260nm in the M501 single beam scanning UV/Visible spectrophotometer Camspec (Biochrom, UK).

The biological activity was determined by using the maximum linear rate. The Kunitz of DNase I per ml was calculated according to the following equation:

$$\frac{\text{units}}{\text{ml}} = \frac{\Delta A_{260\text{nm}}/\text{min Std} - \Delta A_{260\text{nm}}/\text{min Blank}}{0.001 \times 0.5} \times (3) \times (df) \dots \dots \text{Equation 5}$$

3 = Volume (millilitres) of the assay.

0.5 = volume of enzyme used in each test.

df = dilution factor.

0.001 = $\Delta A_{260\text{nm}}$ per the unit definition.

The percentage of the maintained activity of the encapsulated protein was measured according to the following equation:

$$\mathbf{Biological\ activity\ \% = \frac{Units/ml\ (Encapsulated\ DNase\ I)}{Units/ml\ (Fresh\ DNase\ I)} \dots\dots\dots \mathbf{Equation\ 6}}$$

2.5. Preparation and characterisation of polymeric nanocapsules containing lysozyme and trypsin: intended for oral route delivery (Chapter six Methodologies)

2.5.1. Polymeric Nanocapsules (PNCs) Preparation

The polymeric nanocapsules are a novel protein delivery system, where the proteins are being confined inside the polymeric shell. In the literature, several methods are applied to prepare the PNCs containing different therapeutic e.g. nanoprecipitation [141], emulsion diffusion [142], polymer coating [143], layer by layer [144], emulsion coacervation [145], and double emulsion [146].

In the current research, the polymeric nanocapsule formulations were prepared by the double emulsion solvent evaporation method via preparing W/O/W emulsion as described in Section 2.4.2. However, some formulations were developed by S/O/W. Selecting the double emulsion method was based on the previous knowledge, the relevant literature, and the performed pre-formulation risk assessment as detailed in Section 2.4.1.3.

2.5.2. Encapsulation efficiency of proteins

Polymeric nanocapsules encapsulation efficiency can be determined by calculating the actual protein amount loaded inside the polymeric shell. Encapsulation efficiencies of the different formulations were measured by applying the previously developed method [147], Figure 2.5.

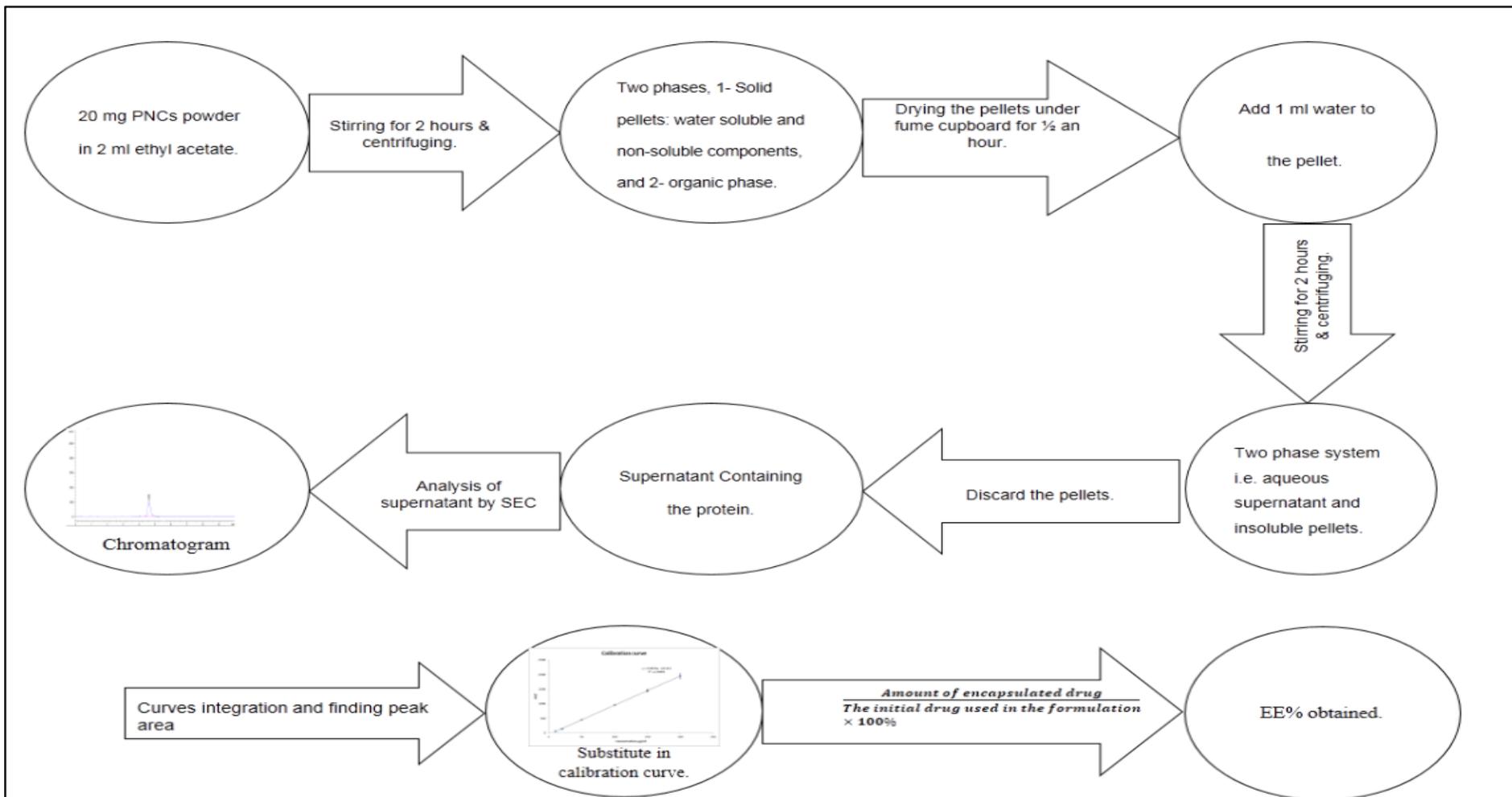


Figure 2.5: Encapsulation efficiency measurement step by step, represented in a schematic diagram.

As illustrated in the schematic diagram in Figure 2.5, the polymeric shell was broken down after a suspension of 1% nanocapsule in 2 ml ethyl acetate has been magnetically stirred under fume cupboard for 2 hours. Then, it was centrifuged for 10 minutes at 10000 rpm; the pellets were collected and left under the fume cupboard for half an hour to dry and then suspended in 1ml 18 MΩ water and stirred for 2 hours until all the protein is dissolved. The suspension was centrifuged using Mikro 220R centrifuge (Hettich centrifuges, Germany) at 10000 × g for 10 min and then the supernatant was analysed by using SEC, to determine the protein concentration in the sample, and then the entrapment efficiency was calculated according to the following formula [148]:

$$EE (w/w\%) = \frac{\text{Amount of encapsulated drug}}{\text{Amount of initial drug used in the formulation}} \times 100\% \dots\dots\dots \text{Equation 7}$$

2.5.3. Particle size of protein polymeric nanocapsules measurement

The polymeric nanocapsules formulation particle size is one of the most important characteristics which should be examined and controlled wisely. Particle size controls the pharmacokinetics characteristics of the formulations (drug distribution, and absorption) by controlling the kinetics of the drug release from the polymeric system [149].

Dynamic light scattering (Zeta PALS, Brookhaven Instruments Corporation, USA) was used to determine the nanocapsules containing proteins particle size. Samples were

prepared by suspending 10 mg of the freeze-dried nanocapsules in 5 ml of Nano pure water ($>\Omega$ 18, Milli-Q). Afterwards, the resulting suspensions were mixed in the vortex for 1 minute and then were left in water bath sonicator for 5 minutes. Nanocapsule diameters were measured in triplicate at 25 °C for particle size distribution analysis by using dynamic light scattering technique.

2.5.4. Microscopic imaging of polymeric nanocapsules using Negative Staining Transmission Electron Microscopy (TEM)

TEM (Hitachi H7000 transmission electron microscope, Japan) was employed to investigate the morphology of PNCs containing proteins; the technique was applied by using negative staining technique 1% (w/v) of sodium silicotungstate solution. A drop of PNCs suspension was applied on 400 mesh Formvar copper grid (supplied by Agar Scientific, UK) on paraffin and the sample was allowed to adhere on the Formvar at room temperature (21 ± 1 °C) for 15 min. The excess suspension was removed, and a drop of 1% (w/v) of sodium silicotungstate solution was applied for 5 minutes. The remaining solution was then removed. The obtained specimen was later observed under the TEM.

2.5.5. In vitro release of trypsin and lysozyme in Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) without enzymes

In order to develop an oral delivery system, the rate of drug release should be studied in simulation conditions. The protein drug release from the nanocapsules was determined in simulation gastric fluids (SIF) and simulation intestinal fluid (SGF) for 4 and 24 hours, respectively, without enzymes. Both SIF and SGF were prepared according to British Pharmacopeia 2014 [150]. However, no digestive enzymes were added. SIF was prepared by mixing 77.0 ml of 0.2 M sodium hydroxide (NaOH) with a 250ml solution of 2.72% w/v Potassium Dihydrogen Phosphate (KH_2PO_4) and 500 ml of Nano Pure water. Then, the mixture was diluted to 1 L with Nano Pure water, and finally, the pH value was adjusted by adding few drops of diluted NaOH to increase it till 6.8 pH. However, SGF was obtained by dissolving 2.0 g of Sodium Chloride (NaCl) in 80 ml of 1 M hydrochloric acid (1 N HCl) and made up the volume up to 1000.0 ml. The obtained SGF solution had a 1.2 pH value [150]. The drug release in SIF was examined over 24 hours at ten different time points; (0.25, 0.5, 1, 2, 4, 6, 8, 12, 16, and 24 hours), while in SGF, the drug release was studied at five time points only (0.25, 0.5, 1, 2, and 4).

Powder nanocapsules containing 1.5 mg entrapped protein, were suspended in 30 ml SIF (pH 6.8) without pancreatin, and in SGF (pH 1.2) without pepsin, then this volume was divided into 10 screw cap Eppendorfs, each contains 2 ml (100 μg protein). The vials were incubated in a shaker water bath under shaking rate of 50 cycles/ minute at 37 °C, and one vial was collected at each time point. All release tests were performed in triplicate.

Eppendorf vials were taken and the samples centrifuged at 10000 rpm for 10 minutes, the pellets were collected, and the shells were broken down. Then the remaining unreleased proteins were collected by applying the methods discussed above, in Section 2.5.2. Protein release from polymeric capsules system was determined by quantification of protein amount remaining in pellets by using SEC after pellets disruption. After that, the amount of protein was determined by difference according to the following equation:

$$\textit{The released protein \%} = \frac{100 \mu\textit{g} - \textit{the remaining}}{100} \times 100\% \dots \textit{Equation 8}$$

2.5.6. Lysozyme stability (from leakage and permeability) after incubation in Simulated Gastric Fluid (SGF) and Simulate Intestinal Fluid (SIF) with the digestive enzymes

The nanocapsules containing protein were incubated in SGF and SIF containing digestive enzymes at 37 °C for 1 hour and 4 hours, respectively. The incubation test was carried out in order to determine the protection of entrapped lysozyme in the presence of gastric and intestinal enzymes.

Pepsin 5 IU/ml in SGF and trypsin 700 IU/ ml in SIF were prepared according to [151], and the incubation test in both solutions was carried out at 37 °C, for 1h and 4h, respectively.

Nanocapsule powder of each lysozyme formulations containing 100 µg of lysozyme was suspended in 2 ml of each enzyme solution in screw cap Eppendorf, in addition to

the control sample of 100 µg of fresh lysozyme that was dissolved in each enzyme solution. After the incubation, enzyme vials were centrifuged at 10000 rpm for 10 minutes. Pellets were collected, and 0.2 ml of 0.05% trifluoroacetic acid (TFA) was added to the pellet to inactivate traces of enzyme present at the nanocapsules surfaces [152]. Subsequently, the pellets were washed 3 times with water, in each time the supernatants were being taken to trace the diminishing of the digestive enzymes, after that, the amount of the remaining lysozyme in pellets were determined by using the SEC after breaking the polymeric shells by adding ethyl acetate.

2.5.7. Effect of the copolymers and the processes on the proteins' biological activity

The effect of the used reagents including the polymers and the encapsulation process on protein biological activities was examined in this study. Protein biological activity was determined after disrupting the polymeric shell by adding ethyl acetate, and then by measuring ΔA_{450} and ΔA_{253} within 5 minutes for lysozyme and trypsin, respectively, as described in Section 2.2.2.2 and Section 2.2.2.3.

2.5.8. The storage stability of the Polymeric Nanocapsule formulations containing lysozyme and trypsin

The encapsulated proteins in the PNCs and the overall formulation stability studies were carried out to examine the withstand protein and the PNCs over the shelf life.

Therefore, all formulations were stored at three different storage conditions; 5 ± 2 °C in desiccator, 22 ± 2 °C at 76% relative humidity, and 40 ± 2 °C. The stored formulations stability was studied to investigate the biological activity, as previously described, and the physical stability of the encapsulated protein. The physical stability of the proteins was examined by analysing the proteins after breaking the shells down by ethyl acetate by SEC, via the pre-developed and validated methods in this research, as described earlier in Section 2.3.2.

Moreover, the water content of the PNCs powder formulations was determined by Karl Fisher Titration (701 KF Titrino 67 with 703 Ti stand, Metrohm, Switzerland).

This is the method that is used to estimate the moisture content in dry protein samples [153]. The principle of this technique, as explained by [38], is based on two successive reactions and the amount of water required to carry on the second reaction gives the estimation of the moisture present in protein samples. First, chemical reaction gives alkyl sulphate as the product by reaction of sulphur dioxide and alcohol, (..... Equation 9):



However, the second reaction is an oxidation reaction that involves water (from the PNCs sample), Iodine and alkyl sulphate (from reagent), Equation 10):



Where R is variable and generally represents an alkyl group.

In this method, dry samples were introduced into titration cell, and the water or moisture reacts with a reagent to carry on reaction. A sample of the PNCs used is always known and normally ranges from 15-35 mg.

In this study, the moisture of PNCs containing trypsin and lysozyme after storage was estimated. All the readings were obtained in triplicate, and the average value was taken. Before getting any reading, the 701 KF titrino-meter with 703 Ti stand (Metrohm, Switzerland) was calibrated with a standard solution of water in methanol.

2.6. Liquid formulations containing lysozyme and trypsin stability study **(Chapter seven Methodologies)**

2.6.1. Design of experiment

For lysozyme, three design of experiment were built to prepare twenty-four liquid formulations with different compositions. Three buffers, phosphate, citrate, and acetate buffers were used, in addition to two separate excipients; sodium ascorbate and trehalose. DOEa is a full factorial design composed of three distinct factors, and each factor has two different levels, no buffers were used in this design. However, DOEb and DOEc fractional designs. Each design contains eight formulations to form a total of twenty-four lysozyme formulations, Table 2.7.

Table 2.7: A total of 24 lysozyme formulations included in three different designs of experiments (DOEa, DOEb and DOEc).

DOE	Formulation ID	Phosphate mM	Citrate mM	pH	Na Ascorbate	Trehalose
DOEa	La1	0	0	4.0	10	10
	La2	0	0	4.5	10	10
	La3	0	0	4.5	50	10
	La4	0	0	4.0	50	10
	La5	0	0	4.0	10	100
	La6	0	0	4.5	10	100
	La7	0	0	4.5	50	100
	La8	0	0	4.0	50	100
DOEb	Lb1	0	0	4.0	10	0
	Lb2	0	0	4.5	10	0
	Lb3	0	0	4.5	50	0
	Lb4	0	0	4.0	50	0
	Lb5	50	0	4.0	50	0
	Lb6	10	0	4.5	50	0
	Lb7	10	0	4.0	10	0
	Lb8	50	0	4.5	10	0
DOEc	Lc1	0	0	4.5	0	10
	Lc2	0	0	8.0	0	10
	Lc3	0	10	4.5	0	10
	Lc4	0	50	8.0	0	10
	Lc5	0	10	8.0	0	100
	Lc6	0	0	4.5	0	100
	Lc7	0	0	8.0	0	100
	Lc8	0	50	4.5	0	100

On the other side, trypsin formulations were prepared according to one full factorial design of the experiment. Four factors were collected together in a one design where each factor has two levels. Each formulation was prepared by either phosphate or citrate buffer at 10 mM or 100 Mm, with Pluronic F127 at two different concentrations 0.2% w/v or 0.02% w/v. Trehalose was also used at 10 mM or none, Table 2.8.

Table 2.8: A total of 16 trypsin formulations included in one full factorial design of experiments.

Formulation	Buffer type	Buffer concentration (mM)	Pluronic F127 % (w/v)	Trehalose (mM)
T1	Phosphate	10	0.02	0
T2	Phosphate	100	0.2	0
T3	Citrate	10	0.02	10
T4	Citrate	10	0.02	0
T5	Phosphate	10	0.02	10
T6	Citrate	100	0.2	10
T7	Citrate	10	0.2	10
T8	Citrate	100	0.02	10
T9	Phosphate	100	0.02	10
T10	Citrate	100	0.02	0
T11	Phosphate	100	0.02	0
T12	Phosphate	10	0.2	0
T13	Citrate	10	0.2	0
T14	Phosphate	10	0.2	10
T15	Citrate	100	0.2	0
T16	Phosphate	100	0.2	10

ANOVA tables were established for each design, in addition to the essential statistics parameters. All DOE were built and analysed by using MODDE 10.1 (Umetrics AB, Umea, Sweden). All Measurements were performed in triplicate.

2.6.2. Samples preparation

All formulations were prepared by dissolving the proteins and excipient in the desired solvent media (Nano pure water (>Ω 18, Milli-Q) or buffers at certain pH values) according to the designs of the experiment as detailed earlier in Table 2.7 and Table 2.8. The final protein concentration for each sample was 4mg/ml. All buffers were prepared according to pharmacopoeia 2014 unless mentioned otherwise. All formulations were filtered by syringe filters and filled in autoclaved glass vials and capped and stored in calibrated cabinets under the ICH-recommended conditions. For the formulations containing ascorbate, amber glass vials were used to avoid the light ascorbate degradation. References for all formulations containing everything but not the proteins were also prepared and stored.

2.6.3. Toolbox used for formulations Quality Control

As part of pharmaceutical development Quality Control (QC), the proteins' stability in all formulations were investigated by a different stability indicating assays. Selection of analytical method is a critical decision during the stability study of protein formulations. Analytical assays selection depends on the protein structure, available data about the protein from the literature, and the formulation compositions. The degradation pathways of the protein in general and model proteins including lysozyme and trypsin were intensively investigated by the researchers. Lysozyme and trypsin undergo different degradation pathways under during the storage time. Lysozyme and trypsin

contain Asparagine in their amino acid backbone which makes them prone to the deamidation and fragmentation chemical degradation. Also, Asparagine is an essential amino acid in their activities. Thus, the chemical degradation will end up with lysozyme and trypsin deactivation. In light of the previous information, potency assays may provide more exact details about both proteins activities and chemical degradation.

Therefore, lysozyme and trypsin biological activities were measured throughout the period of the storage by applying the potency or enzymatic assays described previously in this project, Section 2.2.2.2 and Section 2.2.2.3.

Moreover, the physical stability of both proteins was assessed by two analytical methods; Size Exclusion Chromatography (SEC) and Turbidimetry. The rationale behind using two different analytical methods is that proteins develop two types of aggregates: soluble and non-soluble aggregates. Size Exclusion Chromatography only detect the soluble aggregates due to samples filtration. Although the non-soluble aggregations can be quantified by SEC indirectly, “How big the aggregations?” and “Which formulations became more opaque and turbid?” cannot be answered by SEC method. Hence, Turbidimetry method was employed to analyse the non-soluble aggregations.

The samples were withdrawn from the formulations and analysed immediately by SEC and turbidimetry. SEC method for analysing the proteins was discussed in details previously in this project in Section 2.3.1.

Turbidimeter is a simple, high throughput and non-destructive measurement tool. It is typically used for comparison purposes or the detection of relative changes during stability studies, due to its ability to detect the observable non-soluble aggregates.

Ratio/XR turbidimeter (Hatch company, USA), was used to detect the non-soluble protein aggregates developed into the stored formulations, the turbidity was measured in nephelometric turbidity units (NTU).

The instrument was calibrated to adjust the reading correctly while measuring the turbidity standards. Sample cells, standards, were matched optically to minimise errors due to optical variations in cells. All cells were coated with a thin film of silicon oil to mask slight imperfections in the glass. All sample vials were slowly inverted several times to gently mix the solution before placing the cells into the cell holder. The readings were recorded 15 seconds after placing the samples into the instrument. When necessary, changes in the setting have been made in small increments, allowing enough time between changes for the reading to stabilise.

Furthermore, in order to ensure the safety of the formulations and build larger insight about the degradation pathways, chromatographic analytical assays were developed and validated to analyse the excipients. The excipients were analysed by HPLC by utilising HILIC column as detailed earlier in Section 2.3.3.

2.6.4. Stability study approaches

All the prepared formulations were stored under three different ICH storage conditions. For the conservative approach, the formulations were stored in the fridge, with calibrated temperature $5\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$, for eighteen and twelve months for lysozyme and trypsin formulations, respectively. For intermediate and aggressive approaches, the formulations were stored at the intermediate condition ($25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$)

and accelerated conditions ($40\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $75\% \text{ RH} \pm 5\% \text{ RH}$) for nine and six months, respectively.

2.6.5. Correlation

The power of the aggressive approach in the prediction of long-term stability of lysozyme and trypsin formulations was evaluated by calculating the Pearson's product moment correlation coefficient (R^2) after plotting the ranking of long-term stability of the formulations and the ranking of the accelerated stability of the formulations. The ranking and correlation processes were done for lysozyme and trypsin formulations and for each stability category (Biological activity and physical stability). For example; a plot was generated by the biological activity of lysozyme obtained after a long term of storage in X-axis versus the biological activity of lysozyme obtained after accelerated storage in Y-axis.

**Chapter Three: Quality by
Design (QbD) based
preparation of liquid
lysozyme and trypsin
formulations**

3.1. Introduction

Protein pharmaceuticals have emerged as promising therapeutic agents in recent years. The proteins in these formulations should be in their native conformation throughout the manufacturing and delivery process to be biologically active. However, finding stable formulations and their delivery to the target site are a challenge due to physical and chemical instabilities of proteins, including the most stable refrigerated ones, even during storage. Chemical degradation of a protein refers to several chemical reactions those change the hydrophobic nature of proteins by the formation or destruction of covalent bonds within the structure of protein molecules (e.g., deamidation).

Different analytical methodologies have been used to characterise the proteins and their containing formulations. Differential scanning calorimetry DSC is a technique usually used to characterise liquid and solid protein formulations by structure integrity determination. Micro Differential scanning calorimetry μ -DSC or so-called VP-DSC determines the protein conformational stability in liquid formulations by measuring its ability to keep in the natively unfolded state under heating effects. Denaturation temperature (T_m) is the point where the protein starts unfolding. Therefore, the higher T_m value, the more conformational stable proteins. Many studies have been performed to investigate the ability of DSC to assess the proteins integrity and formulations stability [61].

All new active pharmaceutical ingredients (API) require long term stability studies to determining shelf life; in order to be registered. Therefore, various tests and characterisations should be carried out before storing the samples for stability studies. Wise and systematic base selection of the formulation conditions helps in saving the

resources. FDA launched the Quality by Design (QbD) initiative to produce high-quality products without extensive regulatory oversight. To adopt QbD approach, the product objectives, target profile, and quality attributes should be predefined from the beginning. Therefore, a scientific base risk assessment must be performed based on the prior knowledge and preliminary screening before selection the appropriate formulation desired characteristics. The aim of QbD to generate a quality in the products by applying scientific base rationales including mathematical models instead of testing that later, in order to have a faster and more consistency product process development, accordingly, reduce the production cost. The design of experiment (DOE) methodology is being applied to analyse the factors, which their presence or absence may form a potential risk to the formulations, quantitatively.

3.2. Aims and Objectives

- To investigate the effect of different buffers and excipients on protein integrity.
- To adopt the QbD approach in the formulation process to screen the highest possible number factors in the shortest possible time, in a view to building the quality in the formulations rather than testing it later.
- To optimise the process parameters and materials attributes intending to having optimum formulations.
- To prepare the optimised stable protein formulations which last for long shelf life, with integrated and biologically active protein.

3.3. Results and discussion

3.3.1. Quality by Design implementation

3.3.1.1. Quality Target Product Profiles (QTPPs) and Critical Quality Attributes (CQAs) identification

QTPPs have been identified as the first step of the QbD process implantation, based on the relevant literature and guidelines such as; ICH Q8 guideline. The CQAs have been determined as the next step after QTPP's determination, based on QTPPs and prior knowledge. Table 3.1 illustrates the QTPPs and CQAs of the current study. The CQAs were related to protein integrity represented by thermal analysis using DSC as a capable tool to determine the protein integrity in liquid formulations. The denaturation temperature, T_m , is an indicator that reflects protein ability to resist unfolding under thermal processing.

Table 3.1: The desired Quality Target Product Profiles (QTPPs) and Critical Quality Attributes (CQAs).

QTPPs	Target
Dosage form	Solution
Route of administration	Parenteral
Site of release	Bloodstream
Biological activity	Active and stable over storage at 5+3 °C
Drug	Protein
Cost	Reasonable
CQA	
Trypsin T _m	≥ 68 °C
Lysozyme T _m	≥ 79 °C

3.3.1.2. Risk assessment

As an essential step of QbD process, the risk has been assessed according to a systematic approach. After the QTPPs and CQAs identification, different Critical Process Parameters (CPPs) and Critical Materials Attributes (CMAs) were determined. Then, the risk factors have been identified by listing 37 different factors based on the relevant literature. The selected factors (Process parameters or material attributes) are proposed to have potential risk on the protein integrity. Fishbone (Ishikawa) diagram has been generated to illustrate all identified CPPs, CMAs, operator, and environmental conditions, which have potential risk on lysozyme and trypsin in liquid formulations, Figure 3.1.

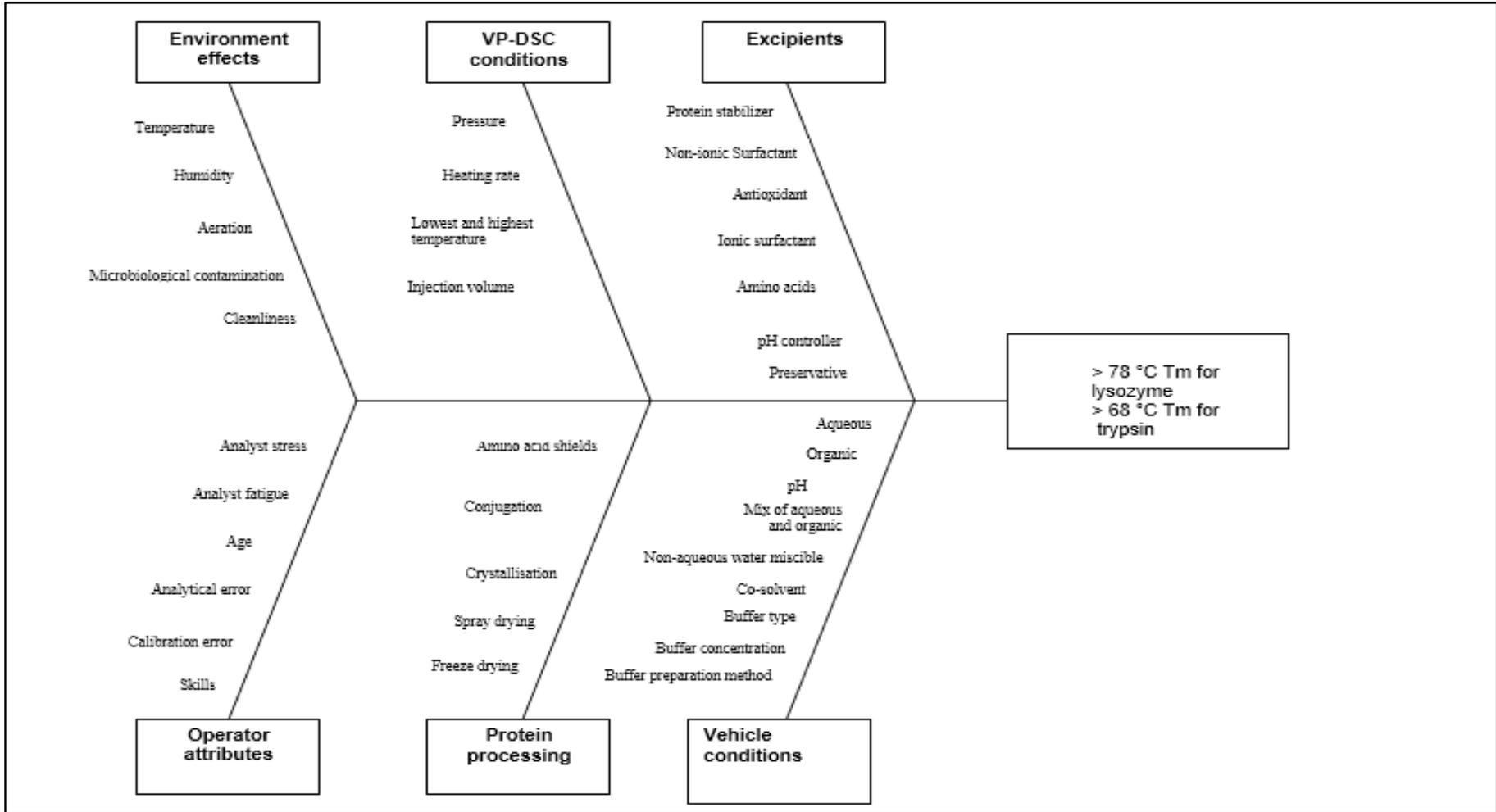
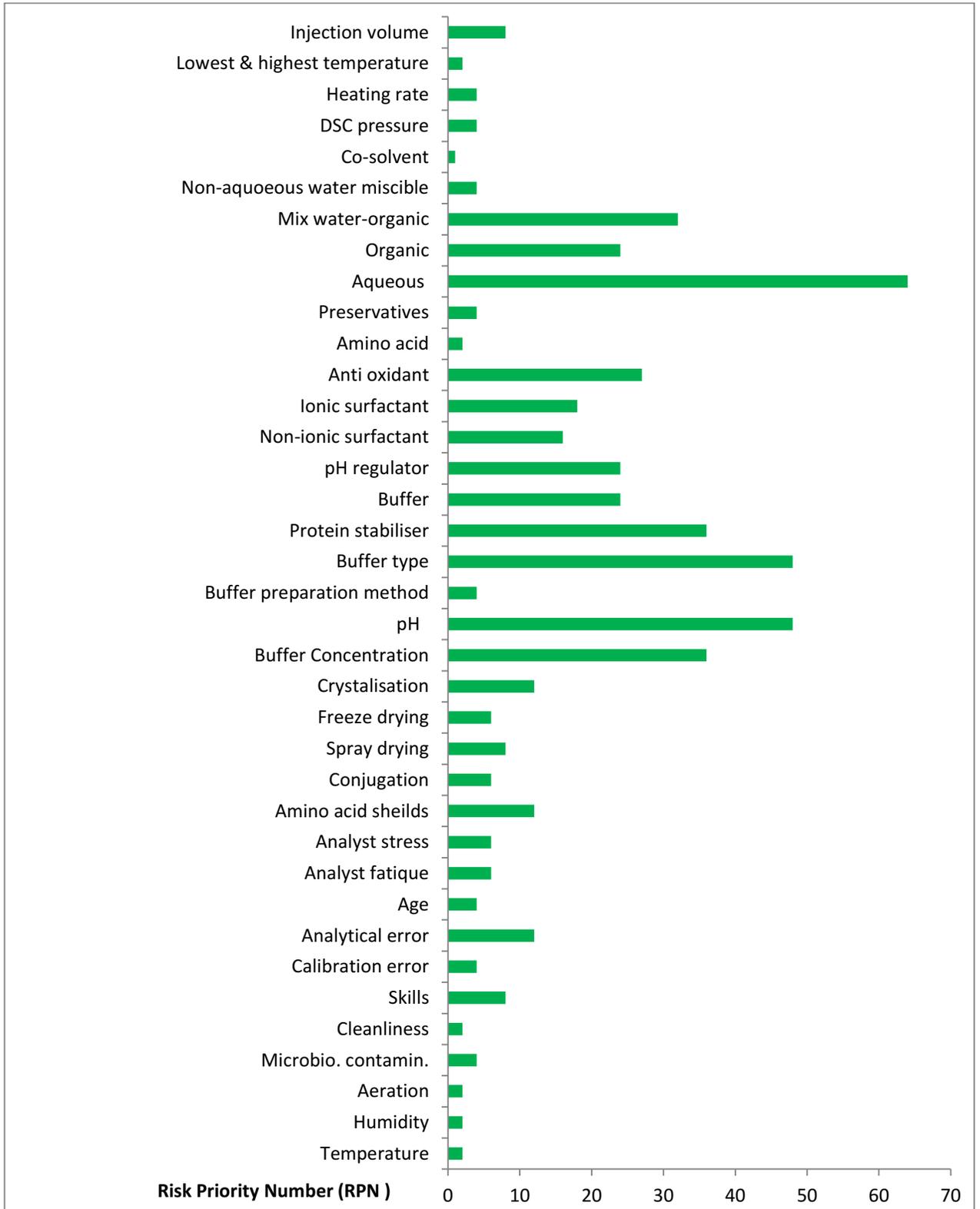


Figure 3.1: Fishbone (Ishikawa) diagram, which illustrates the potential risk causing factors thus affect influence the protein integrity in liquid formulations.

After the potential risks have been identified, risk analysis step has been carried out. The potential hazard was analysed according to ICH Q9 guidelines; by employing Failure Mode Effect Analysis (FMEA), hence, determined the Risk Priority Number (RPN) as described in Section 2.2.1.2. Critical and rigorous risk analysis procedures were performed based on the relevant literature and the preliminary study. According to *Wang et al., 2015*, the factors with RPN more than fifteen, are selected as potentially high-risk factors. Therefore, 12 factors have been found to surpassed 15 RPN from analysed 37 factors, see Figure 3.2. However, not all of these factors were chosen to be included in further models, since some of them have to be fixed such as; the pH value in trypsin case or even excluded such as; using an organic solvent as a vehicle.

In order to evaluate the risk, the potential high-risk factors were classified into three broad categories i.e. factors with more than one level which should be included in the multilevel design, factors with only one value which were fixed, and others which their existence is risky of the protein integrity, and should be excluded.



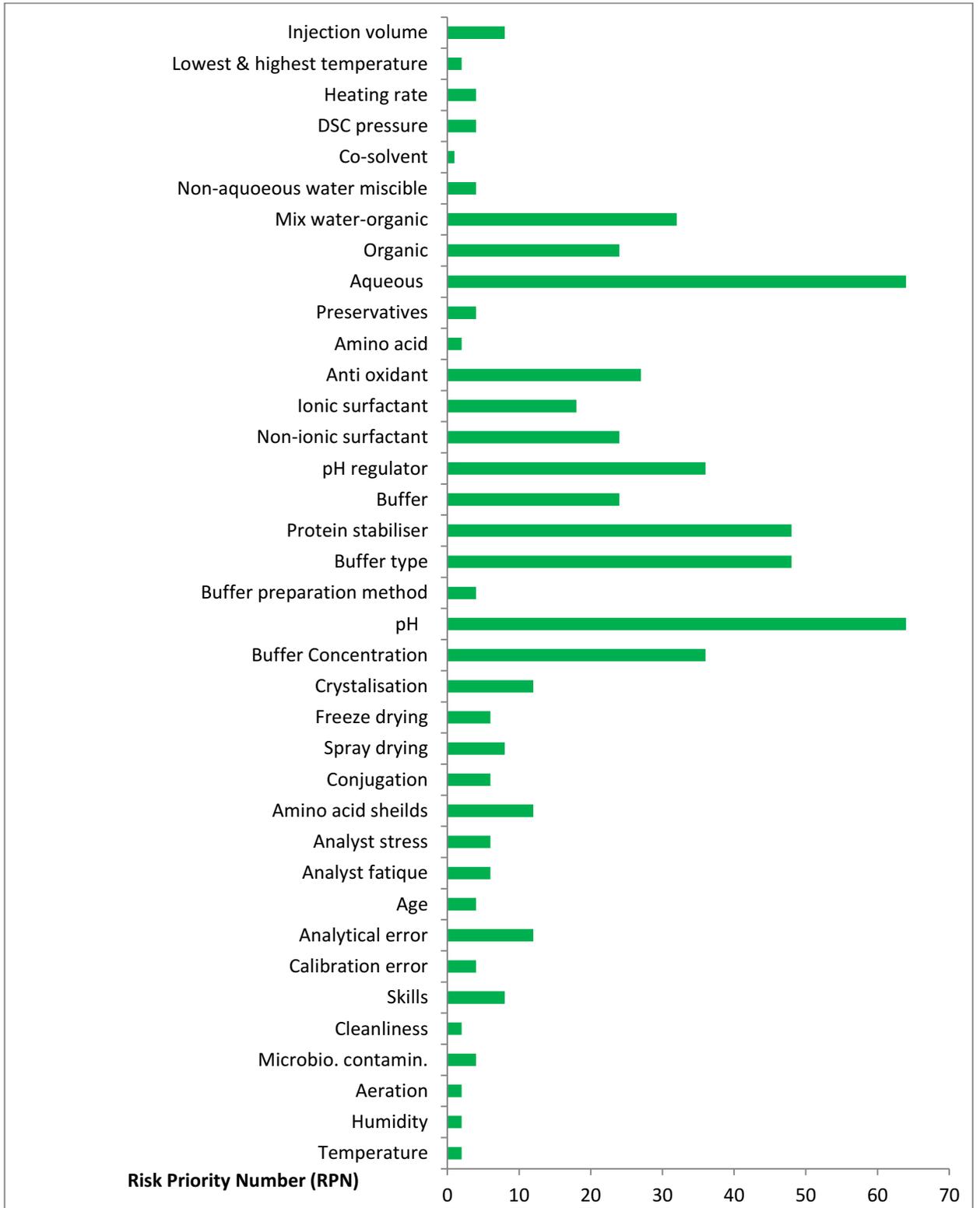


Figure 3.2: Failure Mode Effect Analysis (FMEA) graph is showing the predetermined risk factors and their respective risk priority number (RPN).

In order to evaluate the risk, the potentially high-risk factors were classified into three broad categories i.e. factors with more than one level which should be included in the multilevel design, factors with only one value which were fixed, and others which their existence is risky of the protein integrity, and should be excluded. Table 3.2 reveals the potentially high-risk factors, and how they were classified according to their role in evaluating the risk on both lysozyme and trypsin structural integrity.

Table 3.2: Potentially high-risk factors classified into three different categories; 1- Factors can be used at different levels and should be included into DOE, 2- Factors have only one level or should be employed at fixed level, 3- Factors should be excluded.

Potentially high-risk factors	Lysozyme	Trypsin
Using buffer salts	2	2
Aqueous vehicle	2	2
Buffer concentration	1	1
Buffer type	1	1
pH	1	2
Protein stabiliser	1	1
pH regulator other than buffer	3	3
Non-ionic surfactant	Not high risk	1
Ionic surfactant	3	3
Antioxidant	1	3
Organic vehicle	3	3
Mixture of water organic vehicle	3	3

Based on the described above, different designs of the experiment (DOE) have been built to assess the effect of high-risk factors on the proteins integrity, hence, optimising the levels to obtain stable formulations which contain active and intact protein in the native structure. The details of these designs and their analysis are described in details in the following sections.

3.3.2. Preliminary pH screening

The pH has a significant impact on both chemical and physical stability of proteins. The pH values are limited. Therefore, it is easy to tighten and select a stabilising pH range for protein formulations. The pH screening test was performed by using trypsin and lysozyme as model proteins, at a pH range of 3-10, to find the most stabilising pH range for both of the proteins. The preliminary pH screening was performed to obtain the optimum pH value. Two pH screening tests were carried out: thermal test by using VP-DSC and biological activity by using enzymatic assay methodologies. Both tests concluded that trypsin and lysozyme retained their maximum activity and stability at pH 3 and (4-5), respectively. A significant decrease in the proteins' integrity was recorded with increasing pH, Figure 3.3.

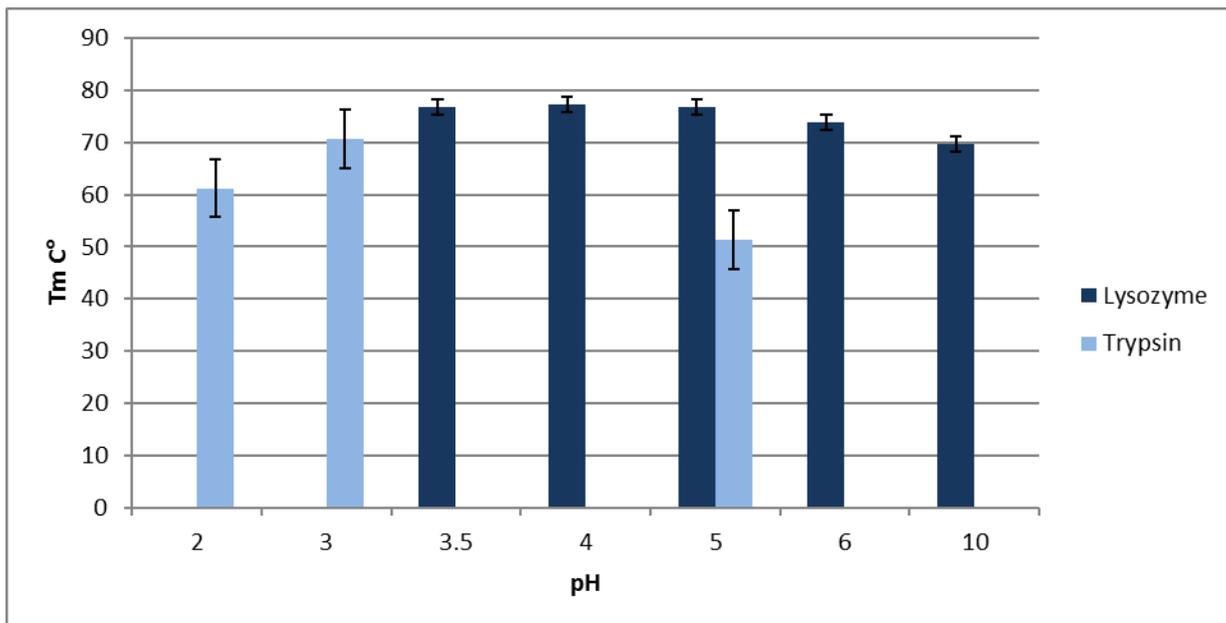


Figure 3.3: Effect of changes in pH value on unfolding temperature for lysozyme and trypsin.

Figure 3.3 shows the relevant T_m values at different pH value, and there is a wide variation of T_m values among the various pH samples. As apparent from Figure 3.3, the thermal stabilities, as expressed by T_m values, of lysozyme and trypsin were the highest at pH 4.5 and 3, respectively. Moreover, biological activity has been affected significantly by changing the pH of the media. The enzymatic assay is the most conclusive test as it determines the biological activity of the protein [154], which is considered a crucial parameter to reflecting protein integrity and stability. Thermal stability showed that the pH value is the most impacting factor on both lysozyme and trypsin conformational stability. The enzymatic assay has been performed to measure the biological activity of lysozyme and trypsin at different pH values. The biological activities of both proteins were expressed as a percentage relative to lysozyme and trypsin at pH 6.24 and 3, respectively, as mentioned in the enzymatic assay guideline.

For example; the biological activity of pH 6.24 lysozyme was 100%. Table 3.3 and Figure 3.4 reveal the effect of pH on the biological activity of both enzymes. The pH values that preserve the protein activity are expected to have percentage biological activity of equal or more than 100%.

Table 3.3: Biological activity of lysozyme and trypsin liquid samples at different pH.

Protein	pH2	pH3	pH4.5	pH6.24	pH8	pH10
Lysozyme	--	100.88%	116.10%	100.00%	87.37%	79.61%
Trypsin	94.25%	100.00%	96.10%	90.34%	79.65%	79.39%

* Data are expressed as a percentage of corresponding values relatively to pH 6.24 and 3 for both lysozyme and trypsin respectively.

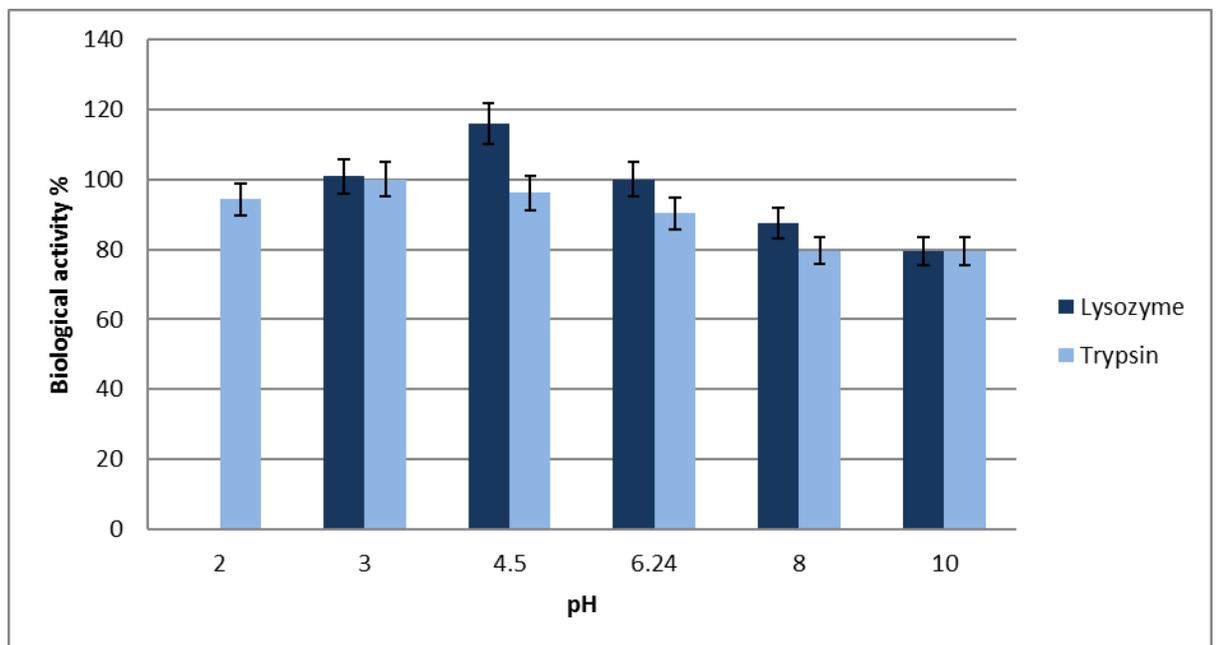


Figure 3.4: Biological activity of lysozyme and trypsin liquid samples at different pH.

Figure 3.4 and Table 3.3 demonstrate the increase in biological activity with decreasing pH values for both proteins. The observed pH dependence is consistent with thermal stability obtained from the DSC. A sharp decline in biological activity (BA%) was observed between pH 6.24 and pH 8 for lysozyme and trypsin (Δ BA% = -12.635 % and -10.69%, respectively). These results can be explained on the basis that, neutral and alkaline media trigger chemical changes in amino acids structures. A reduction of biological activity was reported for a variety of proteins due to deamidation of asparagine and glutamine residues [155]. Deamidation commonly occurs at alkaline and neutral media without an enzymatic catalyst. Asparagine and Aspartate residues usually modified through intramolecular rearrangement, thus converting 75% of asparagine or aspartate into iso-aspartate. Asparagine is playing important roles in lysozyme and trypsin biological activity as a part of the active centre and involved in both enzymes substrates interaction. Also, the changes in pH can affect the protein stability by multiple mechanisms, e.g. hydrogen bond interaction, and charge repulsion effects. Low pH is needed, to maximise repulsive interactions between protein molecules and thus to minimise aggregation and non-aggregation unfolding [156, 157]. These instabilities usually are triggered by chemical decomposition reactions, as protein structure is very sensitive to pH, and can affect chemical and physical degradation. Chemical and physical degradation often come together, and trigger each other.

In conclusion, no significant difference was noticed between T_m values of lysozyme formulations at pH 4, 4.5, and 5. Therefore, these three points were selected as the stabilising conditions to be included in the design.

3.3.3. Buffer screening

Buffers are usually used to control pH and salt content; hence controlling charge repulsion, and accordingly, optimising protein stability and integrity [158]. Therefore, it makes the selection of buffer type a crucial decision in formulations development. Moreover, DOE as a mathematical tool was applied in this study as a part of QbD concept implementation, to evaluate the risk factors, with a view to examining the initial choices with the use of analytical tools. Acetate, citrate, and phosphate buffers are commonly used in parenteral formulations [159, 160], and they cover a broad range of pH values 3-10 [161].

Based on that and the results obtained from pH screening (Section 3.3.2), an initial buffer screening was performed using DSC by applying DOE, at pH (4.0, 4.5, and 5.0) and (3) for both lysozyme and trypsin, respectively, and at buffer concentrations 10, 50, and 100 mM. Full factorial experimental design, with every factor varied at three levels in interaction mode, was created to uncover the relevant factors and their appropriate changes regardless of linear or non-linear dependencies in addition to two factors interactions. The DOE's including factors, levels, and responses are shown in Table 3.4 and Table 3.5. All these factors at different levels have been examined by VP-DSC, to screen their effect on thermal stability of both proteins, thus, optimise according to those results.

Table 3.4: A total of twenty-seven lysozyme formulations included in a design of experiments. All Tms are the mean of triplicate DSC scans $T_m \pm SD$.

Protein	Buffer type	Buffer Concentration (mM)	pH value	T_m	$\pm SD$
Lysozyme	Phosphate	100	5.0	76.75	0.061
	Phosphate	100	4.5	77.09	0.036
	Phosphate	100	4.0	76.87	0.036
	Phosphate	50	5.0	77.12	0.062
	Phosphate	50	4.5	77.45	0.130
	Phosphate	50	4.0	77.23	0.026
	Phosphate	10	5.0	76.87	0.149
	Phosphate	10	4.5	77.19	0.026
	Phosphate	10	4.0	77.04	0.040
	Acetate	100	5.0	76.41	0.026
	Acetate	100	4.5	76.72	0.052
	Acetate	100	4.0	76.23	0.026
	Acetate	50	5.0	76.32	0.020
	Acetate	50	4.5	76.78	0.010
	Acetate	50	4.0	76.21	0.027
	Acetate	10	5.0	76.61	0.046
	Acetate	10	4.5	76.95	0.020
	Acetate	10	4.0	76.63	0.066
	Citrate	100	5.0	74.74	0.043
	Citrate	100	4.5	75.71	0.096
	Citrate	100	4.0	75.14	0.053
	Citrate	50	5.0	75.21	0.030
	Citrate	50	4.5	75.97	0.052
	Citrate	50	4.0	75.23	0.015
	Citrate	10	5.0	75.81	0.089
	Citrate	10	4.5	76.66	0.046
	Citrate	10	4.0	76.01	0.085

Table 3.5: A total of nine trypsin formulations included in a design of the experiment. All Tms are the mean of triplicate DSC scans $T_m \pm SD$.

Protein	Buffer type	Buffer Concentration (mM)	pH value	Tm	$\pm SD$
Trypsin	Phosphate	100	-	68.64	0.053
	Phosphate	50	-	67.93	0.130
	Phosphate	10	-	66.81	0.026
	Acetate	100	-	68.02	0.132
	Acetate	50	-	68.60	0.250
	Acetate	10	-	68.83	0.150
	Citrate	100	-	61.36	0.670
	Citrate	50	-	66.88	0.165
	Citrate	10	-	68.07	0.105

Table 3.4 and Table 3.5 showed the changing in T_m value with changing the levels of each factor. Multi-linear regression fitted T_m values and predicted them by the 3^n models. Models fitting implied valid designs, as R_2 (0.828, 0.94), Q_2 (0.785, 0.908) and reproducibility (0.968, 0.987) for both lysozyme and trypsin, respectively. All of these results are considered high, with little pure error, and Q^2 and R^2 were not separated by more than 0.2-0.3 which reflects accurate models, Table 3.6.

Table 3.6: The statistical parameters (Q², R²) obtained after the trypsin model analysis.

Protein	R ²	Q ²	Reproducibility
Lysozyme	0.828	0.785	0.968
Trypsin	0.94	0.908	0.987

The lysozyme model fitting and analysis showed no significant difference was observed between the different pH values (4, 4.5, and 5) since it was examined at a very narrow range. This observation suggests that; very close T_m values was recorded regardless of at which pH point the formulations were prepared. However, the pH value was fixed at 3 for all the trypsin formulations. Therefore, it was not included in the model analysis.

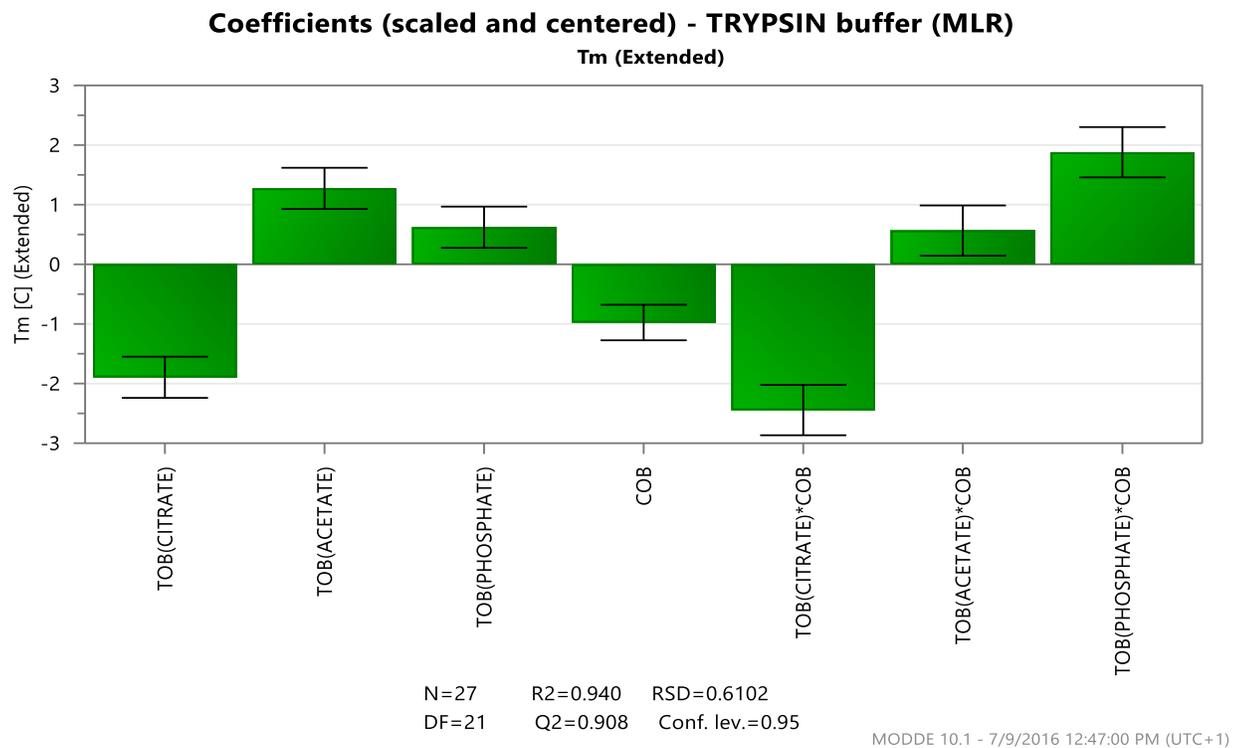
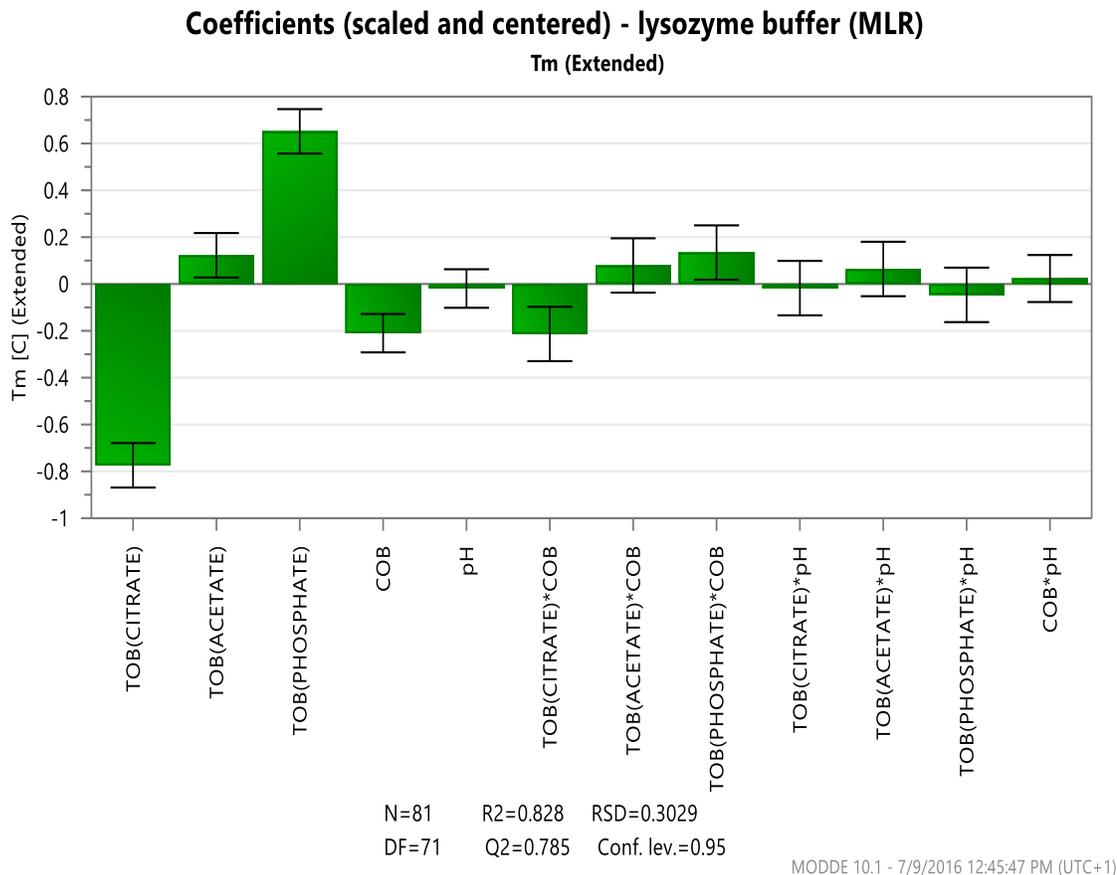


Figure 3.5: Coefficient diagrams are showing the effect of each factor on lysozyme and trypsin T_m values.

An apparent decrease in T_m values was noted for both proteins at high buffer concentrations at all pH value, for all buffers. However, phosphate buffer showed otherwise in trypsin, and a different pattern in lysozyme, hence, the optimum T_m value was reached at concentration 50 mM, while 10, and 100 mM showed close T_m values at all pH, Figure 3.5. The previous results agree with *Chi et al. 2003 and Blumlein et al. 2013* findings [162, 163]; when they concluded that proteins aggregation could be minimised by maximising repulsive interactions between its molecules, which can be achieved in low salt and pH media.

In addition to the concentration of buffer effect, the buffer type showed a significant effect on the unfolding temperature for both of lysozyme and trypsin. The most destabilising buffer was citrate, while phosphate buffer had the best impact on T_m values for both proteins. The observed choice of buffer dependency agrees with the reported conclusion that; both chemical and physical stability of proteins depend on the different buffer ions, which can control the choosing of a buffering agent [158].

All factors were found significant for trypsin, while in the case of lysozyme, pH was not significant, as mentioned earlier. Table 3.7 shows the influence of factors on T_m values, in addition to the interaction between factors, as the interactive and quadratic effects can be evaluated by multivariate experimental design [164].

Table 3.7: Scaled and centred coefficients along with the significance towards Tm.

Protein	Factor	coefficient	significant
Lysozyme	pH	-0.019300	NS
	Acetate	0.122560	significant
	Citrate	-0.774200	significant
	Phosphate	0.651650	significant
	Buffer Concentration(COB)	-0.210040	significant
	Acetate*COB	0.079100	NS
	Citrate*COB	-0.213440	significant
	Phosphate*COB	0.134300	significant
	Acetate*pH	-0.064074	NS
	Citrate*pH	-0.017594	NS
	Phosphate*pH	-0.046480	NS
	COB*pH	0.023385	NS
	Trypsin	Acetate	1.273200
Citrate		-1.895000	significant
Phosphate		0.622000	significant
Buffer Concentration(COB)		-0.976100	significant
Acetate*COB		0.565200	significant
Citrate*COB		-0.244500	significant
Phosphate*COB		1.880000	significant

*NS: Non-Significant.

Table 3.7 and Figure 3.5 show that among two types of protein, trypsin T_m showed more sensitivity to the variances, as those variances had higher coefficients in trypsin case than in lysozyme, except the phosphate buffer which implied similar stabilising effect for both enzymes. The buffer concentrations negatively influenced thermal stability for both of them to a significant degree, this ionic dependency agrees with previously reported data by [165].

Also, it is demonstrated that; the most influencing variable was the citrate buffer since it was reflected by high destabilising coefficients value of -0.7742, -1.895 for lysozyme and trypsin, respectively. Which means the most destabilising buffer was citrate, as it had the most negative impact on T_m values, Figure 3.6.

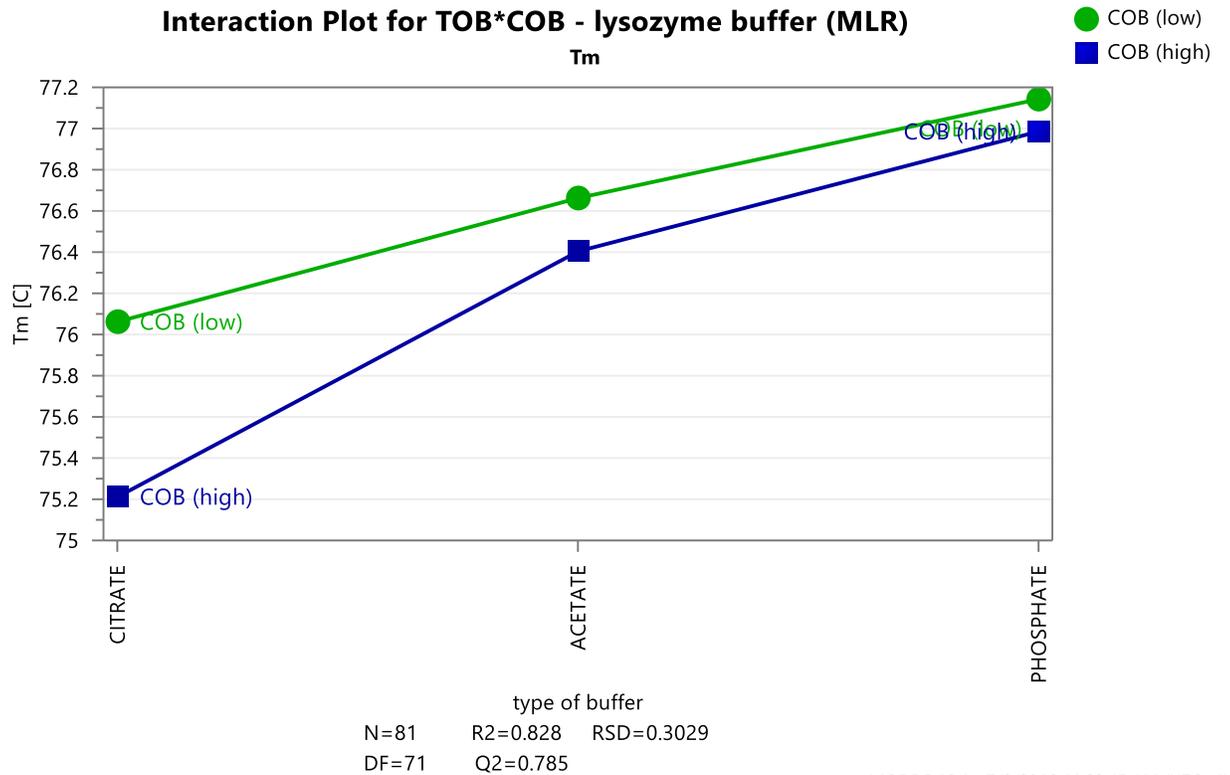


Figure 3.6: The comparison between the effects of buffer type on lysozyme formulations T_m values.

Figure 3.6 shows that; citrate has the most destabilising effect on lysozyme stability. However, phosphate buffer had the best effect in terms of thermal stability. Citrate destabilising impact corresponds with previously reported data about the effect multivalent carboxylic buffers like citrate, on monoclonal antibodies by [166].

DOE as a method is capable of evaluating the interactive effect between factors. The interaction between factors illuminated different results. Hence, the apparent interaction was observed between phosphate buffer, and the concentration of buffer which impacted trypsin T_m values positively on T_m value of trypsin with coefficient reached 1.88, and significant effect, following by acetate, while it was a negative coefficient in citrate, concentration variable interaction case. Significant interaction

between citrate and phosphate as variables with buffer concentration was noticed, for lysozyme formulations, while the rest of interactions were non-significant.

Contour plots, Figure 3.7, were generated for lysozyme. The response contour plots were illustrated in increments of 0.05 °C, 0.05 °C, and .1 °C for phosphate, acetate, and citrate buffers, respectively, with an individual colour determined to each Tm increase. The red contour shows conditions of highest Tm and the darkest blue silhouette shows for lowest Tm.

Response Contour Plot - modde (MLR)

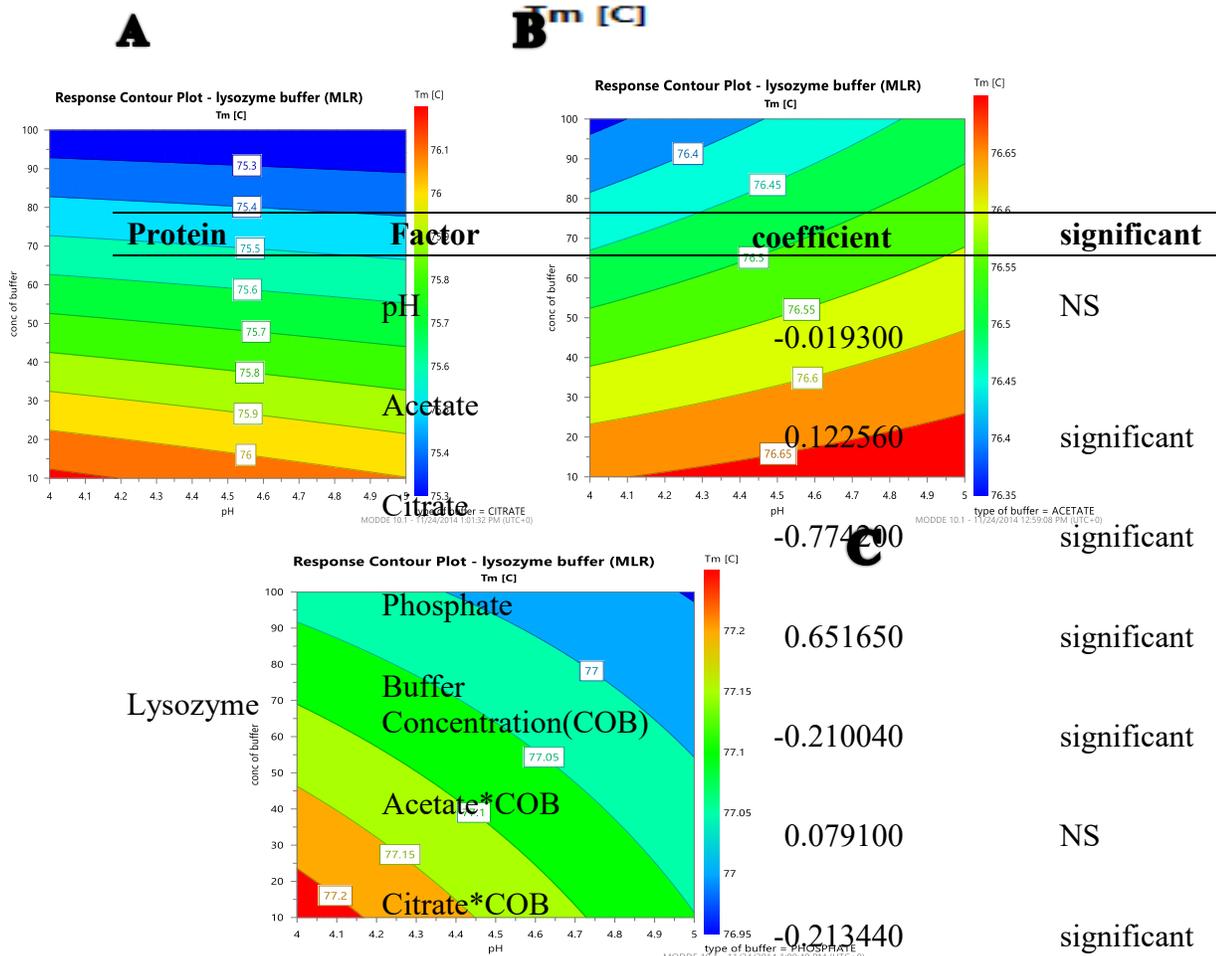


Figure 3.7: (A) Response contour plot of Lysozyme unfolding temperature (Tm) in Citrate buffer, (B) response contour plot of Lysozyme unfolding temperature (Tm) in acetate buffer, and (C) Response contour plot of Lysozyme unfolding temperature (Tm) in Phosphate buffer. The x-axis and y-axis represent pH values and buffer concentration, respectively. Contour lines are labelled with their Tm values.

The generated contour silhouette confirmed the same results concluded above. It is evident that; the T_m values were reversely proportional with the buffers concentrations. Phosphate plot had the highest T_m value since even the blue area reflected T_m values above 77 °C. However, the red areas in acetate and citrate plots, which represent the highest reached T_m , were for $T_m < 76.7$. The concluded findings from Figure 3.7 suggests that phosphate can maintain lysozyme structure integrity more than citrate and acetate.

Moreover, phosphate contour plot illustrated small blue area with wider lines than other buffers. Wide lines mean that; any minor changes in phosphate concentration may result in no impact or little effect on lysozyme T_m values. Thus, lysozyme can maintain its native folded structure up to higher heating degrees even when phosphate concentrations are changed.

The current models have indicated a high level of predictability. Thus the model's goodness was high as reflected by Q^2 . Q^2 is a statistical parameter that indicates how well the model can estimate the ultimate prediction precision.

Q^2 should be and greater than 0.5 for a good design model. Q^2 is the best and most sensitive indicator. Linear correlation plots between the predicted and observed responses for both lysozyme and trypsin were generated and demonstrated high values of " Q^2 "; 0.78 and 0.9, respectively, Figure 3.8.

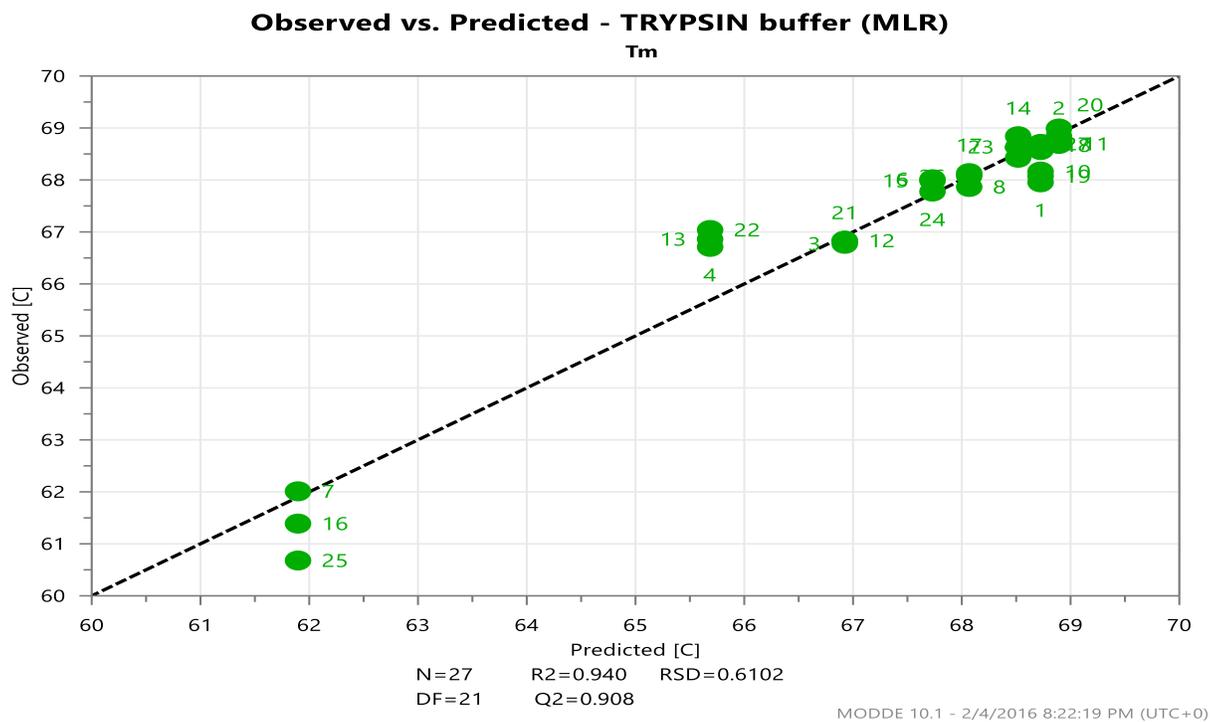
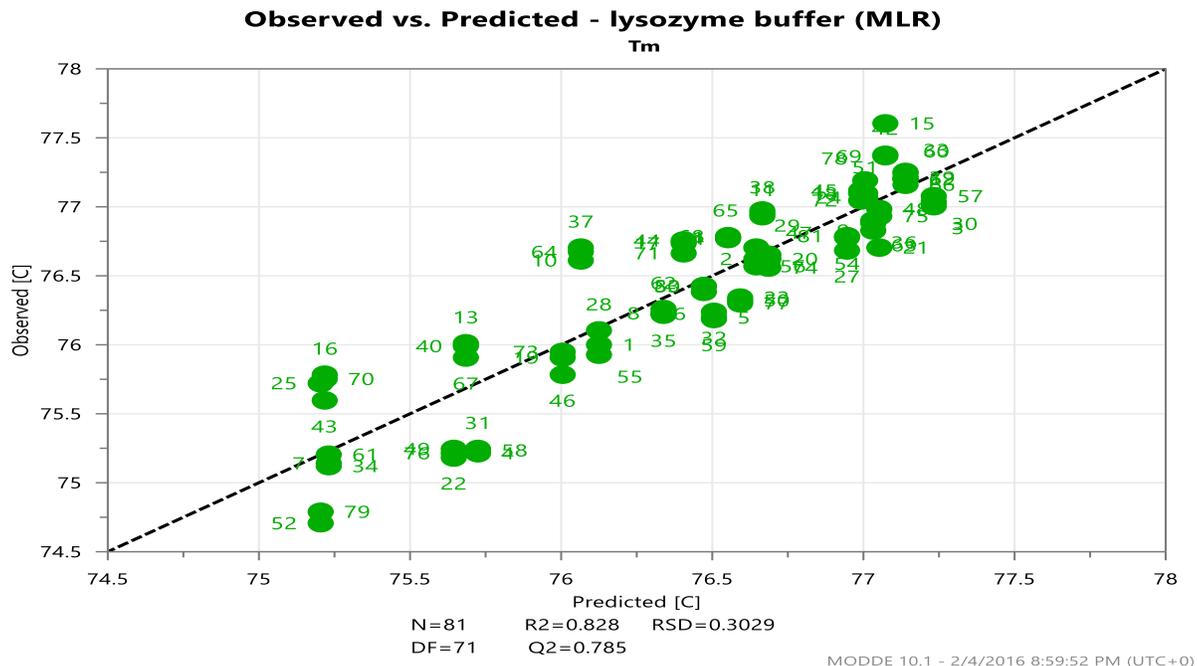


Figure 3.8: Observed versus predicted plot illustrating the prediction power of the lysozyme and protein models.

The corresponding residual plots also confirmed nearly uniform, and randomly scatter of most of the points around the zero axis within the range of -2 and 3, which is ruling out any implicit trends and patterns, thus indicated a high degree of reliability of the QbD based studies, Figure 3.9.

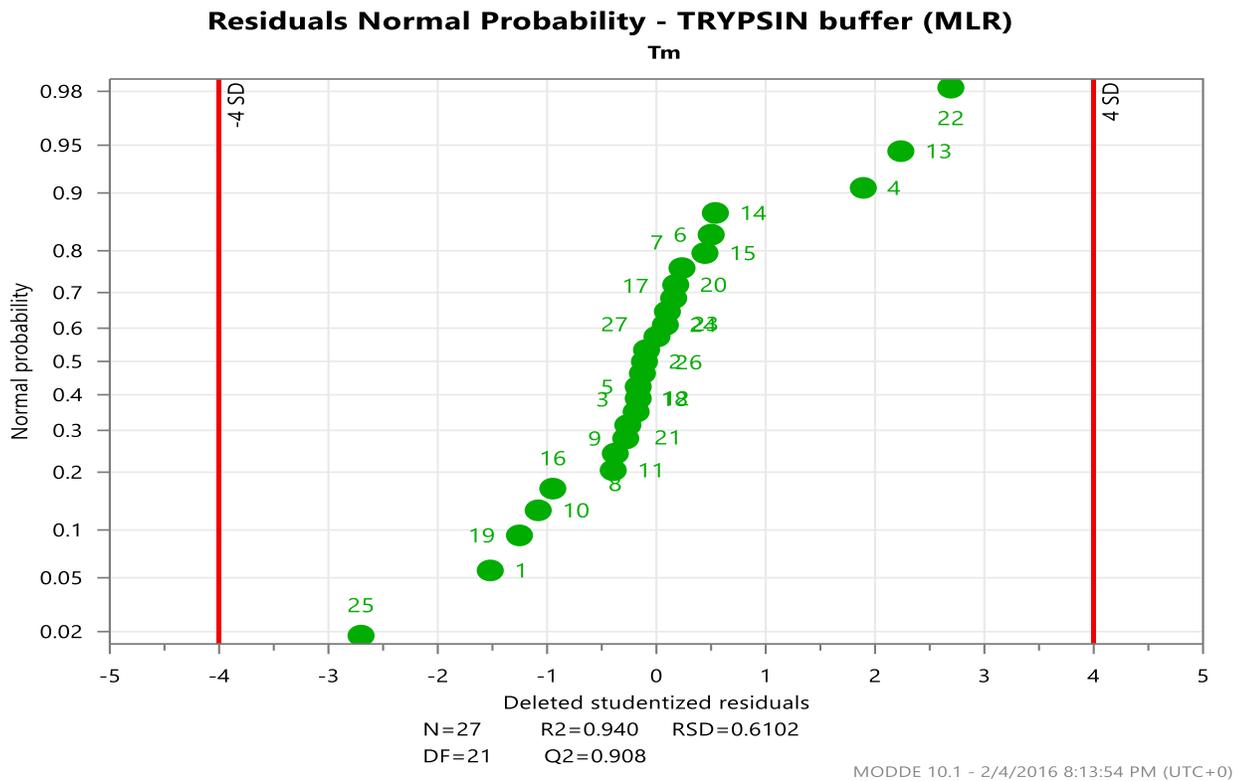
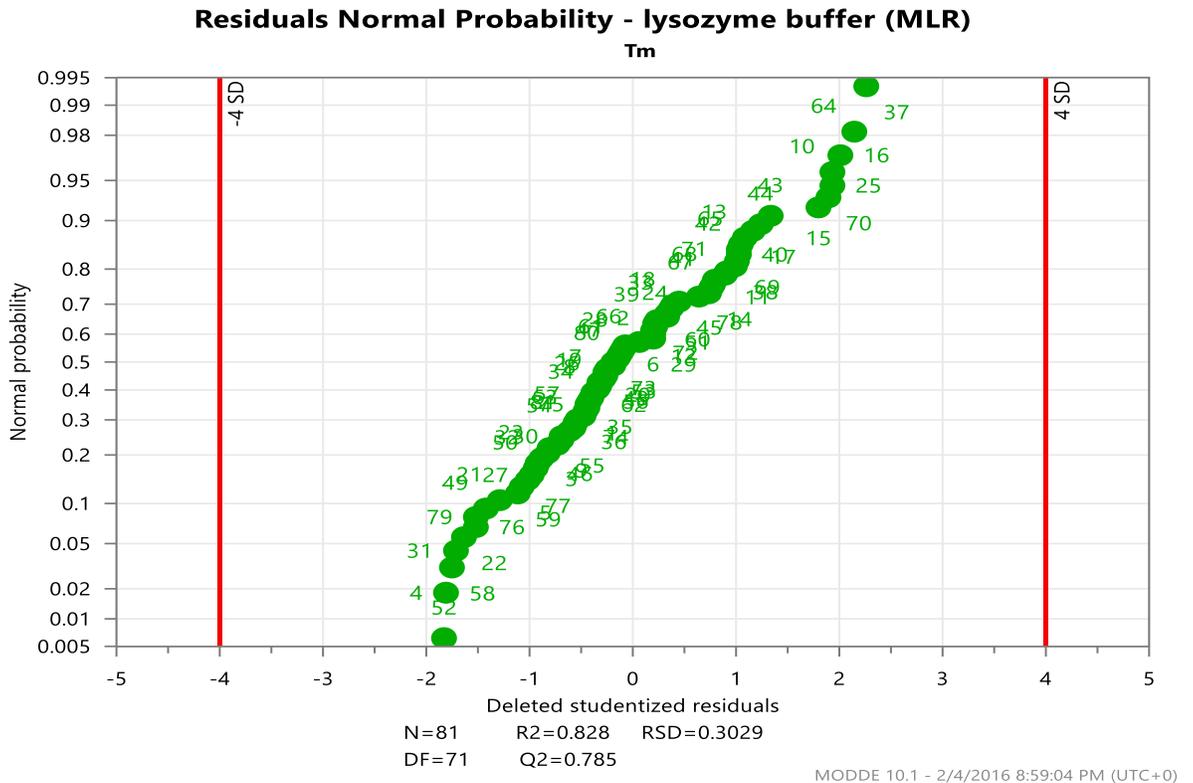


Figure 3.9: Residual normal probability plots for lysozyme and trypsin.

This example of employment of DOE is considered as a valid method to practice QbD in protein formulations development, which can help in the final formulations.

Based on the efficient and reliable models, the optimum formulations were selected according to the desired CQAs, which were demonstrated by the thermal stability, T_m 79 °C, and 68 °C for lysozyme and trypsin, respectively. Phosphate buffer acted as the stabilising buffer for both proteins, and it was chosen as the suitable buffer to prepare the optimised formulations. Two different set points were selected for each protein, by applying combinations, through mathematical optimisation desirability functions. The tuned set points are shown in Table 3.8 and then chosen to prepare the protein formulations with excipients as the following screening steps in the next section.

Table 3.8: The optimised buffer conditions as obtained from analysing and fitting the buffers models, generated by Design of Experiment (DoE) methodology.

Protein	pH	Buffer	Concentration mM
Lysozyme	4.2	Phosphate	10
	4.2	Phosphate	50
Trypsin	3	Phosphate	65
	3	Phosphate	100

3.3.4. Excipients screening

Based on the primary buffer screening and determination of optimum buffer conditions, a set of three different excipients (trehalose, sodium ascorbate, and Pluronic F127) out of three different chemical groups were selected for formulation conditions optimisation, as explained in Section 2.2.4. One full factorial design was built for each protein to

examine the impact of the excipients on unfolding temperature, in addition to interactive effects, thus optimising the models, in order to obtain stable formulations. Three variables were included in every enzyme DOE, two quantitative (buffer concentration, and concentration of excipient) and one qualitative (excipient). Two full factorial designs, T_m values, and statistical parameters are shown in Table 3.9.

Table 3.9: A total of 12 Lysozyme and 12 trypsin formulations included in two different designs of experiments. All Tms are the mean of triplicate DSC scans $T_m \pm SD$.

Protein	Phosphate concentration(mM)	Excipient	Excipient concentration	T_m	$\pm SD$
Lysozyme	10	Trehalose	10	77.29	0.046
	50	Trehalose	10	77.93	0.078
	10	Ascorbate	10	76.81	0.072
	50	Ascorbate	10	76.50	0.075
	10	Trehalose	50	77.35	0.035
	50	Trehalose	50	77.79	0.020
	10	Ascorbate	50	76.87	0.026
	50	Ascorbate	50	76.53	0.061
	10	Trehalose	100	77.44	0.053
	50	Trehalose	100	77.80	0.035
	10	Ascorbate	100	76.87	0.026
	50	Ascorbate	100	76.61	0.050
Trypsin	65	Pluronic	0.02	68.12	0.100
	100	Pluronic	0.02	68.15	0.101
	65	Trehalose	10	68.55	0.062
	100	Trehalose	10	68.19	0.044
	65	Pluronic	0.1	68.73	0.050
	100	Pluronic	0.1	67.78	0.078
	65	Trehalose	50	68.54	0.066
	100	Trehalose	50	68.23	0.085
	65	Pluronic	0.2	68.96	0.164
	100	Pluronic	0.2	67.52	0.101
	65	Trehalose	100	69.41	0.096
	100	Trehalose	100	69.09	0.147

The effect of trehalose and sodium ascorbate on lysozyme thermal stability in this study. However, trypsin thermal stability in the presence of trehalose and Pluronic F 127 was evaluated.

Trehalose is usually added to biological specimens, as extromolyte sugar, in order to protect these samples against extremely harsh conditions; e.g. high temperature. Sodium ascorbate was not used with trypsin, because of its effect on pH, especially, under thermal treatment, and trypsin is highly sensitive to pH changes as implicated in the preliminary pH screening. Thus, Pluronic F 127, as a non-ionic surfactant, was used instead due to its ability to reduce adsorption on hydrophobic surfaces.

These models have predicted and fitted all T_m values by applying multiple linear regression equations. R^2 was more than (0.99 and 0.83), and Q^2 was more than (0.98 and 0.73) for lysozyme and trypsin, respectively. Q^2 and R^2 values were not separated by more than 0.2 -0.3 for each protein, with reproducibility values more than 0.9 for all designs models, which reflects good models.

Table 3.10: The statistical parameters (R^2 , Q^2) obtained after the trypsin model analysis.

Protein	R^2	Q^2
Lysozyme	0.99	0.98
Trypsin	0.83	0.73

Table 3.11: Scaled and centred coefficients along with P values for Tm.

Protein	Factor	coefficient	P-value	Significance
Lysozyme	Trehalose	0.450994	<0.000	Significant
	Ascorbate	-0.450994	<0.000	Significant
	Excipient concentration	0.0240055	0.079	N/S
	Phosphate	0.0433769	<0.000	Significant
	Phosphate*trehalose	0.19611	<0.009	Significant
	Phosphate*Ascorbate	-0.19611	<0.009	Significant
	Trehalose concentration *	-0.0181697	0.178	N/S
	Ascorbate concentration *	0.0181697	0.178	N/S
Trypsin	Trehalose	0.23722	<0.000	Significant
	Pluronic	-0.23722	<0.000	Significant
	Excipient concentration	0.251437	<0.000	Significant
	Phosphate	-0.286781	<0.000	Significant
	Phosphate*trehalose	0.115277	<0.009	Significant
	Phosphate*Pluronic	- 0.115277	<0.009	Significant
	Trehalose concentration *	0.202434	<0.000	Significant
	Pluronic * concentration	- 0.202434	<0.000	Significant

As clear in the table above (Table 3.11), that lysozyme Tm value was significantly affected by the type of added excipients. Therefore, a significant difference between trehalose and ascorbate was observed as higher Tm values were recorded for trehalose containing formulations. However, the effect of excipients was not concentration dependent as the change in trehalose and ascorbate concentration did not modify the Tm values significantly, Figure 3.10 and Figure 3.11 .

Trehalose stabilising effect on proteins and biologics is consistent with what was concluded previously by [167] [114] and [167]. *Kaushik et al. 2003* revealed that; trehalose acted as an exceptional protein stabiliser, as it has increased T_m values for five different proteins, including lysozyme, at various pH points, significantly. Trehalose stabilises proteins in liquid formulations because of higher preferential hydration of the unfolded proteins which eventually leads to decrease the proteins unfolding [167]. The effect of excipients on lysozyme T_m was not concentration dependent as the change in trehalose and ascorbate concentration did not change the T_m values significantly, however, in trypsin, a significant difference was recorded between different excipients concentrations.

Factorial design methodology can evaluate interactive effect between factors. The interaction between excipients and phosphate buffer concentrations showed different patterns depending on excipient type; in trehalose formulations, the interaction with buffer concentration was significantly stabilising, that means the buffer stabilising effect increased synergistically in the presence of trehalose, while it decreased in the presence of ascorbate. This interaction effect relates to the presence of excipients themselves rather than their concentrations since no significant effect was observed for the interaction between the buffer and the excipients concentrations, Table 3.11.

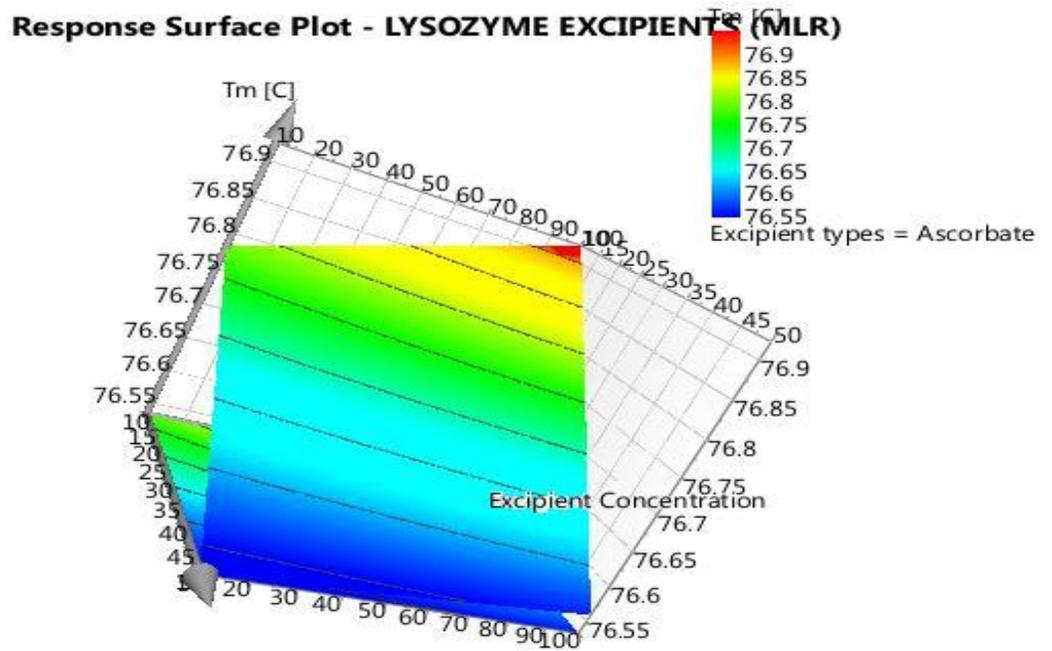
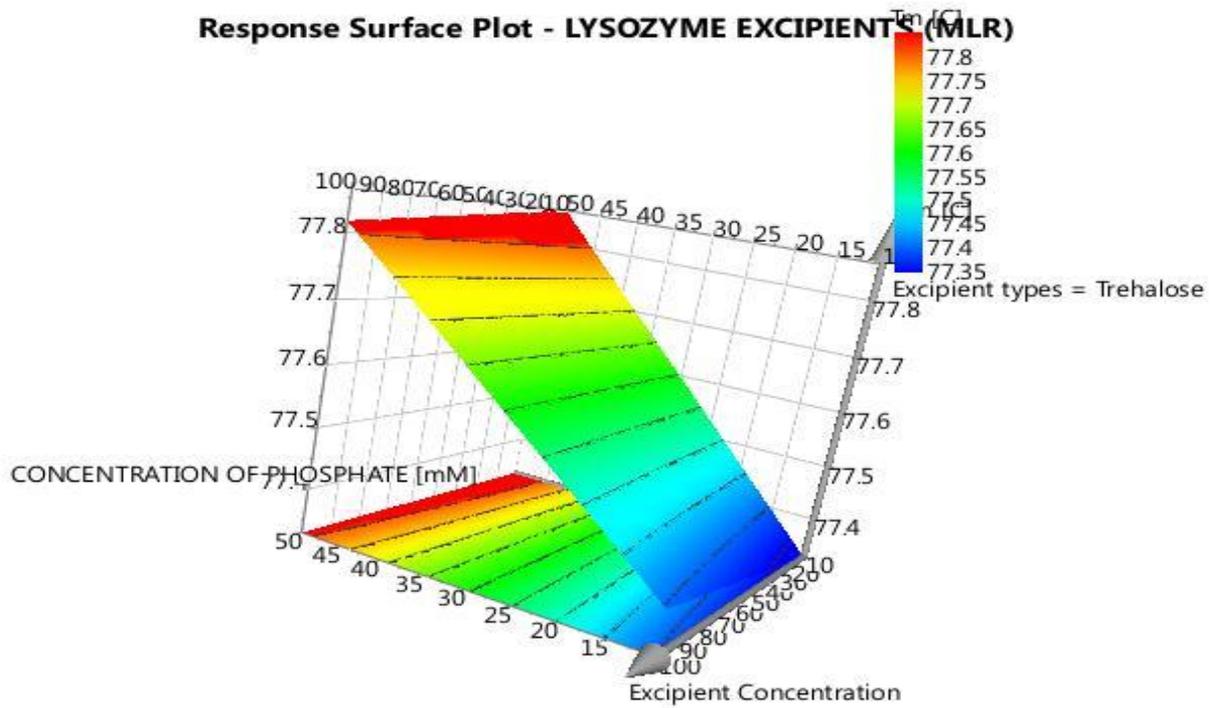
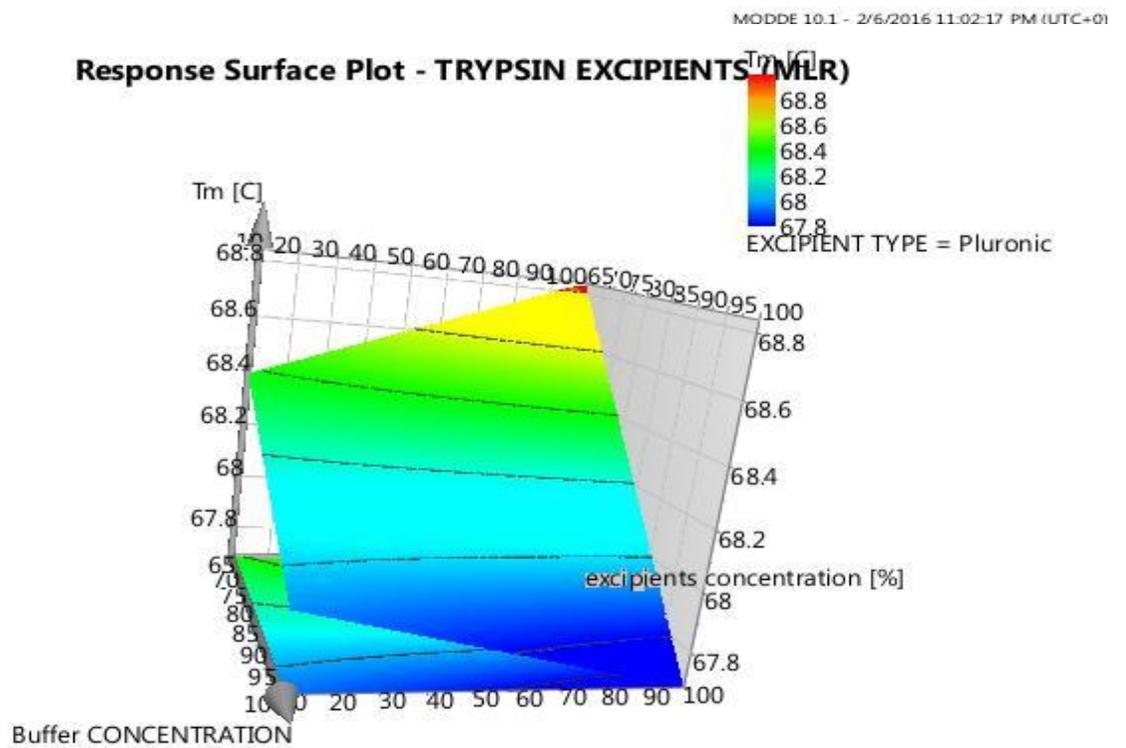
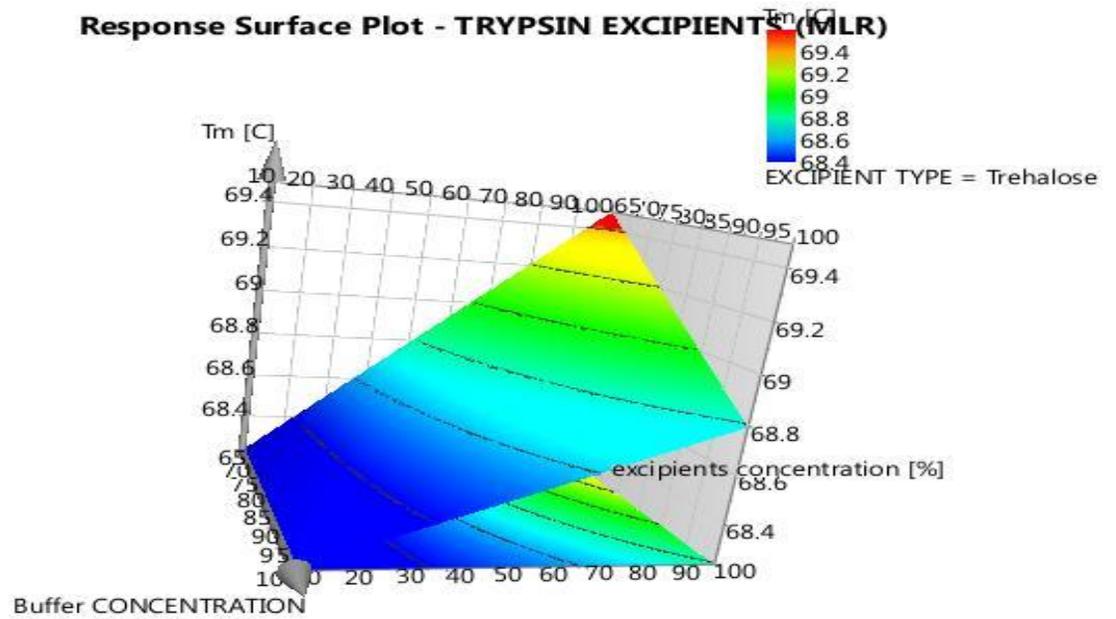


Figure 3.10: Response surface plot of Lysozyme unfolding temperature (T_m) in phosphate in the presence of trehalose and ascorbate. The x-axis and y-axis represent pH values and buffer concentration, respectively. Surface lines are labelled with their T_m values.

On the other side, increasing buffer concentration had destabilised trypsin in the presence of excipients significantly. Like in lysozyme case, trehalose stabilised trypsin significantly over Pluronic F12. Trehalose stabilising effect was concentration dependent, the higher level, the higher T_m value, Figure 3.10. Furthermore, the interaction between buffer concentration and excipient was also significant, the effect of phosphate buffer was even increased in the presence of trehalose and increased with increasing in trehalose concentration. The interaction between factors should be considered even if a single factor or both of them are not significant [168].



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Figure 3.11: Response surface plot of trypsin unfolding temperature (T_m) in phosphate in the presence of trehalose and Pluronic F127. The x-axis and y-axis represent pH values and buffer concentration, respectively. Surface lines are labelled with their T_m values.

Good models have been generated to screen the excipients effect, which has been reflected by high Q^2 values >0.7 . Q^2 values were obtained after drawing the linear correlation between the predicted and observed responses for both lysozyme and trypsin, Figure 3.12. Predictability degree of the models was high; thereby the optimisation process and determination of set points are reliable. The corresponding residual plots also demonstrated almost uniform, and randomly scatter of most of the points around the zero axis, with a range of values between -2 and 3, which is ruling out any implicit trends and patterns, and confirmed a high degree of reliability of the QbD based studies, Figure 3.13.

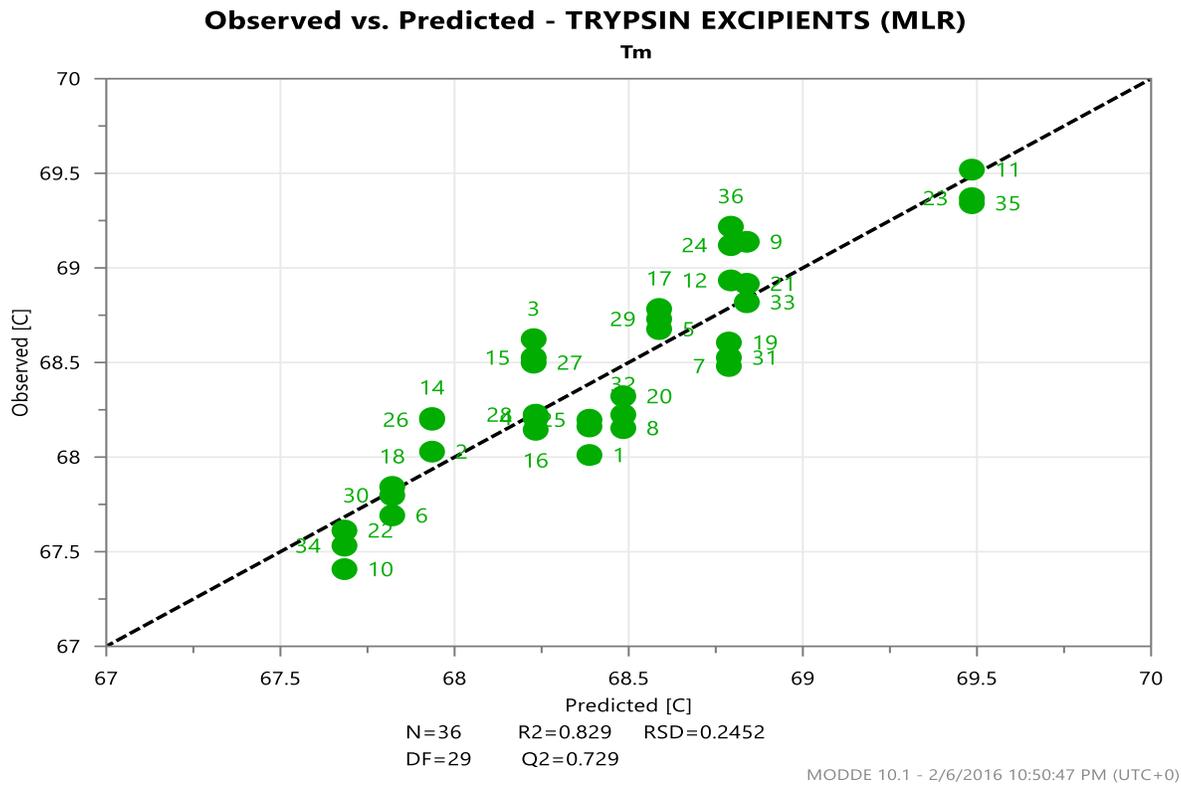
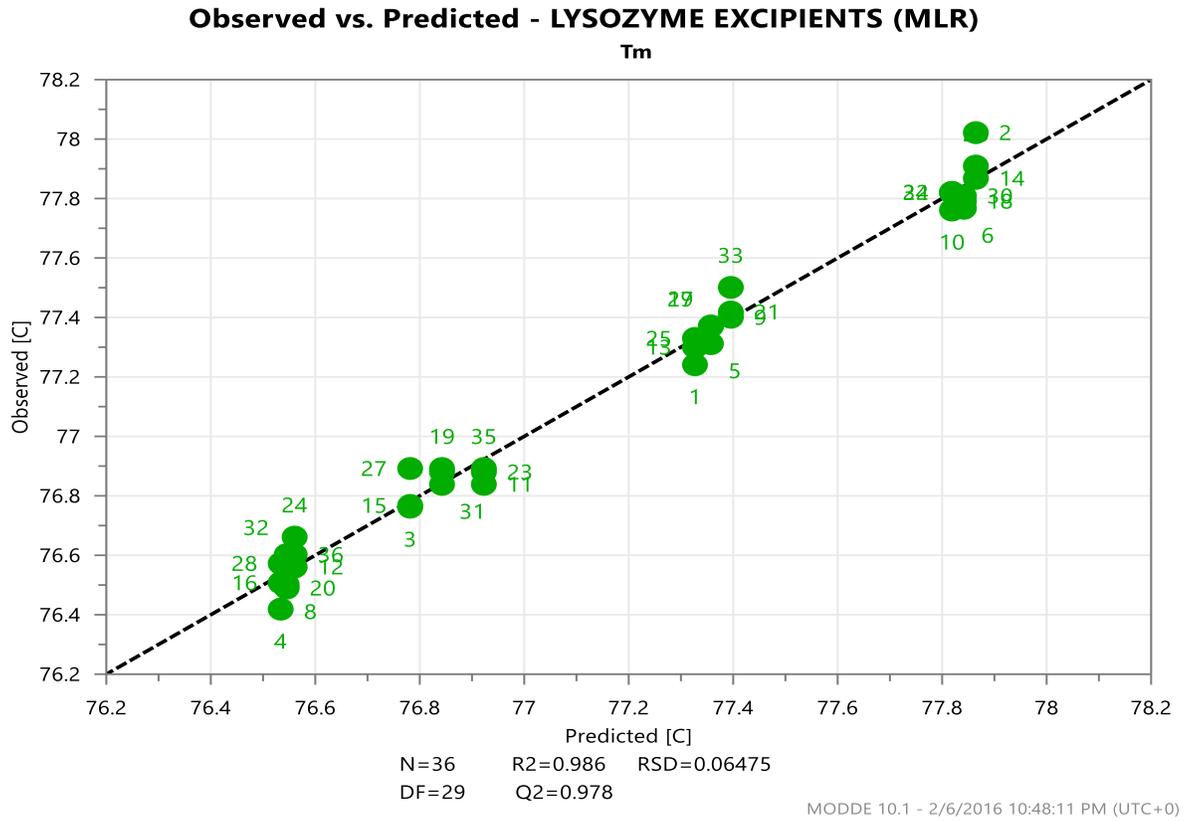
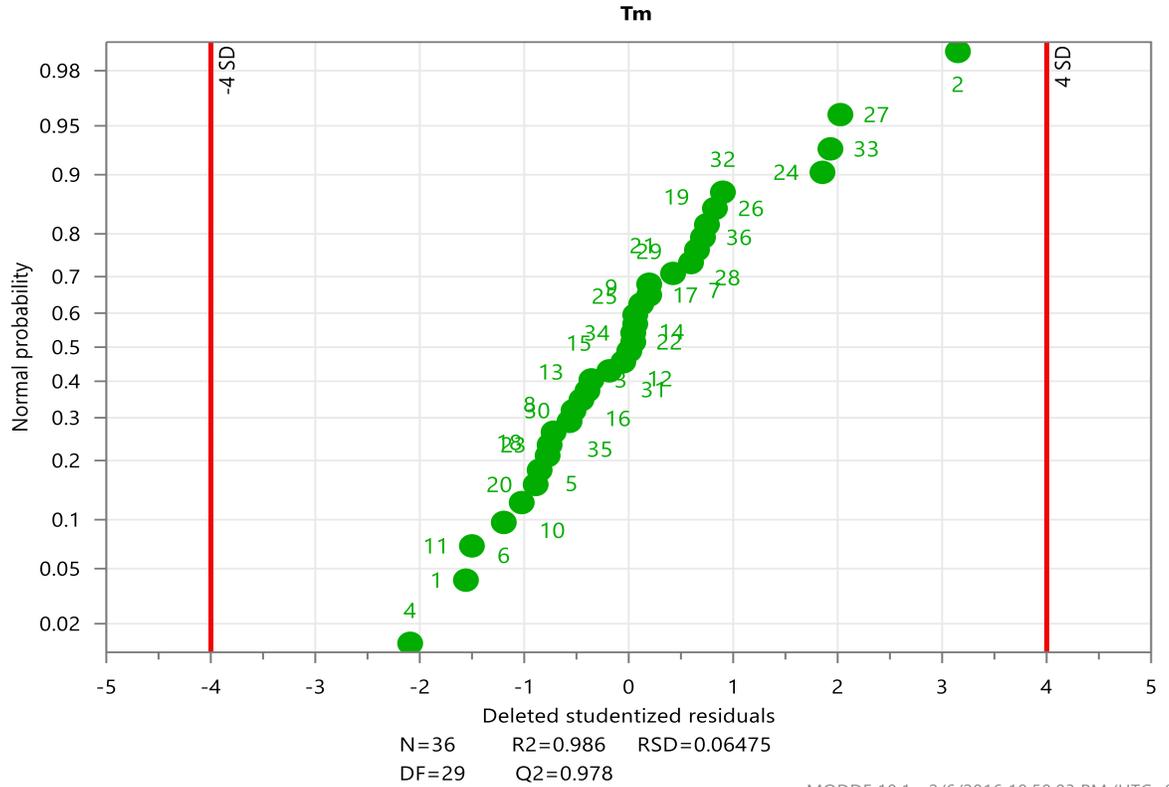


Figure 3.12: Observed vs predicted plot illustrating the prediction power of the lysozyme and protein models.

Residuals Normal Probability - LYSOZYME EXCIPIENTS (MLR)



Residuals Normal Probability - TRYPSIN EXCIPIENTS (MLR)

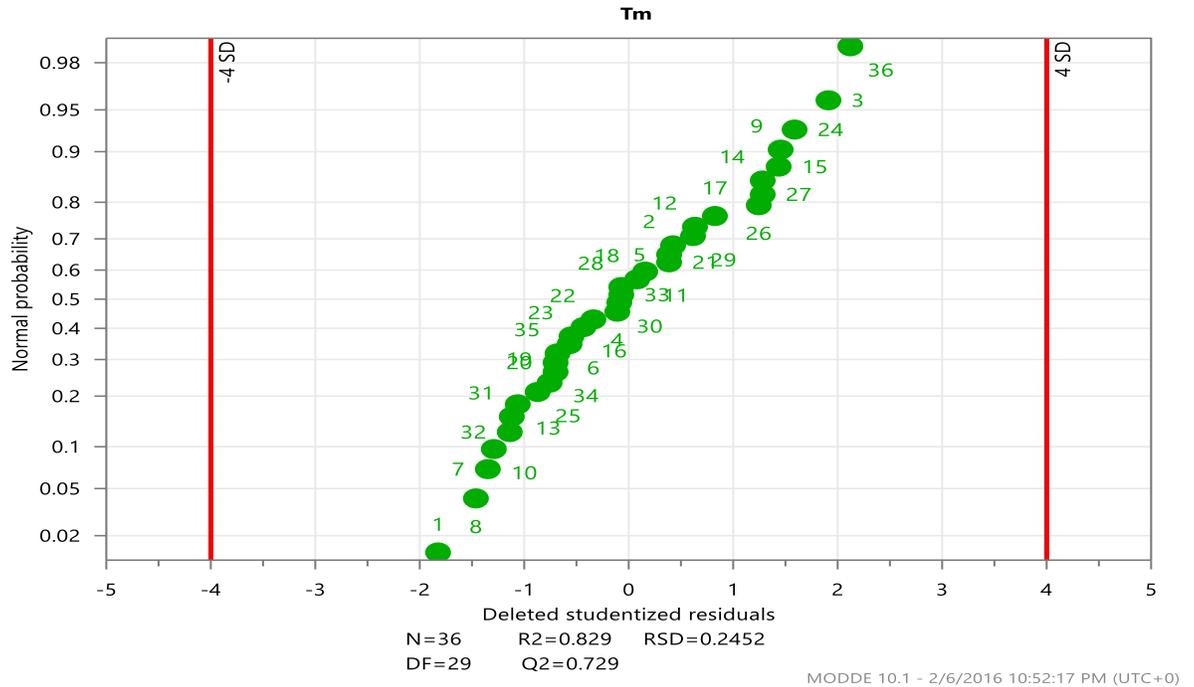


Figure 3.13: Residual normal probability plots for lysozyme and trypsin.

Experimental designs help to optimise the factors at individual levels by mathematical functions. The optimum formulations conditions are illustrated in Table 3.12. These formulations have been further characterised and stored for stability tests.

Table 3.12: The optimised buffer and excipient conditions as obtained from analysing and fitting the excipient models, generated by Design of Experiment (DoE) methodology.

Protein	pH	Buffer	Concentration mM	Excipient	Concentration mM
Lysozyme	4.2	Phosphate	5	Trehalose	50
	4.2	Phosphate	27.5	Trehalose	5
Trypsin	3	Phosphate	69	Trehalose	40
	3	Phosphate	65	Trehalose	10

3.3.5. Characterisation of optimised formulations

3.3.5.1. Thermal stability

The optimum formulations for both proteins were selected, and the conformational stability of each formulation was performed to find the denaturation temperature T_m by using VP-DSC. T_m values for the optimised formulations were recorded and then compared to the predicted values Table 3.13, Figure 3.14. A t-test was carried out to find the significances in T_m difference between the observed and predicted values, no significant difference was recorded, p-value >0.05. The non-significant difference

between the observed and optimised formulations reflected a valid QbD methodology and assent ability of the used mathematical model.

Table 3.13: The observed and predicted T_m of lysozyme and trypsin in the optimised formulations.

Protein	Sample	Observed	Predicted
Trypsin	1	68.43	68.22
	2	70.04	68.65
Lysozyme	1	78.57	77.56
	2	77.75	77.66

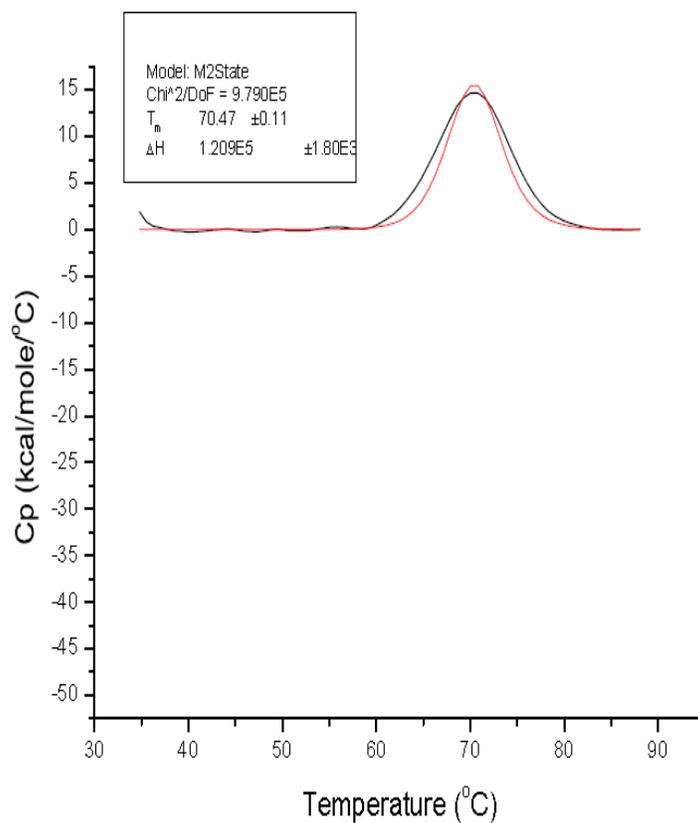


Figure 3.14: HSDSC graph illustrating the T_m value of the optimised trypsin formulation.

3.3.5.2. Biological activity and Storage physical stability

Further to proteins integrity determination by DSC, the optimised formulations biological activity was measured by applying the enzymatic assay methodologies described in Section 2.2.2.2 and Section 2.2.2.3. Biological activity was determined for freshly prepared lysozyme and trypsin samples, and after six month's storage at 5 °C and 25 °C. Significant increase in biological activities has been recorded for the optimised fresh formulations in comparison to fresh formulations containing trypsin and lysozyme at standard conditions i.e. pH 3 and pH 6.24, respectively, see Table 3.14.

Table 3.14: Biological activity of the optimised formulations after and before the storage.

Protein	Sample	BA Fresh	BA after six months 5 °C	BA after six months 25 °C
Lysozyme	pH 6.24	100%	92.6%	79.3%
	pH 4.0	116.1%	104.3%	90.2%
	Optimised formulation 1	121.6%	119.5%	110.9
	Optimised formulation 2 at t0	126.2%	121.9%	116.1%
Trypsin	pH 3	100%	96.4%	86.2%
	Optimised formulation 1	109.3%	106.3%	94.6%
	Optimised formulation 2	107.2%	106.8	88.3

As apparent from Table 3.14, applying QbD approach to optimise the formulation variables, in order to get stable formulations containing stable Active Pharmaceutical Ingredients (API) was found an efficient way to increase the formulation biological activity, and even to retain their activity after storage for the entire periods. Both lysozyme and trypsin optimised formulations maintained more than 100% of their activity after storage in a refrigerator (3-8 °C) for six months. Stability study under the accelerated conditions (25 °C) revealed that the optimised formulations containing either one of the proteins retained (88.3 – 94.6 %) and (110.9 – 116.1%) of their activity when compared to freshly prepared pH 3 and pH 6.24, for lysozyme and trypsin respectively.

This increase in the proteins integrity and activity over the storage period may have happened because of trehalose presence in all formulations in a proper ratio and amount in relative to buffer type, concentration, and pH. Trehalose as sugar works as extremolyte in biologic liquid formulations is able to protect the proteins against the harsh condition e.g. temperature by preferential hydration and exclusion.

3.4. Conclusion

This study has revealed that applying QbD approach and the relevant methodologies i.e. preliminary screening, experimental design, and risk assessment, can predict the formulations factors and variables that have a potential influence on the products quality. Therefore, this prediction aids in the reduction of the early development applied

features, hence, reduce the necessary time, labour, raw material, storage process and overall operation cost. QbD base studies are needed especially in biopharmaceutical formulations when the materials are very expensive and limited with more complicated techniques and skills required since it is more direct to the target. The current study demonstrates two examples to developing protein containing liquid formulations with predefined quality target product profile, and critical quality attributes. Phosphate buffer and trehalose with optimised levels were found to be the best conditions among the screened factors in term of protein integrity.

Next chapter demonstrates the results of developing and validating the analytical and quality control assay methods.

Chapter Four: Analytical methods development and validation

4.1. Introduction

The pharmaceutical analysis is used throughout drug development and including preformulation, formulation, and later stability studies. Therefore, the analytical methodologies play crucial roles in the analysis of pharmaceutical formulation; and they are used for stability indication and formulation characterisation. Stability indicating assays are analytical methods for accurately and precisely analysing the drugs or their relevant formulations in order to assess their capacities to remain within the acceptable range of specifications over the entire period of storage under certain conditions [169]. Furthermore, the analytical methods are required to be reliable and validated in accordance with ICH guidelines to characterise the formulations as evidence to support the selection of the affecting parameters, e.g. excipients, temperature, and the physical state, at different stages of the formulation [170].

Stability indicating assays should assess the overall formulation stability and the drug substances stability. Therefore, the excipients stability and safety profile are crucial and play a critical role in accepting or rejecting the formulation, even if the drug substances are intact. The development of a method to analyse the hydrophilic excipients, e.g. sugar, or salts, when present in the same formulation, is a challenge, as some molecules are structurally related with similar physicochemical characteristics such as; high polars which can hinder their separation by reverse phase chromatography; even if a large proportion of aqueous mobile phases are used with this technique. Hydrophilic interaction liquid chromatography (HILIC), is a type of chromatography in which normal phase stationary phases are used with high organic, but reversed phase, mobile phase.

HILIC technique aids in solving the reproducibility and poor separation problems of other techniques.

Protein formulations are considered as one of the most interesting among pharmaceuticals. Proteins are a diverse group of large molecules that behave differently from other small drug molecules. Proteins require special formulation, characterisation, stability assays and storage conditions [171]. In order to quantify the intact proteins within formulations; size exclusion chromatography (SEC) is commonly used. SEC is a robust, high-throughput analytical method, able to observe the protein aggregates and estimate the percentage of high molecular weight species directly such as; soluble protein aggregates. Moreover, SEC is also able to quantify the fraction of large aggregates indirectly, as a decrease of total peak area [172].

In order to improve the resolution between the drug therapeutic protein, its aggregates/degrades, excipients, and impurities; and to make the method more robust, different parameters should be optimised and controlled in every analytical method. The validation is a crucial part to ensure that the SEC method is suitable for the desired assay.

The regulatory agencies, such as; International Conference on Harmonization (ICH), and European Medicines Agency (EMA) released several documents defining the guidelines, requirements, and key steps of method validation for any analytical test which allows the manufacturers and researchers to adopt a consistent, systematic analytical approach to the validation or analytical methods.

Any method for the analysis of biologics, including proteins, should demonstrate the robustness and reliability to separate the main analyte and measure its concentration

in a specific biological formulation in the presence of impurities and other compounds. In this study, a SEC method was selected, developed and validated to examine the long-term physical stability and the characterisation of nanocapsule formulations containing lysozyme, trypsin, and DNase I. In addition to a SEC method, HILIC methods were developed and validated to assess the long-term stability of excipients.

The methods were assessed and validated in term of specificity, linearity and range, accuracy, precision, lower limit of detection (LLOD), lower limit of quantitation (LLOQ), and robustness. A fractional factorial design of experiment (DOE) was used to investigate each variable's role in the assay result.

4.2. Aims and Objectives

The aim of this chapter is to develop and validate accurate, precise, and robust analytical methods, which they are able to separate the proteins, lysozyme and trypsin, from other formulation components such as; excipients and protein by- products. The purpose of protein formulations analysis is to evaluate the long-term stability of the liquid formulations and the characterisation of nanocapsules containing proteins. Moreover, analytical methods to assess the liquid formulation excipients, trehalose, ascorbate, and Pluronic F127, stability were also developed and validated with ensured accuracy, precision and robustness.

4.3. Results and discussion

4.3.1. Development and validation of Size Exclusion Chromatography (SEC) method as stability indicating assay

The separation method was developed by utilising Size Exclusion Chromatography SEC column to assess the physical stability of the used proteins. The analytical parameters should be wisely selected, controlled and adjusted to obtain an efficient separation and acceptable resolution. In the current study, the analytical parameters were selected to suit the nature of the proteins of interest and to achieve the desired purpose. The stationary phase, column pore size, mobile phase type, pH of the mobile phase, and salt concentration in the mobile phase are the factors determine the separation of the protein molecules. The column was selected to have an insert and silica based stationary phase with no interaction with no undesired interaction with the protein. Moreover, the pore size of the column was selected to fit the molecular weight of the proteins under investigation. Hence, 100 Å pore size was selected as it is suitable for the protein with molecular weight between 0.1 – 100 KDa. The molecular weights of lysozyme, trypsin, and DNase I are within the mentioned range.

Phosphate buffer at pH 7 was selected as a mobile phase for analysing the proteins as a close to the physiological characteristics. The concentration of phosphate buffer (150 mM) was selected to provide high salt content, as high salt content in the mobile phase reduces the interaction of the protein with the stationary phase.

4.3.1.1. Specificity

The specificity of the bioanalytical assay is the ability to assess the therapeutic protein unequivocally in the presence of potential interferences such as; inactive ingredients in the formulated products and the degradation and aggregation products. The specificity of the SEC method was examined qualitatively by lysozyme/trypsin peak area. In order to demonstrate the specificity, lysozyme samples were analysed in the presence of excipients, Sodium ascorbate, trehalose, or both, while trypsin samples were analysed in the presence of trehalose, Pluronic F-127 or both. No interferences appeared at the retention time of each protein in the presence of any other potential excipients, Figure 4.1.

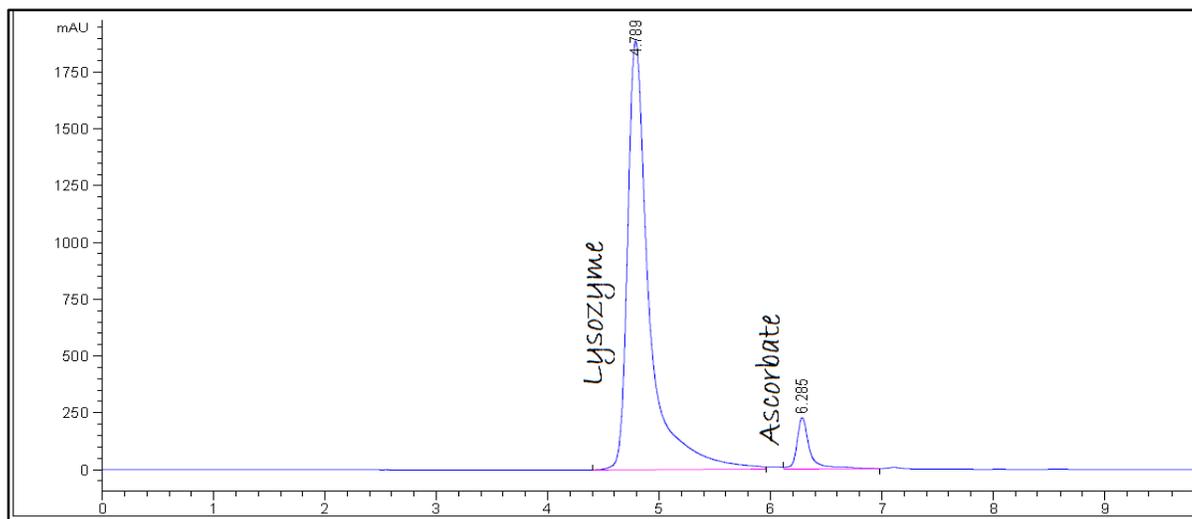


Figure 4.1: SEC chromatogram for lysozyme with sodium ascorbate and trehalose lysozyme 4.9 minute, ascorbate 6.3 minute. The conditions were mobile phase: 150 mM phosphate buffer pH 7, and column: SEC-5,100 Å, 7.8x150mm, with internal temperature 25 °C. a flow rate was 1 ml/min and 1 µl injection volume.

Both proteins were exposed to stress conditions to generate their degradation products. Heating, extreme acidity, extreme alkaline conditions, and denaturants such as urea are a suitable option to force degrade the protein.

Lysozyme and trypsin samples (4mg/ml) were prepared in alkaline conditions, 0.1 mole/L Sodium hydroxide solution, heated to boiling point, left to cool down, and then examined by using SEC. The chromatograms showed late eluting peaks, demonstrating protein degradation products, which were smaller than the original proteins sizes (Figure 4.2).

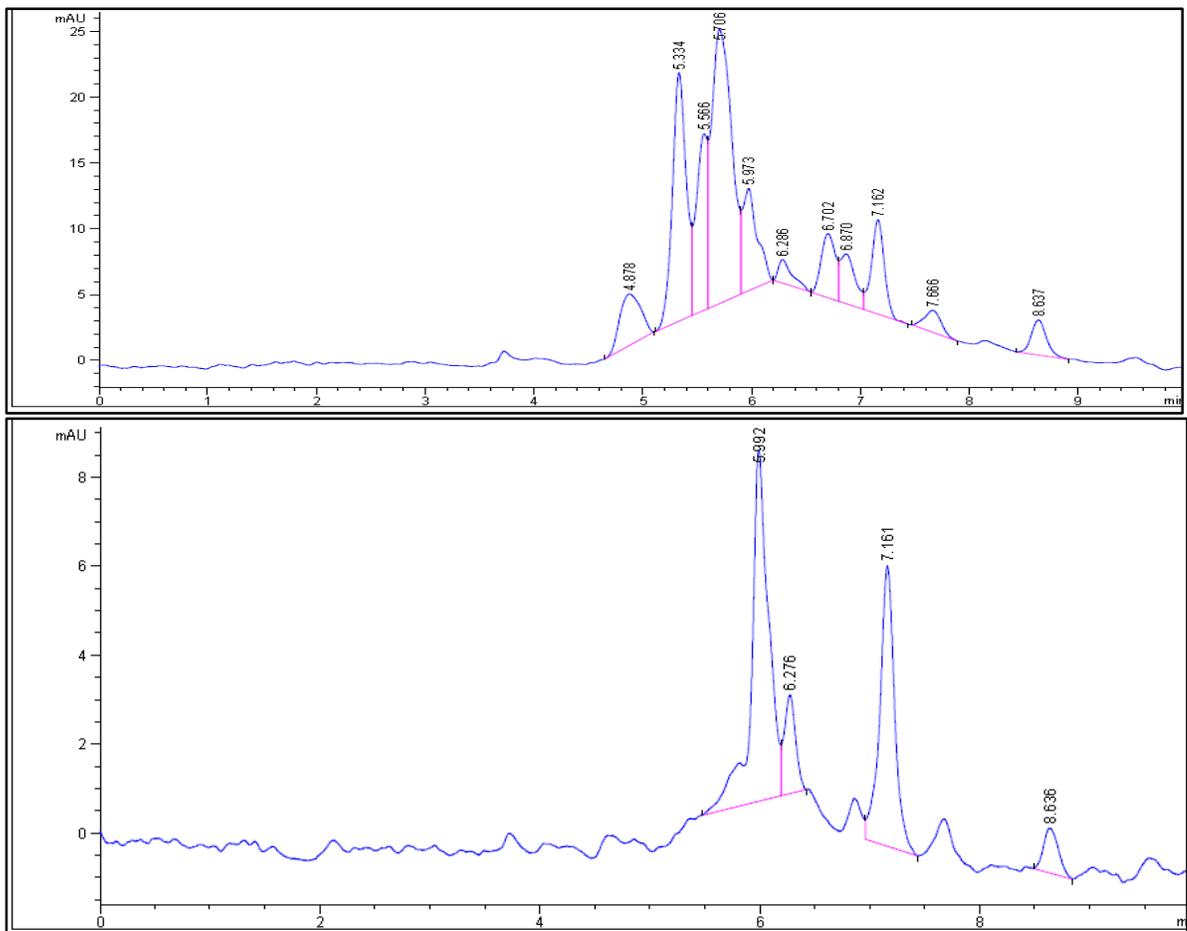


Figure 4.2: SEC chromatogram for trypsin (above) and lysozyme (below) after heating in alkaline media. The conditions were mobile phase: 150 mM phosphate buffer pH 7, and column: SEC-5,100 Å, 7.8x150mm, with internal temperature 25 °C, with a flow rate was 1 ml/min and 1 µl injection volume.

The aggregation products were generated by forming concentrated proteins solutions (50mg/ml) in urea and stored at room temperature for one week to enhance the aggregation process.

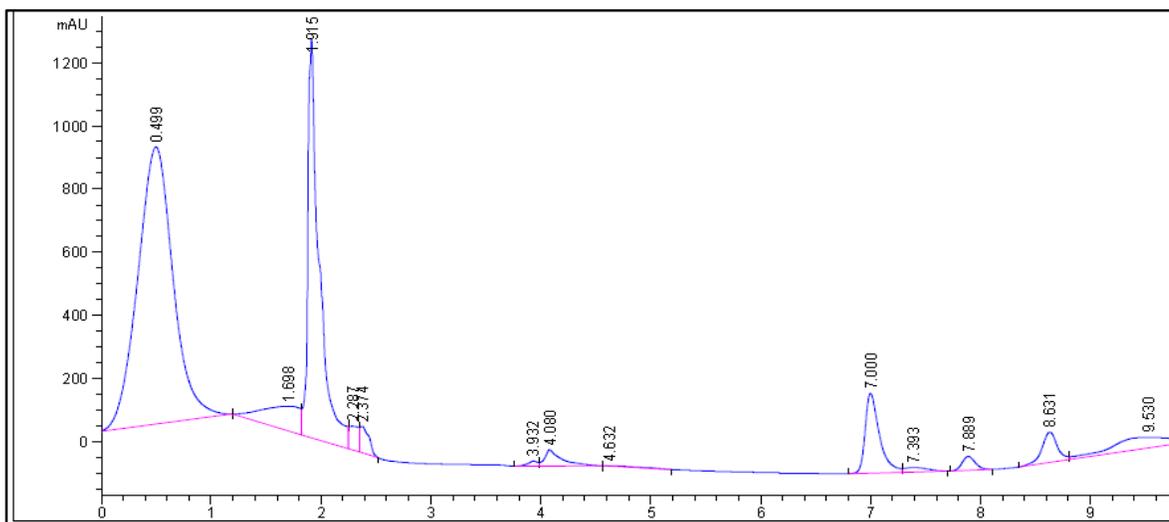


Figure 4.3: A Size Exclusion chromatogram for aggregated trypsin. The conditions were mobile phase: 150 mM phosphate buffer pH 7, and column: SEC-5,100 Å, 7.8x150mm, with internal temperature 25 °C, with a flow rate was 1 ml/min and 1 µl injection volume.

The results of which demonstrates aggregation molecules at 0.5 and 1.915 minutes, (Figure 4.3).

The method was specific and able to separate lysozyme and trypsin from other formulation ingredients. In addition to a well-separated protein, peak was observed at the same retention time.

4.3.1.2. Linear range

The relationship between analyte concentration and the response is called calibration curve. The calibration curve is considered the way of expressing the linearity. The concentration range should be pre-decided before starting method validation, as a lower limit should be LLOQ, and the upper limit should be at least 120% of analytical concentration (4mg/ml).

In this study, eight standards were prepared from 50 µg/ml to 8 mg/ml to characterise linearity. Each concentration level was freshly analysed in triplicate. The linear regression and correlation coefficient were found from the plotted graphs between peak area versus concentration which were then represented by least square regression. High correlation coefficients were found to be 0.9998 and 0.999 for trypsin and lysozyme, respectively, (Figure 4.4 and Figure 4.5).

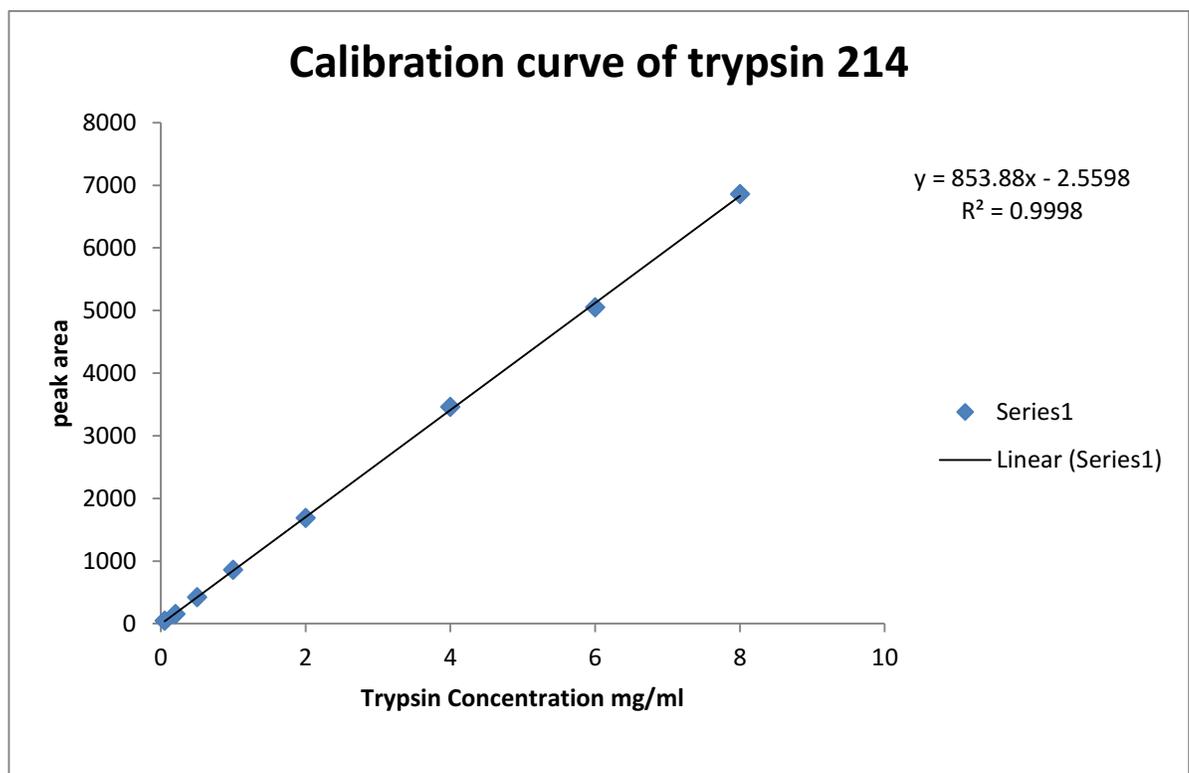


Figure 4.4: Calibration curve of trypsin, the curves correlate the samples concentration with an area under the curve over the detected range. Error bars are included into the line.

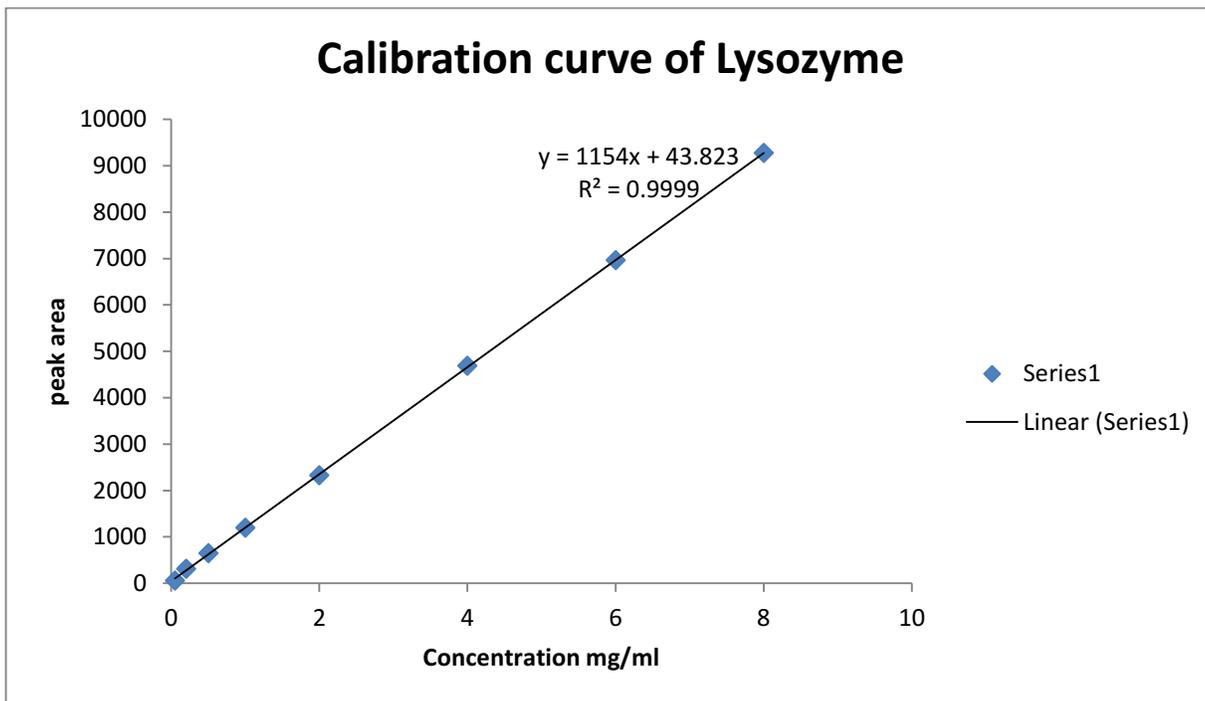


Figure 4.5: calibration curve of lysozyme, the curves correlate the samples concentration with an area under the curve over the detected range. Error bars are included into the line.

4.3.1.3. Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

The limit of detection and the limit of quantitation were determined by the peak-to-noise method. The limits of detection and the limit of quantitation were found to be 0.05 mg/mL and 0.02 mg/mL respectively for both proteins, (Figure 4.6).

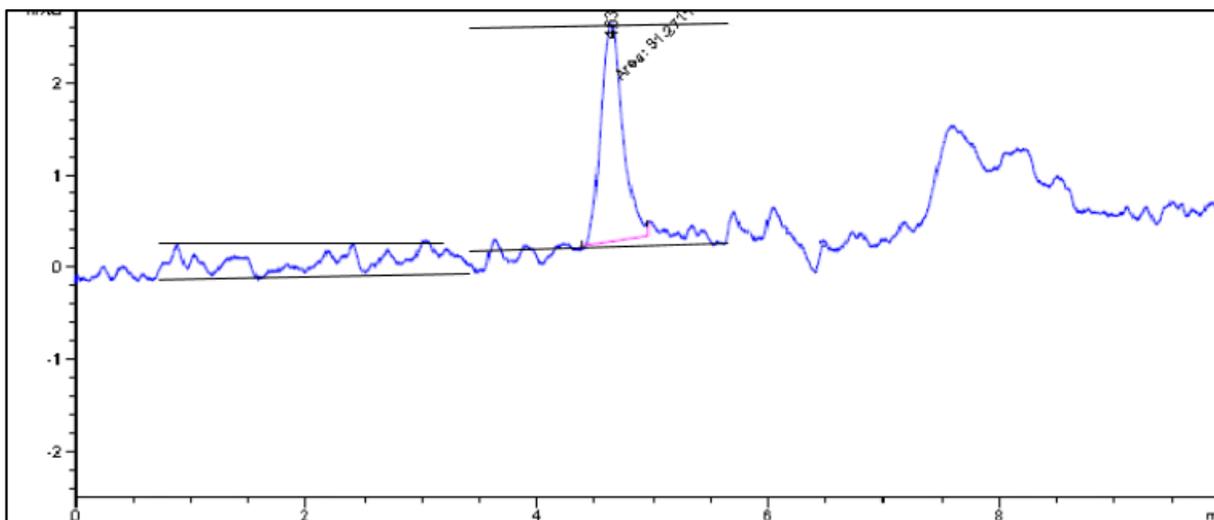


Figure 4.6: SEC chromatogram showing the Lower Limit of Detection (LLOQ) of Trypsin sample (50 µg/ml). The conditions were mobile phase: 150 mM phosphate buffer pH 7, and column: SEC-5,100 Å, 7.8x150mm, with internal temperature 25 °C. a flow rate was 1 ml/min and 1 µl injection volume.

4.3.1.4. Accuracy

The accuracy study has been performed by analysing QC samples at four levels for five samples per level on three different days (two samples per day). In order to determine within-run accuracy the values of their averages was calculated by dividing the average of six samples of each level by the nominal value obtained from calibration curve equation, and between-run accuracy was calculated by dividing each value of six samples of each level by the nominal value. Table 4.1 and Table 4.2 show the between-run and within-run accuracy values. It is clear that the percent recovery of the average of each level at low, medium, and high values was found in range 101.71 -103.08 and 94.41 – 101.02 of nominal value for trypsin and lysozyme, respectively. The recovery values for LLOQ samples were 95.75% and 96.17% for trypsin and lysozyme, respectively. The between-run accuracy showed 91.48% - 109.63% for trypsin and

90.60% – 103.65% for lysozyme. QC samples at LLOQ concentration also demonstrated accurate results within 20% of nominal values.

This method showed accurate results as all QC samples were within 15% of nominal value, and 20% at LLOQ based on.

Table 4.1: Analysis of lysozyme and trypsin between run accuracy.

Protein	Concentration (mg/ml)	Assay Results
Trypsin	6.00	103.08 %
	4.00	102.81%
	0.20	101.71%
	0.05	95.75%
Lysozyme	6.00	101.03%
	4.00	98.55%
	0.20	94.41%
	0.05	96.17%

Table 4.2: Analysis of lysozyme and trypsin within run accuracy.

Level	Concentration mg/ml	Trypsin	Lysozyme
High	6	100.013%	101.78%
	6	102.16%	100.25%
	6	105.24%	101.57%
	6	105.93%	104.46%
	6	102.08%	98.77%
Medium	4	106.57%	100.08%
	4	109.45%	96.79%
	4	95.05%	100.31%
	4	98.38%	100.47%
	4	97.78%	97.83%
Low	0.2	97.90%	97.77%
	0.2	104.21%	103.16%
	0.2	91.48%	90.80%
	0.2	105.88%	93.26%
	0.2	101.93%	90.60%
LLOQ	0.05	102.68%	95.96%
	0.05	109.99%	98.21%
	0.05	90.90%	97.23%
	0.05	86.37%	102.83%
	0.05	88.83%	96.86%

4.3.1.5. Precision

The precision of newly developed method was evaluated by intra-day and inter-day precision and was expressed by percent relative standard deviations of peak area. Intra-day and inter-day precision were carried out by performing six replicates of LLOQ, 0.2 mg/ml, 4 mg/ml and 8 mg/ml. The %RSD of the peak area of intra-day and inter-day precision results were calculated. The % RSD value of intra-day precision (all four concentrations) was found to be less than 3.1% and %RSD value of inter-day precision, all three concentrations, was found to be <6%.

4.3.1.6. Robustness

The robustness is a crucial procedure during method validation, to ensure that the results are not significantly affected if any analytical parameters are subject to small variations.

In order to assess the method robustness; mobile phase pH, mobile phase concentration, and flow rate, were changed within 5% interval and combined into the fractional design of experiment (DOE). Each DOE consists of nine different conditions in addition to centre point for each protein design, as described in Section 2.3.1.6. Quality control samples of each protein at 4mg/ml concentration were prepared to be analysed in triplicate at these conditions.

DOE analysis demonstrated no significant effect on either peak area or on retention time had been occurred when changing any parameter by $\pm 5\%$ range, or by the interaction of changed parameters.

It has shown by calculating the relative percent of peak area of QC after changing the conditions from the nominal value that were obtained by the method without any parameter variations (calibration curve), that the recovery percent under all changed conditions were between 94.7% - 100.4%, and 99.0% -105.9% for trypsin and lysozyme, respectively. The changing in the retention time was within a very narrow range for both proteins, it was around $\pm 8\%$ under the most extreme conditions, this result was noticed with lysozyme when three parameters were changed at the same time. The above results revealed, that validated SEC method demonstrates a good robustness and no significant effect can happen if small variations in analytical parameters happen.

4.3.2. Validation of Size Exclusion method to characterise nanocapsule formulations containing proteins

In order to characterise nanocapsule formulations and quantify the amount of encapsulated proteins an accurate, robust and reliable analytical methods should be applied. In current study, proteins were quantified by applying the previously validated SEC method, Section 2.3.1. The previous method was validated as the stability indicating assay for lysozyme and trypsin liquid formulations. Therefore, partial validation procedures are required to be performed to suit the method for the characterisation purposes. However, a full validation was also conducted to characterise the encapsulated DNase I in the polymeric nanocapsules. The injection

volume for the current method was 10 µl rather than 1 µl for the previously used in liquid formulations. The rationale behind increasing the injection volume is to decrease the value of LLOQ which in turns, increased the method detection and quantitation sensitivity since the method is to be applied at much-diluted scale.

4.3.2.1. Partial validation of Size Exclusion method to characterise polymeric nanocapsules containing lysozyme and trypsin

In order to increase the sensitivity of applied method injection volume was raised from 1 µl to 10 µl. The increase in injection volume changed the instrument response values and, thus, changed the linearity range. Therefore, partial validation of the method is required to achieve the analytical purposes. Specificity test was performed due to the different expected materials in the samples, and linearity was studied by plotting new calibration curves fit to the new concentrations.

4.3.2.1.1. Specificity

Specificity tests were carried out by analysing a sample of dissolution aliquot, which should contain the expected substances. After analysing the aliquot, the system was able to separate the proteins from other expected substances, (Figure 4.7).

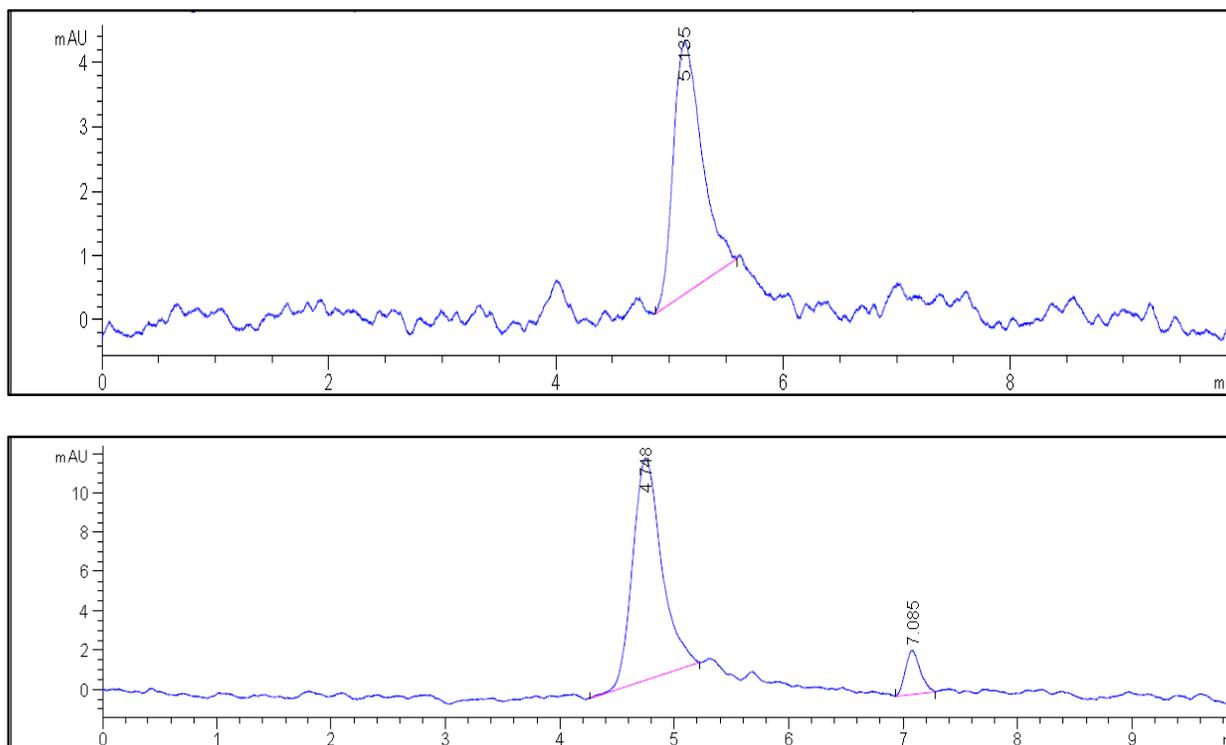


Figure 4.7: SEC chromatogram demonstrating the specificity of the assay to characterise lysozyme and trypsin in nanocapsule formulations.

Figure 4.7 illustrates the ability of the analytical method to separate lysozyme and trypsin from other potential substances. Lysozyme and trypsin were eluted at their retention time and their well separated nice shape peaks were observed without any interference with other peaks.

4.3.2.1.1. Linear ranges

Changing the injection volume affect the concentration of eluted substances from the column and therefore changes the amounts of detected materials by the detector; UV and the values of peak area. Increasing the injection volume from 1 μ l to 10 μ l increased the peaks height and peak area, and increased method sensitivity by decreasing the LLOQ. The highest expected value for the nanocapsule aliquot is 100 μ g/ml, so the

upper limit of the calibration curve was 200µg/ml. Calibration curves were generated over a range of LLOQ- 200µg/ml for both lysozyme and trypsin, with a correlation coefficient (R^2) 0.9999, Figure 4.8 and Figure 4.9. LLOQ values were 10 µg/ml and 20 µg/ml for lysozyme and trypsin, respectively. Whilst, lysozyme and trypsin have been detected down to 5 µg/ml and 10 µg/ml, respectively.

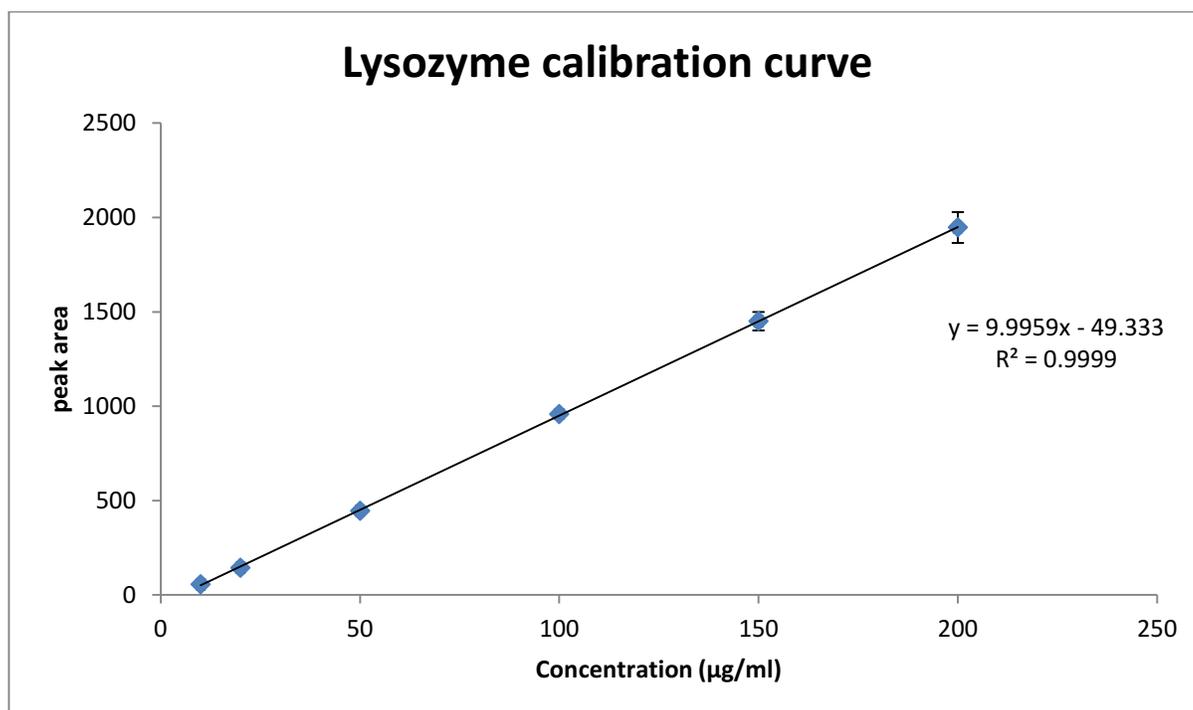


Figure 4.8: Lysozyme calibration curve at concentrations fit nanocapsule formulations. Error bars are included into the line.

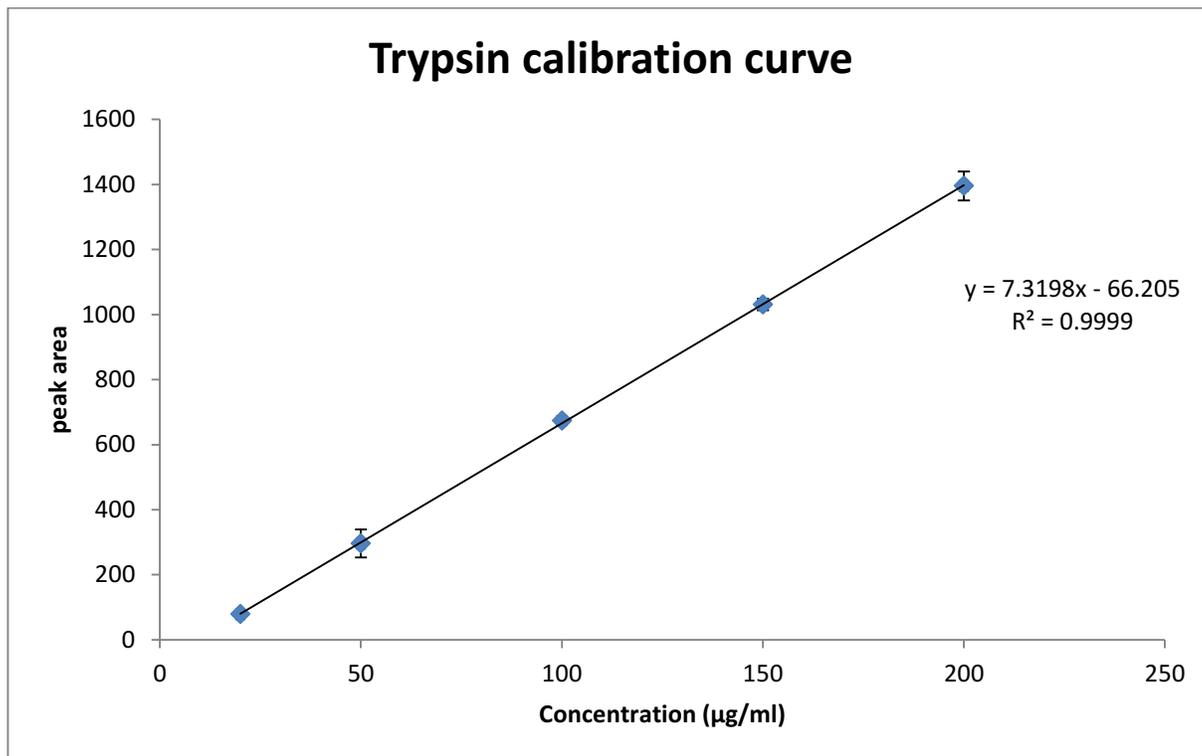


Figure 4.9: Trypsin calibration curve at concentrations fit to nanocapsule formulations. Error bars are included into the line.

4.3.2.2. Validation of SEC method to characterise polymeric nanocapsules containing Deoxyribonuclease I (DNase I)

Deoxyribonuclease I DNase I is a therapeutic enzyme in the treatment of respiratory disorders, like cystic fibrosis and taken in inhalation dosage forms. However, in order to reduce the side effect associated with this enzyme and to improve the patient compliance an oral dosage form could be considered as one the promising approach). In this study, DNase I containing nanocapsule formulations were prepared characterised by using pre-validated analytical approaches and analytical methods are required to characterising these formulations. The applied method for characterising lysozyme and trypsin was adapted to quantify DNase I. Therefore, full validation procedures were performed in order to ensure using specific and robust method able to quantify the encapsulated DNase I accurately, and precisely.

4.3.2.2.1. Specificity

Specificity test is a qualitative test to assess the system ability to separate the desired substances from other ingredients by applying certain method. Specificity was carried out by analysing DNase I containing nanocapsules aliquot. A well separated DNase I peak eluted early when compared to lysozyme and trypsin, due to its larger size and molecular weight, Figure 4.10.

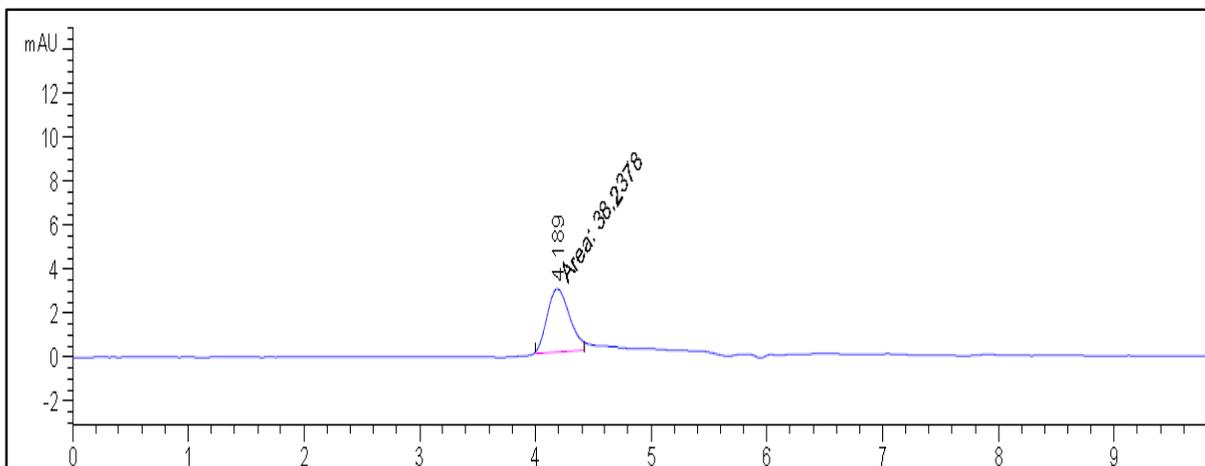


Figure 4.10: SEC chromatogram demonstrating the specificity of the assay to characterise Deoxyribonuclease I in nanocapsule formulations.

4.3.2.2.2. Linear range

The correlation plot between series concentrations of the enzyme in X axis and area under the curve in Y axis was drawn over a range of (10- 200 $\mu\text{g/ml}$). LLOQ and LLOD were recorded by using signal noise ratio method at (5 $\mu\text{g/ml}$, and 10 $\mu\text{g/ml}$), respectively. A linear relationship with high correlation coefficient and low intercept value were noticed, Figure 4.11.

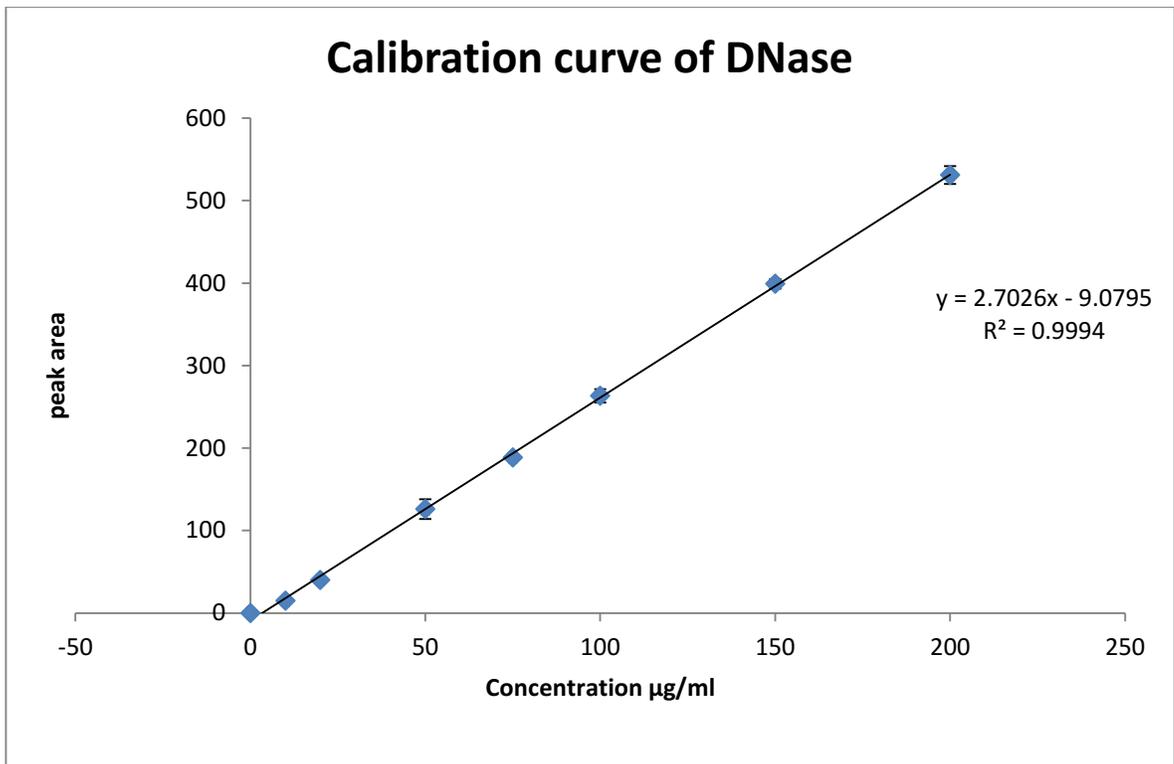


Figure 4.11: Deoxyribonuclease I calibration curve at concentrations fit nanocapsule formulations. Error bars are included into the line.

4.3.2.2.3. Accuracy

In order to assess the reliability of readings, accuracy test is performed to calculate the closeness of the results to nominal values. Four different concentrations of QC samples i.e. LLOQ, low QC, mid QC, and High QC were prepared in triplicate over three consecutive days and inter and intra accuracy were calculated. Inter accuracy was calculated by dividing the averages of each QC concentration by the nominal value of the respective concentration. While intra accuracy is the recovery value of dividing each value over the respective nominal value, inter and intra accuracy values were within $100\% \pm 7\%$ for low, mid and high QC, whilst, the values were between (94.9% –

103.5%) for LLOQ. These results are within the acceptable range for accuracy $\pm 15\%$ and for LLOQ is $\pm 20\%$.

4.3.2.2.4. Precision

Precision was measured to determine the closeness of the results together. Inter and Intra precision were found out by calculating CV after dividing the averages by the standard deviations as described in section. The method was found to be highly precise with values reached up to 2.64% for inter and intra precision at low, mid QC, and high QC, while the CV was less than 5% for LLOQ. Precision readings were within the regulatory requirements to validate the method in order to analyse the biologics.

4.3.2.2.5. Robustness

The robustness is important, but an optional requirement, in any analytical method and validation procedures in order to assess the ability of the method to maintain the results within a narrow range and with no significant difference when changing the analytical parameters within small variations.

In order to assess the method robustness some analytical parameters have been modified i.e. mobile phase pH, mobile phase concentration and flow rate within 5% interval and combined into the fractional design of experiment (DOE) exactly as have been performed for lysozyme and trypsin. No significant difference was noticed between the results themselves and with the centre points. The results were analysed in term of peak area and retention time.

Calculation the percentage of the peak area of the samples (after changing the chromatographic parameters) to the samples at centre point conditions (pH 7, flow rate 1 ml/min, and buffer concentration 150mM) demonstrated that all the percentages were less than 15%, which is the acceptable value for the protein.

No significant differences in retention times between all conditions were recorded. Furthermore, the percentages of different retention times to centre point retention time were within $\pm 8.6\%$. The developed method for DNase I analysis is robust, and no significant effect is expected if any small changes to the analytical conditions have been occurred either by fault or by deliberate.

4.3.3. Method development and validation of stability indicating and Quality Control (QC) assay of formulation excipients

Stability assays for liquid protein formulations require a number of methods to analyse the protein active ingredient, in addition, the number of excipients. The method for protein analysis by using SEC was validated as shown previously Section 4.3.1.

The three used excipients have different structures, but closely similar physicochemical characteristics, thus, there is a need to develop and validate an associated analytical method to detect the excipients.

When reviewing the validation of analytical methods, 10%, and 5% for LLOQ and other QC samples, respectively, were applied as the acceptable accuracy and precision criteria for the analysis of non-biological molecules.

Method validation was carried out in liquid protein formulations to support the stability indicating assays to identify the excipient impurities over the long term of storage under different conditions or different temperatures.

4.3.3.1. Development of analytical methods to assess excipients stability

Firstly, a gradient reverse phase high performance liquid chromatography (RP-HPLC) was developed by utilising Jupitar C18 column with UV-DAD and ELSD detectors, as discussed in Section 2.3.3. However, an early elution of the ascorbate and trehalose with bad resolution was obtained due to their hydrophilicity properties, and both of them can be detected by ELSD. This problem could not be overcome by changing the analytical parameters. Thus, it restricted the using of RP-HPLC.

Therefore, (Phenomenex HILIC (SILICA) LUNA 3 μ HILIC 200 Å (4.6 × 150 mm, 3 microns) at 30 °C) column was selected to quantitatively analyse the excipients, trehalose, ascorbate, and Pluronic F127.

In order to obtain appropriate chromatographic selectivity and sensitivity the composition and pH of mobile phases, flow rate, injection volume, and detectors conditions were optimised.

The initial step was mobile phase buffer pH optimisation. Silica stationary phase is suitable only with acidic and neutral pH, while alkaline pH could affect or damage the stationary phase. Ammonium acetate buffer was prepared at two different pH levels i.e. 3, and 5.8 in order to assess the effect of pH on peaks resolution, retention time and the overall separation efficiency. The pH screening test has been performed by using

95:5 acetonitrile: water ratio in the weak mobile phase. Using a buffer with pH 3 in mobile phase has helped the molecules to elute very fast, with no Pluronic F127 peak, and very close trehalose and ascorbate peaks. However, ammonium acetate buffer at pH 5.8 has well-separated peaks and provided a Pluronic F127 peak with a reasonable distance from t_0 , Figure 4.12.

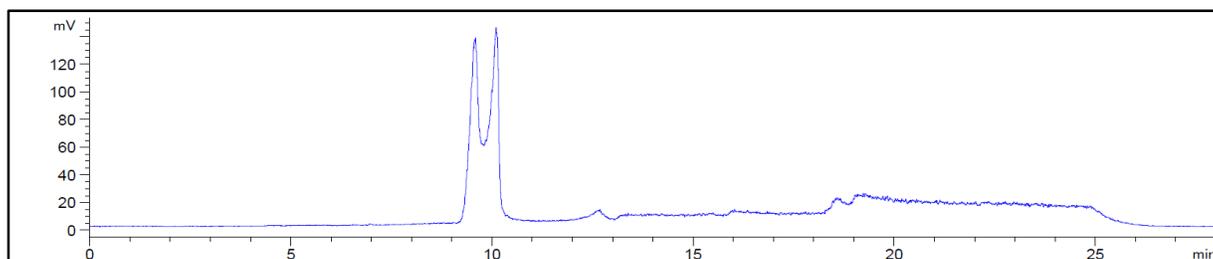


Figure 4.12: HILIC chromatogram is showing the bad resolution between trehalose and ascorbate peaks when the used buffer was at pH 3.

After that, the effect of flow rate as an essential chromatographic parameter on the separation process has been assessed. Even 2 ml/min flow rate eluted the molecules very fast, but the peaks resolution and separation were poor. However, reasonably late and well-separated peaks were obtained at 1 ml/min flow rate.

After fixing the buffer pH at 5.8 and the flow rate at 1 ml/min next step was to pick the best (acetonitrile: buffer) ratio in the weak mobile phase. Different ratios were used i.e. 100:0, 98:2, 95:5, 92:8 and 90:10 in order to get a higher resolution, avoid excipient peaks overlapping and to keep the peaks as far as possible from the t_0 and within a reasonable time. All the ratios demonstrated the benefits and limitations. Higher water ratio in the weak mobile phase gave a faster elution. However, the peaks were too close with a short distance between Pluronic F127 peak and t_0 . In the case of using 100%

and 98% acetonitrile, Pluronic F127 peak was well separated from t_0 with high resolution, Figure 4.13.

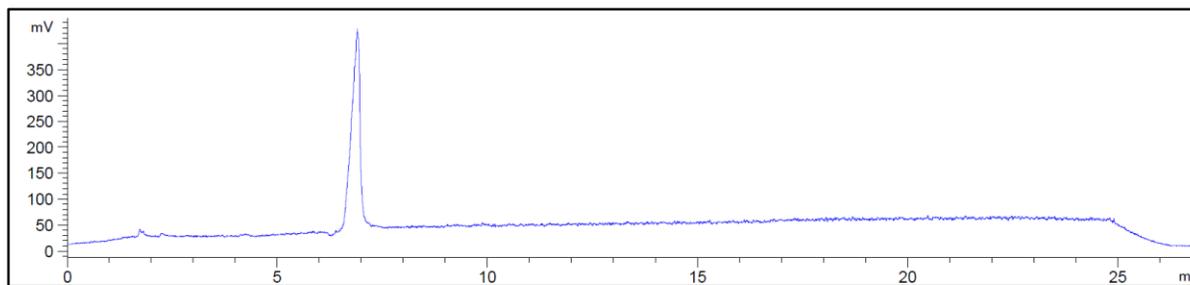


Figure 4.13: HILIC chromatogram is showing the good separation efficiency of Pluronic F127 when mobile phase A was made of 100% acetonitrile.

However, ascorbate and trehalose retention time was more than the time of run, reached more than an hour, thus, it delayed the separation. In addition to long retention time, the peaks of both excipients were not well defined with bad resolution due to their poor solubility in acetonitrile which may have led to their precipitation in the system by using this ratio.

However, the method was developed to satisfy all the required parameters by using 95 and 92 acetonitrile ratios. Both of these ratios gave the satisfactory results in terms of retention time and separated peaks for trehalose and ascorbate. But, using 92% of acetonitrile didn't provide good resolution for Pluronic F127, and its peak was close to t_0 . Nevertheless, by increasing the acetonitrile up to 95 % gave the good resolution of Pluronic F127 that is reasonably apart from t_0 , but then delayed the process by increasing the retention time greater than what observed by using 92%. Therefore, it is concluded that by increasing water content in mobile phase the Pluronic F127 was eluted early and had a peak closer to t_0 but decreased the retention time of ascorbate and trehalose. In order to get a single good method, that can add the benefits of both

approaches. (92% and 95%), the system was kept running for two minutes before starting gradient dilution in order to keep low water content in the system.

Finally, in this method, the system was kept running for two minutes before starting gradient mixing of mobile phase B gave the best results in terms of separation among the peaks, low retention time as well as delayed resolution peak for Pluronic F127 from t_0 , Figure 4.14.

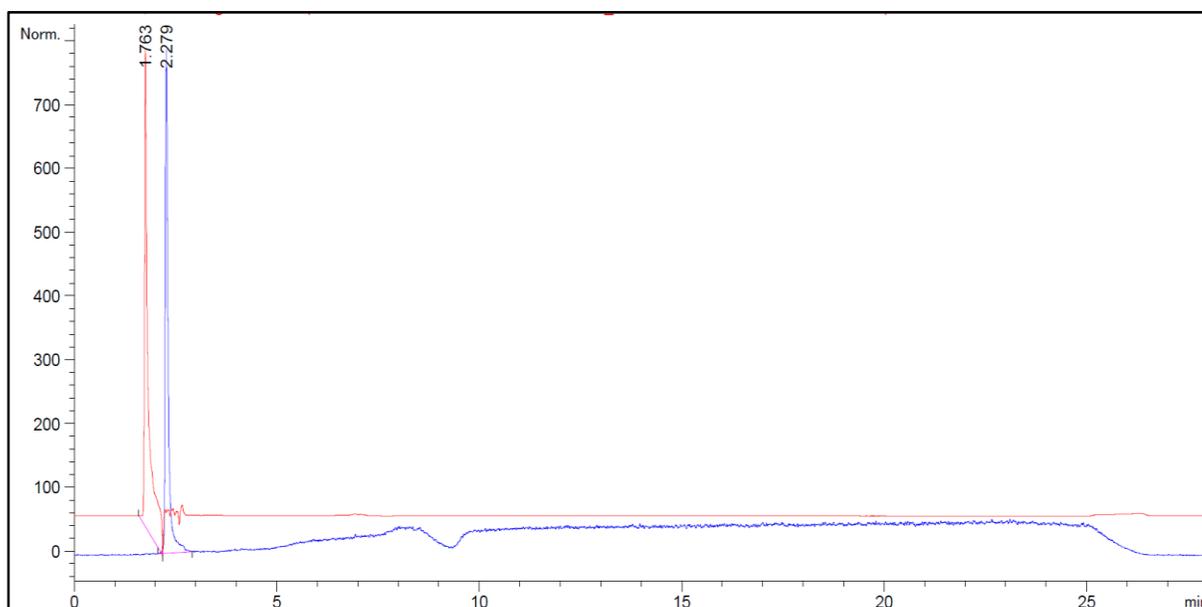


Figure 4.14: HILIC chromatogram showing the separation efficiency of the developed method, as reflected by the distance between Pluronic F127 Peak and t_0 (toluene). Mobile phase A was 92:8 acetonitrile: buffer (pH 5.8), the system was running at 100% mobile phase A for two minutes before starting the mixing with mobile phase B.

The ratio of mixing between mobile phase A and mobile phase B was monitored and the overall gradient system over the run time was demonstrated in Figure 4.15 and Table 4.3.

Table 4.3: The gradient run and the time of mixing mobile phase A and mobile phase B. Mobile phase A composition is (92:8 acetonitrile: Ammonium acetate buffer 100 mM and pH 5.8), while mobile phase B composition is (50:50 acetonitrile: Ammonium acetate buffer 100 mM and pH 5.8).

Time (minute)	Mobile phase A: Mobile phase B
0	100:0
2	100:0
20	70:30
23	70:30
25	100:0
28	100:0

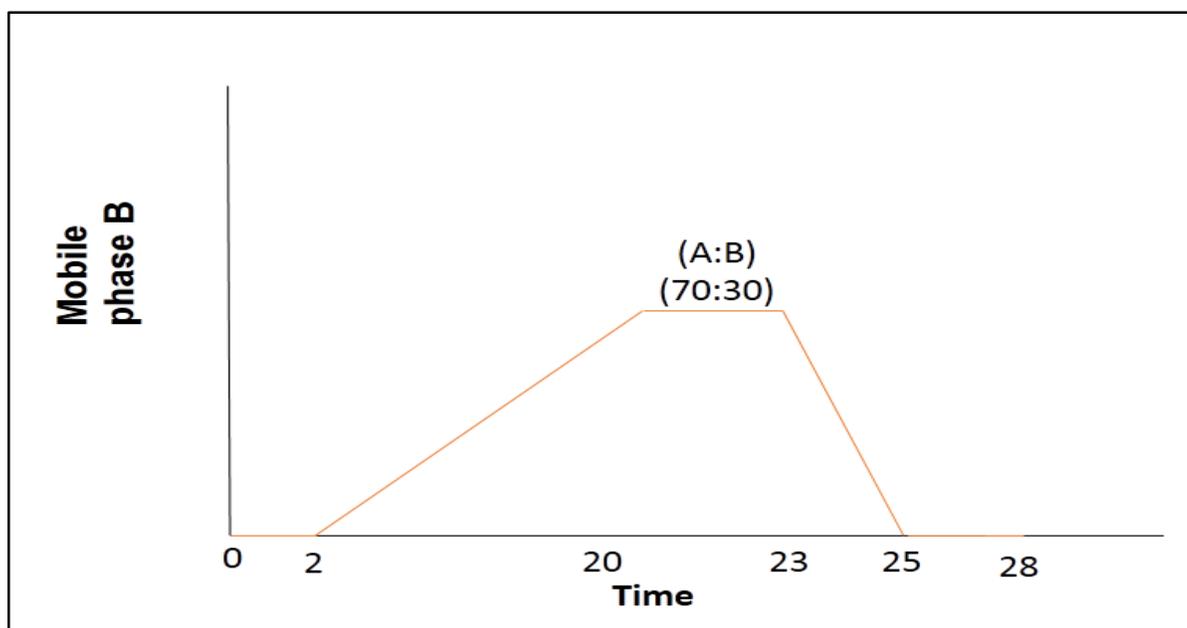


Figure 4.15: Illustrating diagram for the gradient run and the time of mixing mobile phase A and mobile phase B. Mobile phase A composition is 92:8 acetonitrile: Ammonium acetate buffer 100 mM and pH 5.8), while mobile phase B composition is 50:50 acetonitrile: Ammonium acetate buffer 100 mM and pH 5.8).

Injection volume was also optimised. For trehalose and ascorbate injection volume was 10 µl, as the higher volumes gave massive peaks which cannot be quantified and the linearity was missed at the higher concentration. Therefore, at 20 µl injection volume, the linearity obtained up to < 1mM for both substances, which requires about 100 dilution factors for the samples in order to be within the linear range. Whilst the case was different with Pluronic.F127 20 µl was selected as the appropriate injection volume, in a view to reduce LLOQ, and accordingly to increase the system sensitivity and selectivity for impurities. Injecting 20 µl Pluronic F127 resulted in generating a linear calibration curve over the range up to upper limit just before the critical micelles concentration.

The optimum detectors conditions were also selected. Evaporative Light Scattering Detector (ELSD) was chosen to detect trehalose due to its poor detection by the UV-DAD detector. However, ascorbate was better detected by using UV at 260 nm wavelength. The developed was acceptable in excipients separation when the chromatographic parameters were wisely chosen and optimised.

The developed methods at the optimised chromatographic conditions were validated for stability indicating purposes.

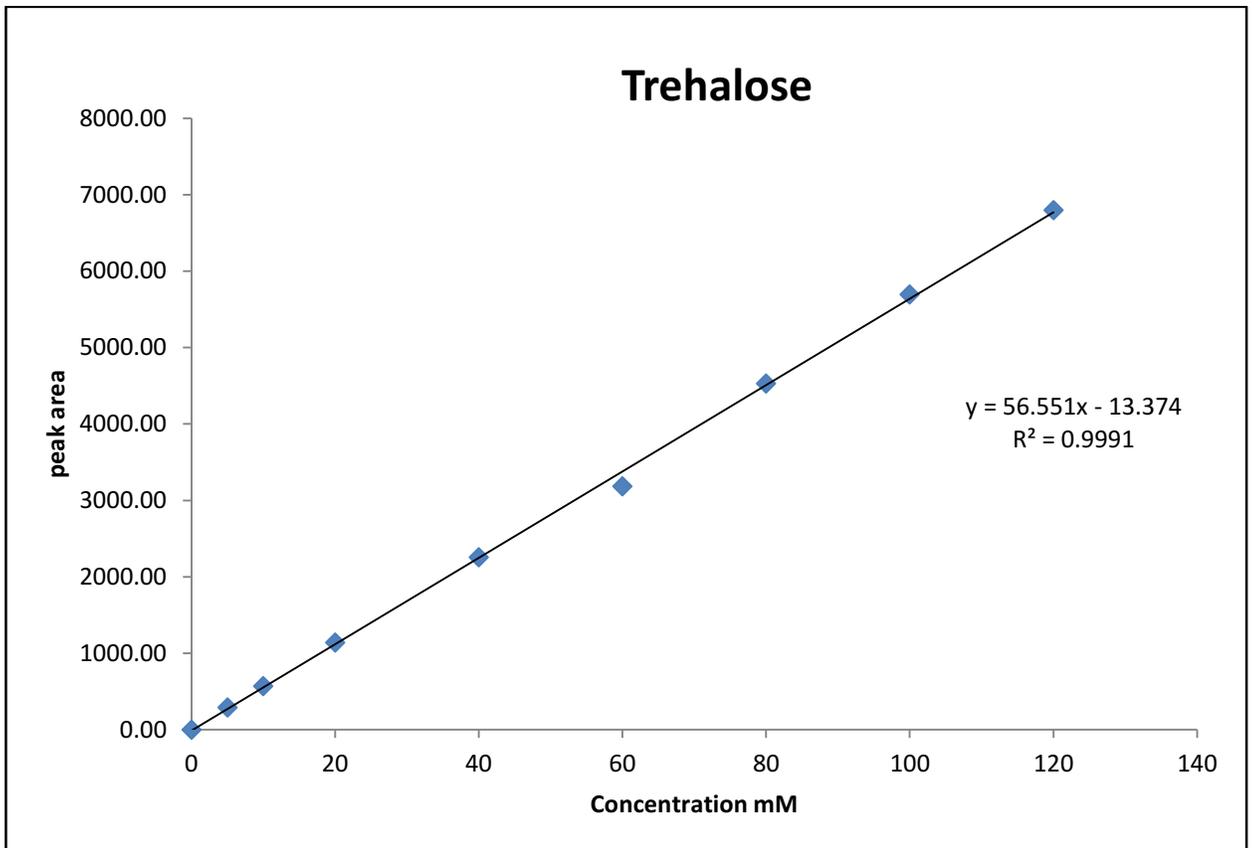
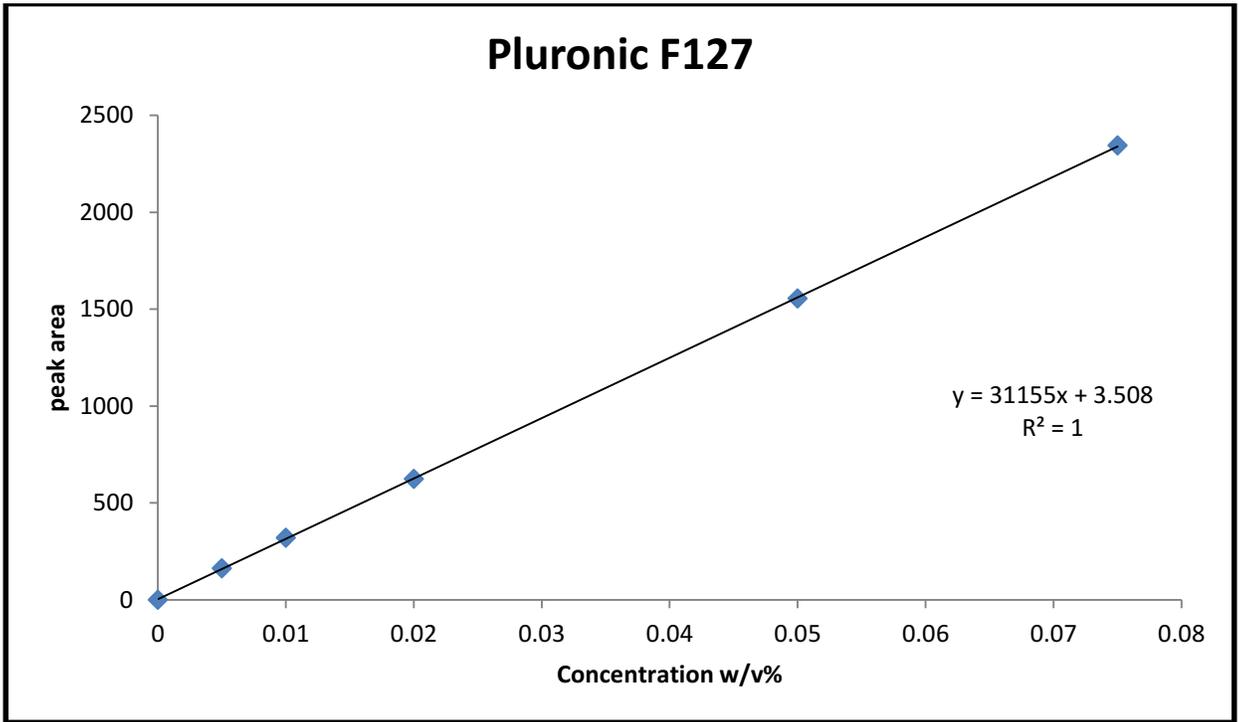
4.3.3.2. Validation of the Developed analytical methods to assess the excipients stability

4.3.3.2.1. Specificity

Specificity test is usually performed in order to evaluate the ability of the analytical method to separate the analyte from other compounds which are expected to present in the sample. Specificity demonstration procedures usually selected based on the goal of the analytical method. In the current study, the procedures were chosen to suit the intended objective of the method which was stability indicating assay. Therefore, combinations of the excipients with proteins were freshly prepared, and the system separation ability was examined. Furthermore, the samples were stored under stress conditions for accelerated degradation. The specificity of the methods to separate the excipients from the degradants and other existing materials was high, and the methods were able to give a well-defined shape peak for each excipient without tailing at different three retention times. The peaks of the excipients in individual samples were similar to the relevant peaks in the mixtures and degraded samples. The developed HILIC method was specific to separate the ascorbate, trehalose, and Pluronic F-127 from other solutions components.

4.3.3.2.2. Linearity

The calibration curves were generated by plotting the concentration of each excipient versus the obtained peak areas. The calibration plots generated for the three excipients were linear over various concentration ranges (Figure 4.16).



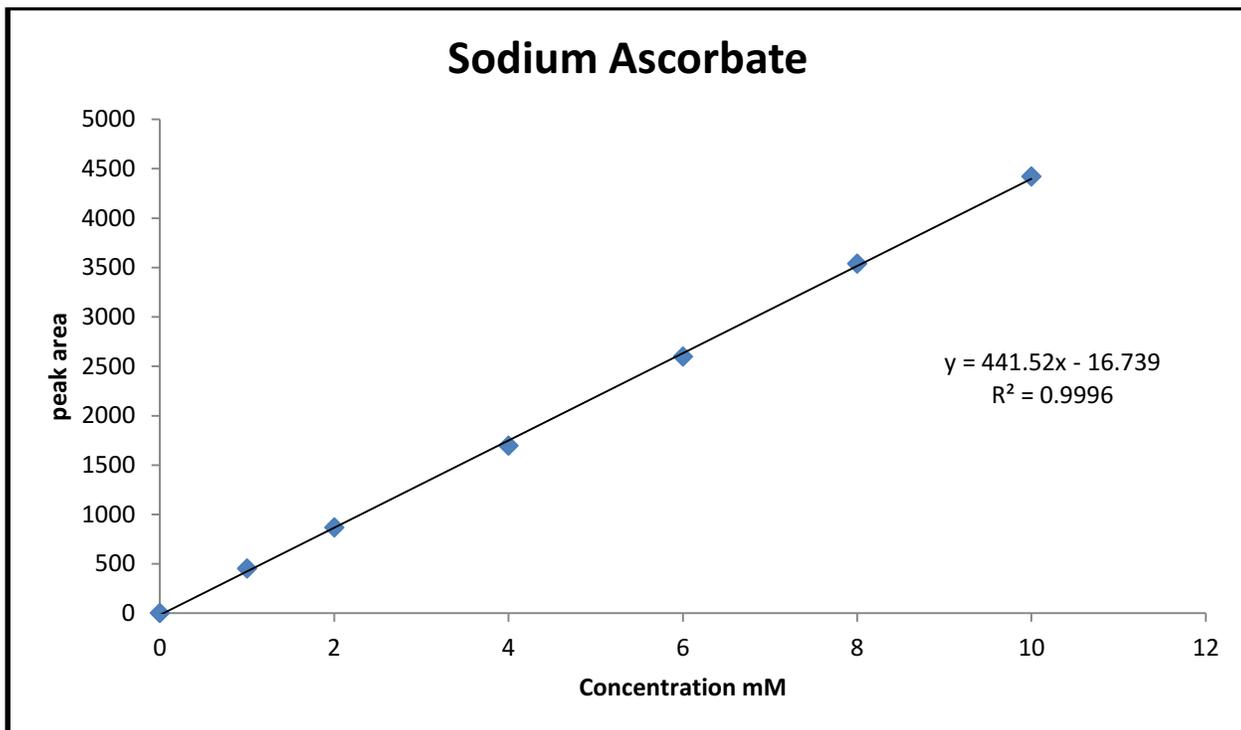


Figure 4.16: Pluronic F127, Trehalose, and Ascorbate calibration curves. X axis is the concentration, while Y axis is the peak area. For the ranges, LLOQ, LLOD refer to Table 4.4. Error bars are included into the line.

As illustrated in Figure 4.16, the correlation coefficients (r^2) were 1, 0.9996, and 0.9991 for Pluronic F 127, trehalose, and ascorbate, respectively. The excipient linear ranges are summarised in Table 4.4. The ranges were selected based on the linearity relationship.

Table 4.4: Pluronic F127, ascorbate, and trehalose ranges, LLOQ, LLOD, and correlation coefficients.

	Pluronic F127	Ascorbate	Trehalose
Range	0.005 – 0.075 %w/v	1 – 10 mM	0.5 – 12 mM
LLOQ*	0.005% w/v	1 mM	0.5 mM
LLOD**	0.002 %	0.5 mM	0.1mM
R ²	1	0.9996	0.9991

*LLOQ: lower limit of quantitation.

**LLOD: lower limit of detection.

Table 4.4 summarises the different three substances' ranges, LLOQs, LLODs, and square Pearson coefficients for the correlation between the concentration and area under the curve.

For Pluronic F127, a non-ionic surfactant with critical micelles concentration (CMC) around 0.1%, the correlation the concentration and the peak area was found linear only at the concentrations below the CMC. Therefore, the Pluronic F127 calibration curve was generated over a range of concentration from (0.005% – 0.075 %) w/v%.

In case of trehalose, a series of samples were prepared over a range of (0.5 – 12 mM), and then was diluted 1:3 sample: weak mobile phase (high acetonitrile proportion), because of poor solubility of trehalose in the pure mobile phase A, which resulted in precipitating trehalose in the column, thus, a separation within the peak was observed, Figure 4.17.

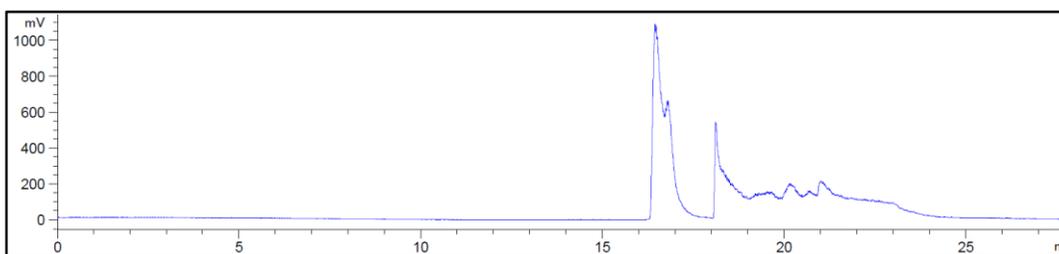


Figure 4.17: HILIC chromatogram is showing the separation within trehalose peak when trehalose sample was prepared in water.

Accordingly, all the QC and stability samples were diluted to fit the entire range. For Ascorbate, the range was (1 – 10 mM) by using water as the blank.

LLOD and LLOQ values were determined based on peak: noise rule. LLOD was the concentration where the peak: noise ratio around 3:1. While the peak: noise ratio was (5-10):1 for LLOQ.

Finally, QC samples containing the upper limit concentration of the substances (as in actual formulations) have been prepared and diluted further to lay within the linear range. The diluted QC samples were then analysed by using the same methods, and then areas under the curve were obtained. The results reveal that high concentration analyte samples, even the ones with concentrations greater than the upper limit of quantification, are possible to be diluted and then quantified.

To avoid any misleading results which may affect the accuracy or precision, carryover was assessed in triplicate by analysing double blank just after the higher limit of quantitation, and the response was recorded. The recorded area under the curve of the blank at the same retention time of the respected analyte was divided by the area under the curve of the ULOQ for the same analyte. The calculated percentages were even less than 5% for all analytes at all analysis times.

4.3.3.2.3. Accuracy

The accuracy is a term describes the closeness of the readings to the initial readings. The accuracy of the HILIC methods was also studied.

Inter and intra run accuracy values of the assay were determined by preparing three different quality control samples over three different days (one sample per a day). The three QC samples have been prepared for each excipient at three different levels, High level, medium level, and LLOQ, using water as a blank.

Analytical methods accuracy calculations demonstrated that; the percent recovery of the average of each level (intra accuracy) at medium and high values has reached up to 99.1%, 97.9%, and 98.6% for Pluronic F127, trehalose, and ascorbate, respectively. However, the accuracy values went down to 93.4% for trehalose at LLOQ level, which is acceptable accuracy value according to ICH and EMA guidelines.

On the counter side, the inter run accuracy values were more various with wider ranges. Nevertheless, the lowest accuracy value for medium and high level of all the excipients was within 5.0% of the nominal value; this value was obtained after having 104.93% recovery value. The accuracy of all analytes readings at LLOQ was within $\pm 10\%$ of the nominal values.

These results of accuracy calculations were compatible with method validation requirements, as required by the regulatory agencies.

4.3.3.2.4. Precision

Methods precision was carried out in six replicate of three QC samples at three different concentrations i.e. LLOQ, mid QC, and high QC, over six consecutive days. Precision values were expressed as a coefficient of variance (CV).

Intra and inter precision calculation revealed that the developed analytical methods were precise to quantify the three different analytes.

The CV of intra- and inter-day precision was by no more than 2.4%, 2.6%, and 1.1% for Pluronic F127, trehalose, and ascorbate, respectively at, mid QC, and the high QC, see Table 4.5. LLOQ readings were also agreement with CV values within $\pm 5.6\%$.

The previous CV readings reflected high precise and reproducible analytical methods had been developed to be as stability indicating assay, Table 4.5.

Table 4.5: Pluronic F127, trehalose, and ascorbate inter and intra precision values for mid and high QC.

	Pluronic F127	Trehalose	Ascorbate
Inter precision	100%± 1.7 – 2.7 %	100%± 2.3 – 2.6 %	100%± 0.6 – 1.1 %
Intra precision	100%± 0.8 – 2.1 %	100%± 1.8 %	100%± 0.9 – 1.2 %

4.3.3.2.5. Robustness

The robust method is the analytical procedure able to provide consistent results with no significant difference when the analytical parameters are changed accidentally or in purpose within a small range.

In the current study, an experimental design was built, and the following set of factors were changed in the methods within an interval of $\pm 5\%$ of flow rate, pH, and initial aqueous ratio in the weak mobile phase were changed. Quality control samples were prepared in triplicate in order to assess the robustness.

The design of experiment analysis revealed that no significant differences in peak areas and retention times were observed when the analytes were studied under different parameter levels. Furthermore, all the peak areas and the retention times were within $\pm 5\%$ of the respective values when the chromatographic parameters were used at the

centre point. These readings mean the applied chromatographic methods are robust, and any significant difference in quantitative analysis is unlikely to occur if any small analytical errors accidentally occurred.

4.3.4. Conclusion

This work proposes stability indicating chromatographic methods to analysing and quantising lysozyme and trypsin liquid formulations, in addition to the present excipients. The methods were developed and validated by applying SEC and HILIC techniques for the same purpose. Furthermore, SEC method was partially validated to suit nanocapsules containing lysozyme and trypsin characterisation and quantification. Additionally, the SEC method was validated to quantify encapsulated DNase I in nanocapsule formulations. The validation process proves that the methods are specific, linear, accurate, precise, and robust, with LLOQ values below the desired levels.

The next chapter presents the results of establishment of strategic approach for development of oral polymeric nanocapsules containing biomolecules. Lysozyme and trypsin were used as model protein and then, as a part of the approach application, DNase I was used as a therapeutic protein.

**Chapter Five: Development
of a strategic approach for
preparation of oral polymeric
nanocapsules containing
biomolecules.**

5.1. Introduction

Product and process development and even product manufacturing were traditionally based on experienced and fixed procedures in the pharmaceutical industry. This situation has been influenced by a rigid regulatory environment which controls the pharmaceutical industries business, hence, which hindered the improvement of the manufacturing technology. which consequently, led to economic problems, due to e.g. products discarding caused by manufacturing deficiencies [173].

Therefore, Food and Drug Association (FDA) established QbD concept in 2004, with a view to building the quality into the product from the beginning of the design, through understanding the relations of product quality and parameters affect it i.e. process and material attributes, instead of testing it later on [174]. Accordingly, QbD can promote faster and more consistent product and process development, thus, to increase flexibility in manufacturing in order to reduce production cost and time. QbD is defined as a “systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control based on sound science and quality risk management” [116].

ICH Q8, Q9, and Q10 guidelines detailed the principles and the tools for the implementation of QbD and continuous improvement and risk management. Adoption of the QbD approach in biopharmaceutical formulations can provide high-quality products without extensive regulatory oversight[74].

Therefore, QbD approach has been adapted in this study to develop biodegradable polymeric nanocapsule formulations containing proteins, intended for oral delivery. A

biodegradable polymeric nanocapsule system (PNC) is defined as a drug containing a reservoir that is surrounded by a polymeric shell [175]. Polymeric nanocapsules PNCs are being used to carry therapeutic proteins in order to protect them from the harsh surrounding environment either inside the body or before the delivery to the patients. In addition, PNCs are compatible with body tissues and cells due to the nature of the biodegradable polymers [129]. Moreover, PNCs can enhance the kinetic properties of drug release [176]. Various factors affect the success of PNC's formulations, e.g. polymer characteristics, surfactant type, preparation method and preparation conditions [177].

However, optimising all of these individual factors is considered a challenge but is required to assure the quality of the final products. Many researchers have performed projects in this area including a lot of efforts to formulate viable polymeric nanocapsules containing protein drugs. However, nothing yet is available in the market. In the formulation of the nanocapsules, the parenteral administration is considered as the most convenient approach because the oral route has the limitations of delayed polymer degradation. However, in the current study, the use of plasticisers (PEG 8000) in PNCs for administration via oral route has demonstrated comparable results, when the drug release percentage has increased after adding PEG 8000 as a release enhancer.

Developing PNC formulations based on the QbD approach and according to DOE, helped to optimise the formulation factors e.g. the used copolymer in order to achieve the desired quality targets.

The results of the optimised PNC formulations characterisation justified the theoretical results as predicted by the models. The developed strategy may form a promising

approach to developing oral therapeutic macromolecules within shorter time with saving development cost.

5.2. Aims and objectives

The aim of this study is to employ the QbD concept to developing a strategy to preparing polymeric nanocapsules containing stable and active macromolecules with reduced processing cost and development time. The strategy should be developed by identifying and analysing of the potential risk factors and evaluating the effect of the critical variables and their interactions on the desired quality attributes of lysozyme and trypsin containing PNCs. Accordingly, to optimise the variables and obtain optimal formulations fulfilling the predetermined QTTPs.

Moreover, to validate how well the mathematical Design of Experiments in screening and optimising the different formulation parameters and identify the desired combination of the overall formulation characteristics.

Applying QbD assisted optimisation of the factors in order to achieve high encapsulation efficiency and high release profile at the same time for biologically active proteins.

To the best of our knowledge, no researchers have added a release enhancer after formulating the PNCs. The rationale behind that is to increase the overall drug release without affecting the encapsulation efficiency.

The developed strategy was validated by preparation of PNCs containing DNase I at the optimised conditions and characterised to suit the intended oral characteristics.

5.3. Results and Discussion

5.3.1. Quality by Design (QbD) implementation

In the current study, the QbD, as a scientific, regulatory approach, has been adapted according to the International Conference of Harmonisation (ICH) Q8, Q9, and Q10 guidelines. The QbD has been applied in the early stage of preparation of the PNCs where the Active Pharmaceutical Ingredients (APIs) were therapeutic proteins i.e. lysozyme and trypsin.

The implementation has been performed as described in details in Section 2.4.1 and the relevant subsections. The author has identified and determined the Quality Target Product Profiles (QTPPs) and has selected the desired Critical Quality Attributes (CQAs) accordingly. Then, the risk assessment has been performed in order to screen the potential risk factors and to further examine them for optimisation study by three steps i.e. risk identification, risk analysis, and risk evaluation, according to [116]. Risk identification was performed by listing the potential risk factors; then the risk analysis has taken place by Failure Mode Effect Analysis (FMEA). Finally, the risk evaluation has occurred by building a mathematical design of experiment in order to evaluate the effect of several variables on the desired CQAs and then obtain optimised PNC formulations.

QbD implementation process will be discussed in details in the following sections.

5.3.1.1. Quality Target Product Profiles (QTPPs) and Critical Quality Attributes

CQAs identification

As mentioned above, the ultimate goal is to develop stable polymeric nanocapsule formulations containing therapeutic protein and intended for oral drug delivery. Hence, QTPPs have been identified, as the first step of the QbD implementation process, based on the scientific knowledge, and relevant previous literature in a view to achieve the ultimate purpose of the formulations. Moreover, the QTPPs are selected to suit the patient relevant characteristics.

In order to achieve the desired QTPPs, the CQAs were identified based on the prior knowledge of the relevant literature and author's suggestions to influence the quality of the final product.

Table 5.1 lists the QTPPs, CQAs, their desired targets, and justifications behind the elements selection. The selection process of QTPPs was in agreement with what was recommended in the ICH Q8 guideline as it has to be based on the characteristics related to the patients i.e. safety, efficacy, and quality [76]. However, the CQAs were determined in accordance to the drug product and the used materials relevant characteristics. The CQAs were selected based on the identified QTPPs, and they have influences on the product properties and qualities for example; drug release and particle size.

Table 5.1: The desired QTPPs, CQAs, their targets, and justifications.

	Target	Justification
QTPPs		
Dosage form	Nanocapsule	To protect the protein from the proteolytic digestive enzymes. Also nanoscale sizes to enhance the pharmacokinetic properties.
Route of administration	Oral	Easy dosage form, no need for professional help like the parenteral dosage form, fewer complications.
Site of release	Intestine	To avoid the gastric enzymes, and the harsh pH gradient.
Stability	One year at 5+3 °C	To protect the proteins from the chemical degradation e.g. oxidation and physical degradation e.g. aggregation, in order to avoid the immunological reactions.
Drug	Protein	Therapeutic category for the treatment of various diseases, uncommon oral delivery, and sensitive structures.
Bulk size	Reasonable	To be able to deliver the daily required dose in a single oral capsule or tablet. To be convenient for swallowing by the patient.
Cost	Reasonable	To be economically effective for the patient and the health care provider.

Table 5.1 (continue): The desired QTPPs, CQAs, their targets, and justifications.

CQA		
Overall release within 24 hours	>75%	To reduce the waste and overall cost. In addition to delivering the proper dose for the patients within a predefined time.
Nanocapsule Size	≤500	Large surface area, increase the drug release, reduce the bulk size.
Active protein	>90%	To reduce the aggregation and the deactivation of the protein. The aggregate may cause immunological reactions.
Encapsulation efficiency	>75%	To reduce the waste and overall cost. In addition to delivering the proper dose for the patients, with low bulk size.
Accelerated Stability	>95%	To reflect the entire shelf life stability. Hence, reducing the degradation and saving time.
Resistance to gastric enzymes	>90%	To avoid the degradation by the gastric enzymes and reduce the permeability of the polymeric shell.
Resistance to intestinal enzymes	>90%	To avoid the degradation by the intestinal enzymes and reduce the permeability of the polymeric shell.

As clear in Table 5.1, the ultimate target profile is to prepare oral nanocapsules solid dosage form, which is able to retain the stability of the encapsulated proteins for a long time after storage at refrigerated conditions. This dosage form must be able to keep the

proteins protected from degradation by gastric enzymes. The bulk size of the dosage forms and the cost of the production have also been taken into considerations, and all of these criteria are in consistence with the patient related qualities.

On the other hand, the newly developed formulations must have the following attributes; release more than 75% of proteins over 24 hours in intestinal fluids, protect the proteins from the mechanical stress induced during encapsulation process, retain their biological activity, encapsulate at least 75% of the proteins, and to aid more than 90% of the encapsulated proteins to remain intact after incubation in both SGF and SIF.

5.3.1.2. Risk Assessment

After QTPPs and CQAs identification, the risk assessment step has been carried out. The risk assessment has been performed in three consecutive steps; i.e. risk identification, risk analysis, and risk evaluation.

Therefore, potential risk factors have been assessed and critically evaluated to comply with the QbD requirements, in order to achieve the predefined QTPPs and CQAs. Potentially risk factors including Critical Process Parameters (CPPs) and Critical Materials Attributes (CMAs) were illustrated in a fishbone (Ishikawa) diagram to understand their influence on the desired QTPPs and CQAs well and to identify the risk, see Figure 5.1.

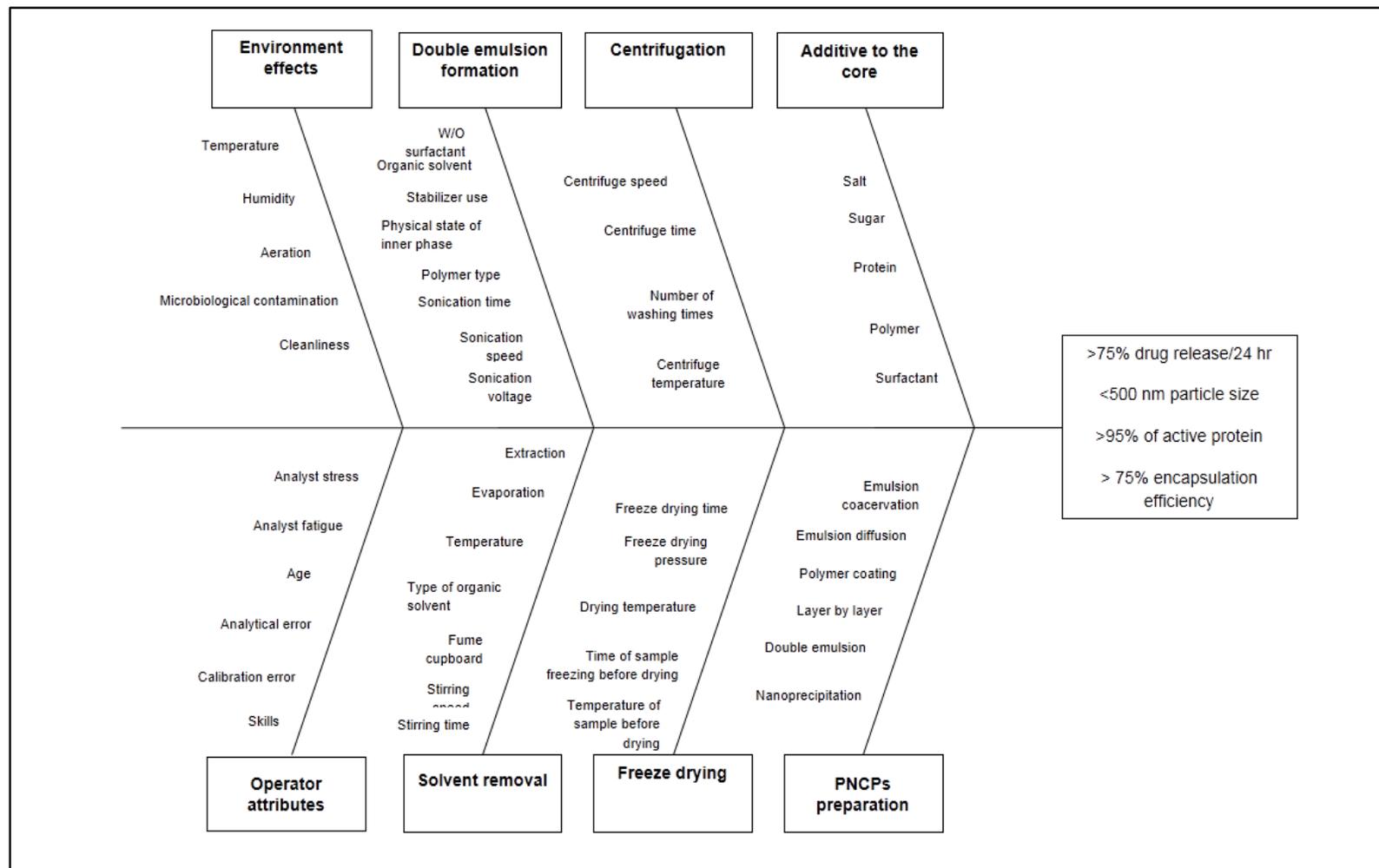


Figure 5.1: Fishbone (Ishikawa) diagram, which illustrates the potentially risk factors i.e. material attributes, process parameters, and environmental factors which influence the desired QTPs and CQAs.

Furthermore, all the identified potentially risk factors (44 factors) in the above fishbone diagram (Figure 5.1) have been listed and critically analysed by applying FMEA as described in Section 2.4.1.3. Figure 5.2 illustrates these factors and their relevant Risk Priority Numbers (RPN), only 17 factors have exceeded the 15 RPN value as a threshold for the factors to be candidates for further investigations. Moreover, these 17 factors are listed and classified into four different categories, as shown in Table 5.2.

The first category includes the factors which their existence has a potential risk effect, but their optimum level is not well defined. Therefore they will be incorporated into a mathematical design of experiment in order to optimise their levels. The second category covers the factors when their existence has a potential hazard, so they have to be excluded e.g. using nanoprecipitation method to preparing the PNCs containing protein. Then, the third factors category represents the factors which they have to be taken into account and fixed at a certain value or level, which previously, identified in the literature or had preliminary screening e.g. type of organic solvent when ethyl acetate has been chosen in the current study as it has less negative impact on the protein structure. The last category contains the unmeasurable parameters when they can be determined by the analyst before commencing the experiments, or their impact can appear and be monitored during the study e.g. analyst skills and analytical error.

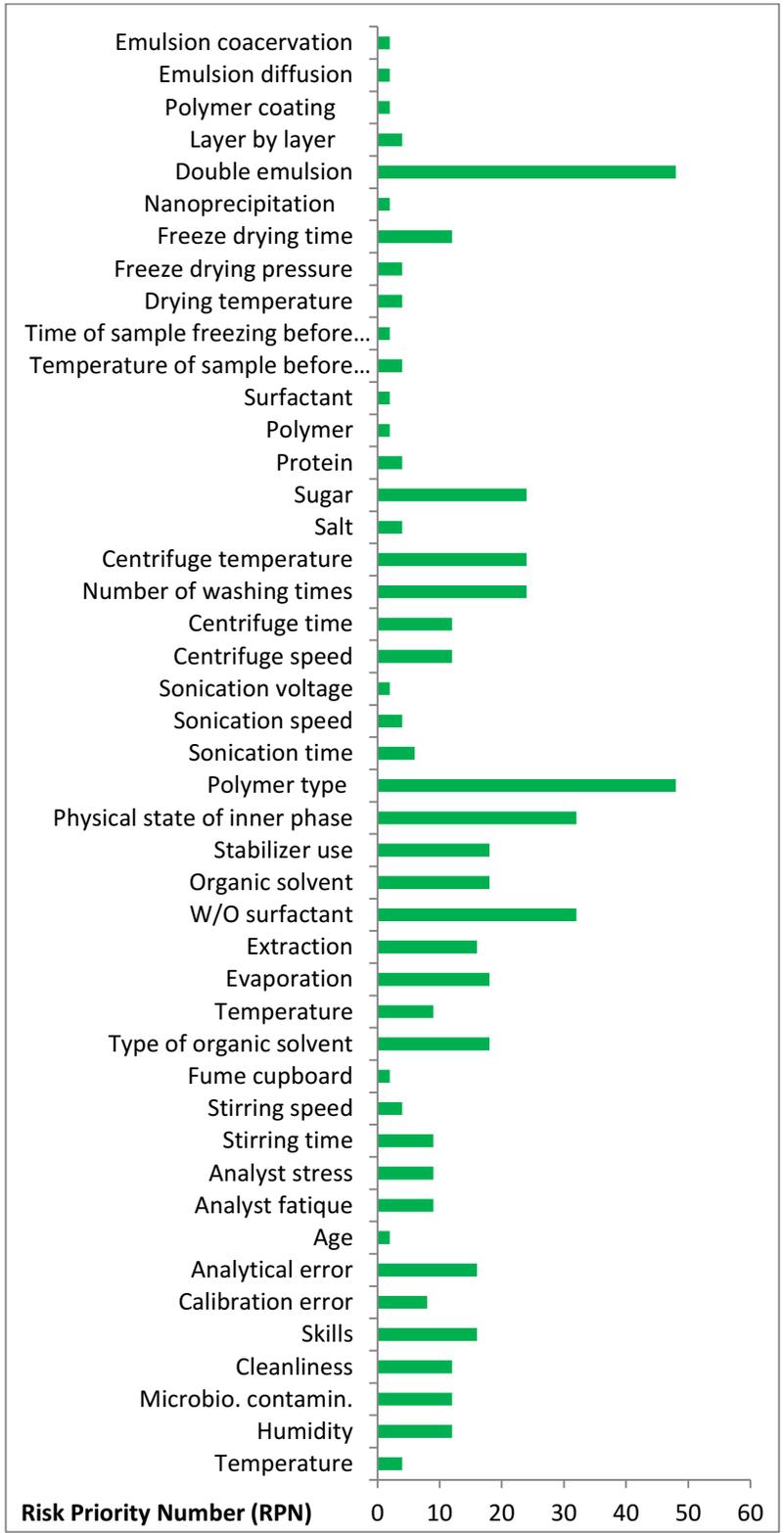


Figure 5.2: FMEA graph showing the predetermined risk factors and their respective risk priority number (RPN).

Table 5.2: potentially high-risk factors classified into three different categories; 1- The factors which can be used at different levels and should be included into DOE, 2- The factors have only one level or should be employed at fixed level, 3- The factors should be excluded 4- The factors which their effects appear and monitored during the study.

Factor number	Potentially high-risk factors	Category
1	Analyst skills	4
2	Analytical error	4
3	Type of organic solvent	2
4	Temperature	3
5	Evaporation	2
6	Extraction	3
7	W/O surfactant	2
8	Organic solvent	2
9	Stabilizer use	2
10	Physical state of inner phase	1
11	Polymer type	1
12	Number of washing times	2
13	Centrifuge temperature	2
14	Salt	3
15	Sugar	1
16	Nanoprecipitation	3
17	Double emulsion	2

Following to the risk identification and risk analysis procedures, the potentially risk factors classified as category one were lists and included into designs of an experiment in order to determine the factor levels interval and then optimise these levels to achieve the desired targets and CQAs.

The above risk assessment was carried out to suit the conditions of preparation of PNCs containing proteins i.e. lysozyme and trypsin, and if any difference between these two proteins in term of encapsulation appears later in the study, it will be taken into account in the optimisation stage.

Table 5.3 shows these two experimental designs which have been built to prepare, characterise and then optimise PNC's formulations containing lysozyme and trypsin.

Table 5.3: A total 16 different PNCs formulations 8 for each protein; lysozyme (L) and trypsin (T), along with their factors and levels.

Formulation	Polymer type	Core Physical state	Trehalose mM
L1	86:14*	Liquid	0
L2	40:60**	Liquid	0
L3	86:14	Solid	0
L4	40:60	Solid	0
L5	86:14	Liquid	10
L6	40:60	Liquid	10
L7	86:14	Solid	10
L8	40:60	Solid	10
T1	86:14	Liquid	0
T2	40:60	Liquid	0
T3	86:14	Solid	0
T4	40:60	Solid	0
T5	86:14	Liquid	10
T6	40:60	Liquid	10
T7	86:14	Solid	10
T8	40:60	Solid	10

86:14*: Poly (D, L- Lactide-co- caprolactone) 14:86 ratio

40:60**: Poly (D, L- Lactide-co- caprolactone) 40:60 ratio

As clear from Table 5.3, lysozyme and trypsin have the same formulations conditions, and these 16 different formulations have been prepared in triplicate, and further details about the formulations and their preparation are mentioned and discussed in the following section.

5.3.2. Preparation of polymeric nanocapsules

Polymeric nanocapsules containing lysozyme and trypsin have been prepared and formulated by applying double emulsion solvent evaporation method as described in Section 2.4.2. Preparation method selection was based on applying QbD approach by the initial risk assessment processes, as described in Section 2.4.2, to compare between the different methods and was based on previous knowledge and relevant literature in order to select the best one amongst the preparation methodologies. A total of 16 different W/O/W and S/O/W emulsions have been prepared in triplicate, by probe sonication, as explained previously in Section 2.4.2. The physical appearance of each emulsion was evaluated, and some differences have been reported. Therefore, no phase separation or emulsion cracking was observed on standing for all the preparations, and only one homogeneous layer emulsions have been formed. However, the liquids colour was varied from formulation to another. Among the sixteen formulations shown in Table 5.3, the formulations with trehalose had whiter and more opaque colour. However, formulations with small particle sizes had a transparent appearance.



Figure 5.3: Two different double emulsions, showing the different in the emulsions transparency.

The previous differences in the emulsion appearances, as seen in Figure 5.3, are due to the presence of trehalose in the inner phase and may be related to the droplet sizes formed. The observations are consistent with what was mentioned in [178]. Gillian 2007 stated that; the emulsion with droplet size in sub-micron and nanoscale usually have a transparent to slightly milky appearance in comparison to the ones with large droplet size which has an opaque appearance.

After solvent evaporation process by overnight stirring at the room temperature ($22\text{ }^{\circ}\text{C} \pm 2$), the organic solvent was evaporated, and the particles were solidified. Thus, suspensions of polymeric nanocapsules were developed. Then the resulted suspensions have been washed in triplicate by centrifugation to remove all the organic

solvent residues. Afterwards, the PNC formulations have been dried by lyophilisation for 48 hours. Fluffy white and fine powders have been obtained after freeze drying.

PNCs have been prepared according to the optimise design of experiments, as described above. Firstly, 8 formulations formulated for each of trypsin and lysozyme, and then the models have been mathematically analysed, and all responses were fitted and analysed by multi-linear regression analysis method. After the data analysis, all factors levels were optimised according to a function of equation applied by MODDE 10.1 Software to find out the set points which fit the target or the desired CQAs. Combining a double emulsion method, a PNCs preparation method suitable for encapsulation of hydrophilic drug, with DOE methodology helped to study the overall appropriate conditions of PNCs containing proteins and determination the pros and cons of these conditions. And then, assisted in obtaining PNCs containing stable proteins with characteristics suit the oral delivery systems, as will be shown later.

5.3.3. Polymeric nanocapsules characterisation

PNC formulations were characterised in order to evaluate them and the changes in factors, hence optimising the materials attribute and process parameters. The characterisations include measuring the PNCs encapsulation efficiency, drug release in SIF and SGF, particle size, biological activity of the encapsulated proteins, the permeability of PNCs shell to the digestive enzymes, accelerated PNCs formulations stability, and imaging by TEM. Also, all the characterisation methods were adapted

from the published literature, see Section 2.5 and the subsections underneath it. Nevertheless, in order to quantify and analyse the encapsulated proteins, the polymeric shells should be broken down by an organic solvent and the encapsulated proteins should be collected. However, the protein structures are sensitive to the processes and the used organic solvent. Therefore, to avoid any denaturation caused by the organic solvent during the characterisation, the effect of three different organic solvents i.e. acetonitrile, isopropyl alcohol, and ethyl acetate on lysozyme and trypsin structures were evaluated, and water was used as a control in the cases. Lysozyme and trypsin were suspended in the three organic solvents, centrifuged, collected, and then quantified by using size exclusion chromatography (SEC).

Table 5.4 shows the retention percentage of the both proteins after suspending in three different organic solvents, in addition to the control (water).

Table 5.4: three different organic solvents their effect on lysozyme and trypsin after suspending them in 100 µg/ml.

Organic solvent	Lysozyme retention% ± SD	P-value	Trypsin	
			retention% ± SD	P-value
Ethyl acetate	99.09% ± 1.91	0.199919	98.67 ± 1.41	0.068845
Acetonitrile	94.38% ± 2.05	0.020822	98.73 ± 2.11	0.196052
Isopropyl alcohol	88.36% ± 2.65	0.012845	92.71 ± 2.11	0.025012
Water (control)	100.45% ± 1.73	-----	101.30% ± 2.27	-----

It is shown from Table 5.4, the use of ethyl acetate and acetonitrile have almost the same impact on trypsin, as no significant differences were observed between them (P-value >0.05). However, in the case of lysozyme, ethyl acetate gave good recovery as

compared to acetonitrile. By using Isopropyl alcohol, the overall recovery of both the proteins reduced in comparison with ethyl acetate, acetonitrile, and even with water. It is shown in the table that use of IPA has more impact on lysozyme as compared to trypsin. No significant difference was recorded between using the water and using ethyl acetate for both proteins; P-values are shown in Table 5.4.

The selection of ethyl acetate for trypsin was considered as a choice to use one method for both of the proteins as methyl acetate was selected for lysozyme. This high yield percentage may be due to the low denaturation properties of ethyl acetate against the proteins that lead to the selection of methyl acetate as an organic solvent to break the polymeric shell in order to quantify the encapsulated proteins. All the characterisation results and their discussion are shown in details in this research, chapter six.

The characterisation results have been statistically analysed and fitted by applying multi-linear regression analysis (MLR) by using MODDE 10.0 software for this purpose.

The analysis of the designs of the experiment (DOEs), shown in

Table 5.3, concluded that the used models are good models with the lack of error.

Table 5.5 and Table 5.6 show the statistical parameters (Q^2 , and R^2) obtained after the models' analysis.

Table 5.5: The statistical parameters (Q^2 , R^2) obtained after the trypsin model analysis.

Parameters	Encapsulation efficiency (EE)	Release % in SIF	Particle size	Biological activity
R^2	0.999942	0.999155	0.99747	0.999995
Q^2	0.996289	0.945898	0.838085	0.999656

Table 5.6: The statistical parameters (Q^2 , R^2) obtained after the lysozyme model analysis.

Parameters	Encapsulation efficiency (EE)	Release % in SIF	Particle size	Biological activity
R^2	0.999738	0.990427	0.995673	0.999773
Q^2	0.983209	0.65804	0.723055	0.98546

As seen in the tables above, most of the R^2 and Q^2 were higher than 0.9, and the difference between R^2 and Q^2 within the same response was less than 0.2 -0.3, where R^2 reflects the model goodness of fit and Q^2 imitates the goodness of the model prediction [179]. R^2 and Q^2 values are always between 0 and 1, and the closer to 1 are more reflecting excellent models with high predictive power especially when R^2 and Q^2 are not separated by more than 0.2 – 0.3, [180]. In light of the results and based on what has been mentioned above, the developed models are reliable and excellent in fitting and prediction, with no lack of fit of the model.

All the factors have been given coefficients to reflect their effect on the responses along with P-values to estimate the significance of these effects. Table 5.7, Table 5.8, Figure 5.4, and Figure 5.5 show the coefficients of the factors' effect on the response for the responses which have been affected at least by one significant factor.

Table 5.7: The coefficients of the factors affecting the lysozyme formulations characteristics, their interaction along with P values for each factor.

	EE		Release in SIF		Particle Size		BA	
	Coefficient	P	Coefficient	P	Coefficient	P	Coefficient	P
Polymer ratio	14.975	0.011	-17.223	0.047	7.149	0.659	-0.205	0.631
Trehalose	0.500	0.295	0.802	0.643	154.6	0.049	18.846	0.011
Core physical state (Solid)	0.775	0.198	-0.226877	0.881	-93.775	0.081	8.602	0.023
Polymer ratio * Trehalose	0.150	0.656	1.667	0.416	7.725	0.638	0.153	0.710
Polymer ratio * Core physical state (Solid)	-3.575	0.044	0.908	0.607	-13.900	0.455	1.261	0.155
Trehalose * Core physical state (Solid)	0.500	0.295	1.676	0.415	-23.450	0.303	0.544	0.333

*P= P-value.

**Red colour for significant factors.

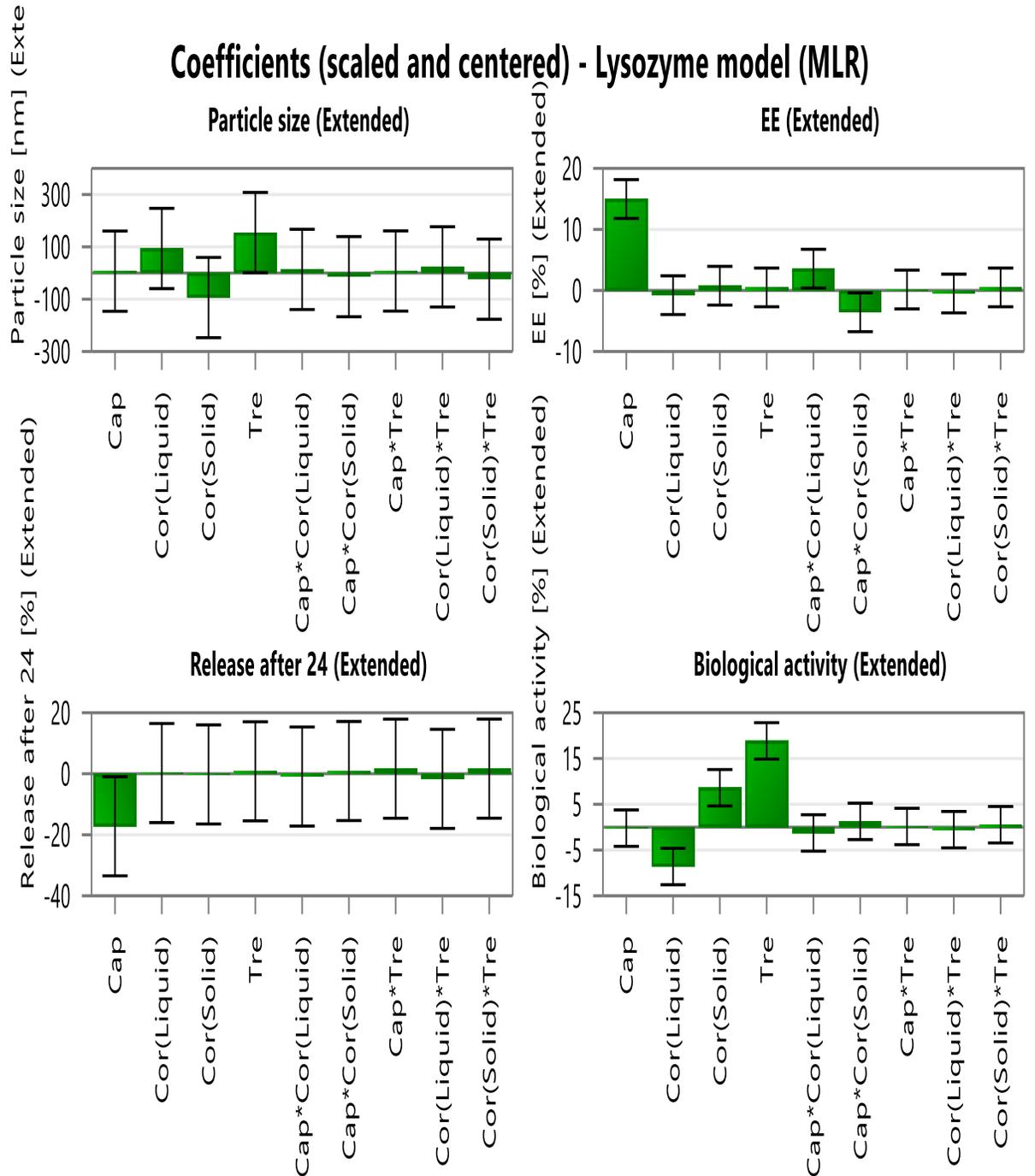
Table 5.8: The coefficients of the factors affecting the trypsin formulations characteristics, their interaction along with P values for each factor.

	EE		Release in SIF		Particle Size		BA	
	Coefficient	P	Coefficient	P	Coefficient	P	Coefficient	P
Polymer ratio	17.263	0.005	-14.392	0.019	1.600	0.912	0.084	0.363
Trehalose	2.788	0.051	-0.065	0.906	199.625	0.036	20.141	0.002
Core physical state (Solid)	-0.163	0.447	1.873	0.145	-106.025	0.068	11.386	0.003
Polymer ratio * Trehalose	3.163	0.028	0.328	0.589	22.400	0.301	1.431	0.023
Polymer ratio * Core physical state (Solid)	-1.488	0.059	0.790	0.320	0.450	0.975	0.021	0.760
Trehalose * Core physical state (Solid)	2.838	0.031	3.503	0.079	-9.675	0.553	-0.081	0.372

*P= P-value.

**Red colour for significant factors.

Coefficients (scaled and centered) - Lysozyme model (MLR)



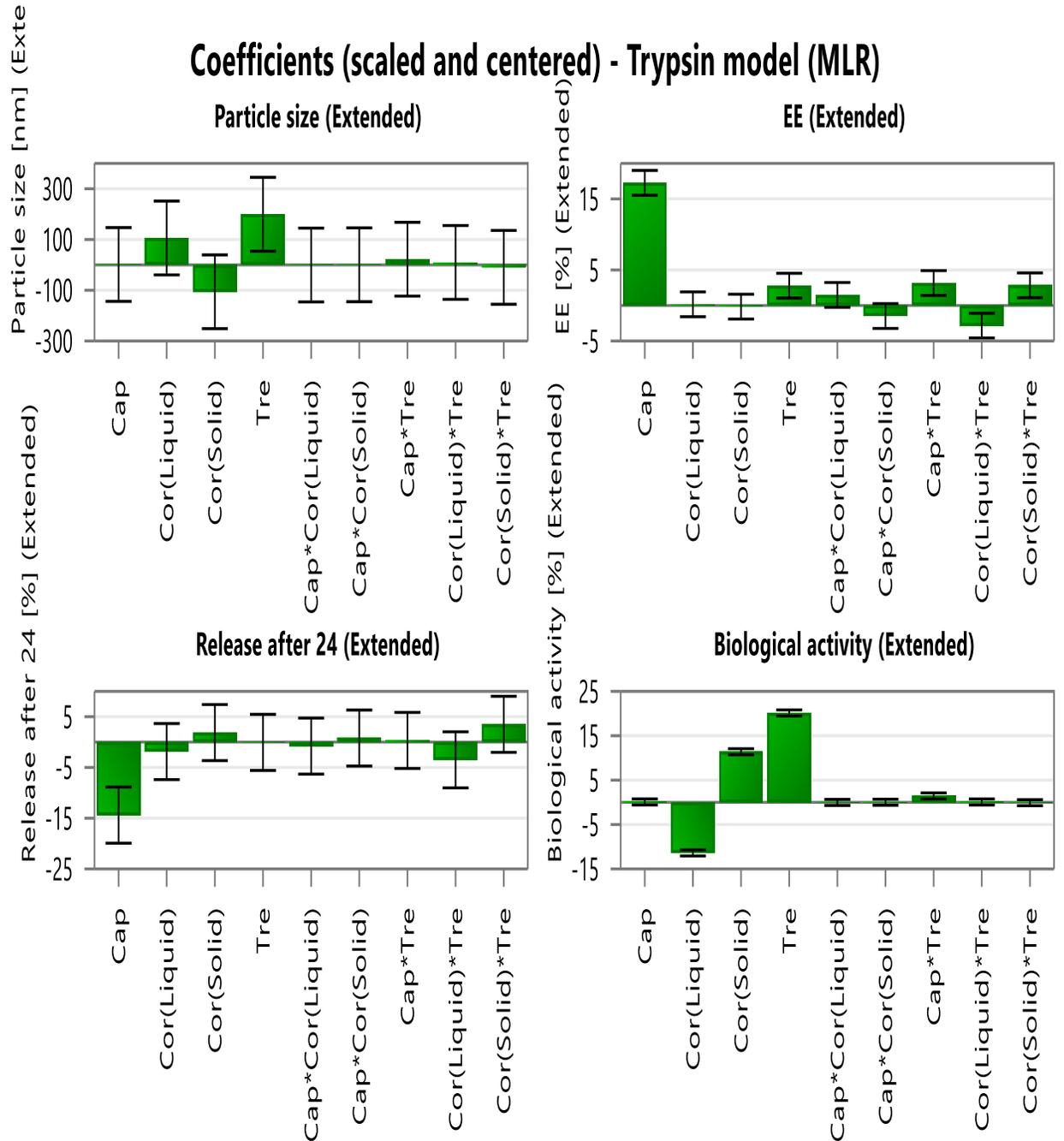
Particle size (N=8; DF=1; R2=1.00), EE (N=8; DF=1; R2=1.00), Release after 24 (N=8; DF=1; R2=0.99),
 Biological activity (N=8; DF=1; R2=1.00) Conf. lev.=0.95

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One cap end is within the green area and the other is outside: significant model term .

Both cap ends are out of the green area: non-significant model term .

Figure 5.4: The coefficients graphs for lysozyme.



Particle size (N=8; DF=1; R2=1.00), EE (N=8; DF=1; R2=1.00), Release after 24 (N=8; DF=1; R2=1.00),
 Biological activity (N=8; DF=1; R2=1.00) Conf. lev.=0.95

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One cap end is within the green area and the other is outside: significant model term .
 Both cap ends are out of the green area: non-significant model term .

Figure 5.5: The coefficients graphs for trypsin.

The coefficient graphs and tables conclude that; the formulation characteristics have been affected significantly by different factors. Moreover, the interaction between the factors had a significant effect on the characteristics, as well. The percentage encapsulation efficiency and drug release in SIF for both of lysozyme and trypsin PNCs have been affected significantly by the ratio between Caprolactone and Lactide blocks in the copolymer. On the other hand, trehalose has a significant effect on the formulations' particle size and the biological activity of the encapsulated proteins. In addition to trehalose effect on the biological activity, significant differences between the solid and liquid encapsulated proteins, in terms of biological activity, were observed. Since the biological activities of S/O/W formulations were, significantly, higher than the activities for the formulations prepared by W/O/W. All the results and the discussions behind the mentioned effects will be detailed in Chapter six in the current study.

In light of the previous results and taking into consideration the desired CQAs (responses target), all the factors were optimised by using the MODDE 10.1 software. The software predicted the proper levels required to fulfil the desired qualities and characteristics, provided values for the factors, and suggested the optimal formulations compositions.

The following section discusses all the optimisation process the optimum formulations preparation and characterisation.

5.3.4. Preparation and characterisation of polymeric nanocapsules at the optimised conditions

5.3.4.1. Preparation of polymeric nanocapsules containing lysozyme and trypsin at the optimised conditions

Applying smart mathematical and statistical models for the design of the experiment helps to reduce cost, effort, and time through reducing the number of experiments needed and prepared formulations by varying all the formulation factors at the same time for product development. Thus, generating clear strategies to provide reliable solutions after performing the experiments, and then optimising the formulation factors and their relevant levels in order to achieve the desired quality attributes. After the designs fitting an optimising equation describing the best fitted model can be applied to optimise factors in order to find the best compromise responses values. Table 5.9 elucidates the optimum conditions and formulation factors levels and their relevance in predicting the desirable formulations attributes including: particle size, encapsulation efficiency, drug release in SIF, and proteins biological activity.

Table 5.9: The optimised formulations conditions and their relevant predicted responses.

Formulation	protein	Polymer ratio ^{***}	Trehalose (mM)	Core physical state	Particle size (nm)	EE [*]	BA ^{**}	Drug release
OL1	Lysozyme	40:60	9.0	Solid	587.35	69.1	93.8	36.5
OL2	Lysozyme	36.2:63.8	9.0	Solid	587.21	7.02	94.1	32.84
OT1	Trypsin	42.4:57.6	11.7	Solid	840.82	80.9	91.6	40.66
OT2	Trypsin	41.3:58.7	11.0	Solid	821.41	81.1	89.5	39.17

^{*} Encapsulation efficiency.

^{**} Biological Activity.

^{***} Poly (D, L- Lactide-co- caprolactone) ratio.

Optimising the factors to achieve all the desired CQAs was not a possible task. Therefore, the selected optimised formulations are predicted to achieve all the desired CQAs except the drug release in SIF. Both the desired drug release or encapsulation efficiency is expected not to be achieved in the optimised formulations as a balance between the encapsulation efficiency, and the drug release could not be achieved. Since drug release and encapsulation efficiency were showing opposite effect. Therefore, the optimised formulations have been prepared to achieve the desired encapsulation efficiency, and further strategies will be applied in order to enhance the drug release profiles.

Certain techniques have been implemented to enhance the polymer ability to release high percentage of the protein within 24 hours during dissolution test in SIF medium.

The optimised formulations have been prepared using the same method used to prepare the previous formulations, with small changes, have been applied to accommodate the new formulations to increase the drug release within a reasonable time, to suit the oral delivery. Previous studies had added plasticisers and release enhancers to the used polymers [181, 182], or blended the used hydrophobic polymer with hydrophilic polymers such as PEG or poly ethyl oxide [183]. *Lu et al. 1999* have concluded that; blending the used hydrophobic polymer with a hydrophilic polymer to develop polymeric nanocapsules resulted in increasing of the leakage of the encapsulated protein into the outer aqueous media. Consequently, this will increase the drug release and reduces the encapsulated efficiency.

In the current study, a release enhancer has been added to the formulations just before the freeze drying process. Adding polyethylene glycol (PEG) 8000, a water soluble the plasticiser, to the formed nanocapsules just before the lyophilization may help to avoid any leakage of the entrapped proteins out the polymeric shell during the formulation process. Thus, no effect on protein confining ability has occurred, and the drop in the drug encapsulation efficiency was avoided.

Also, PEG 8000 had an influence on drug release from PNCs and enhanced the protein release, which attributed to plasticising of the biodegradable polymers. Therefore, different concentrations of PEG 8000 have been added to OL1 to study the effect of the plasticiser concentration on protein release profiles from PNCs. Hence, optimising the concentration to achieve the release target without any anti plasticising effect.

Table 5.10 shows the effect of different PEG concentrations on the protein release profile from OL1 formulation (taken as a model to optimise the polymer: plasticiser weight ratio) in SGF and SIF. Moreover, the relationship between PEG 8000: polymer weight ratio and the overall lysozyme release in SIF after 24 hours is illustrated in a phase diagram, Figure 5.6.

Table 5.10: Total mean released lysozyme from OL1 formulation in Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) in the presence of different PEG concentrations. For formulation composition, refer to Table 5.9.

Formulation	Ratio (plasticizer: polymer)	Release % in SGF (4 hours) ± SD	Release % in SIF (24 hours) ± SD
F1	0	13.81 ±	34.15 ±
F2	10	14.12 ±	55.02 ±
F3	15	21.06 ±	81.07 ±
F4	25	60.02 ±	92.31 ±
F5	50	19.03 ±	25.33 ±

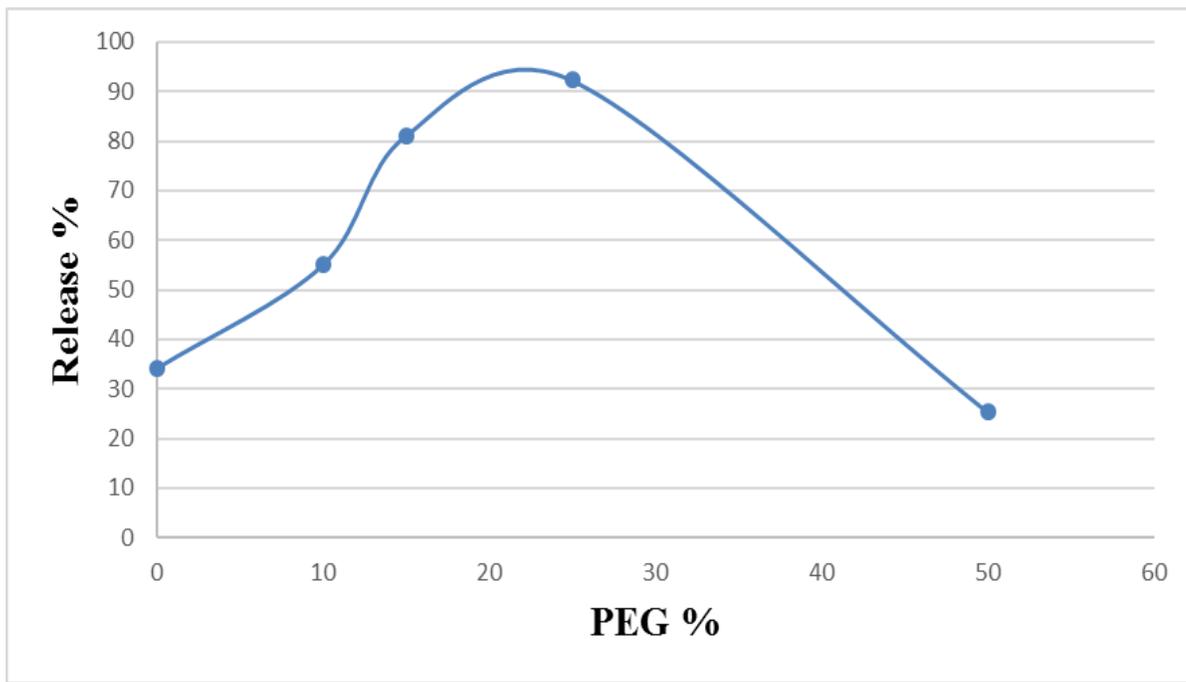


Figure 5.6: Phase diagram of the total amount of released lysozyme from the nanocapsules in SIF in the presence of different PEG concentration.

As seen in Table 5.10 and Figure 5.6, after determining lysozyme release at the four different plasticiser concentrations and at the control (without plasticiser), it was concluded that; the highest drug release in SIF was observed for the formulation containing 25% PEG, with the value reached more than 92%. However, F4 formulation (with 25% PEG) will not be able to protect the most of the encapsulated proteins from the degradation by the gastric enzymes and the sharp pH gradient, as the mean drug release in SGF was 60.02%. Nevertheless, although the drug release from F3 formulation (15% PEG) in SIF, which is 81.06%, was less than the observed release for F4; 15% w/w PEG 8000 weight was selected to prepare the further PNCs since the drug release in SGF was 21.06%.

Accordingly, balancing between high percentage of drug release in SIF and lower percentage of drug release in SGF helped to deciding 15% w/w PEG as a suitable concentration to be used to prepare the optimised formulations, in order to increase the percentage of drug release in SIF with a low percentage of drug release in SGF.

Figure 5.7, and Figure 5.8 illustrate the drug release patterns over four hours and twenty-four hours in SGF and SIF, respectively. The drug release in SGF and SIF was measured and quantified, at five and nine different time points, via analysing the released protein by SEC.

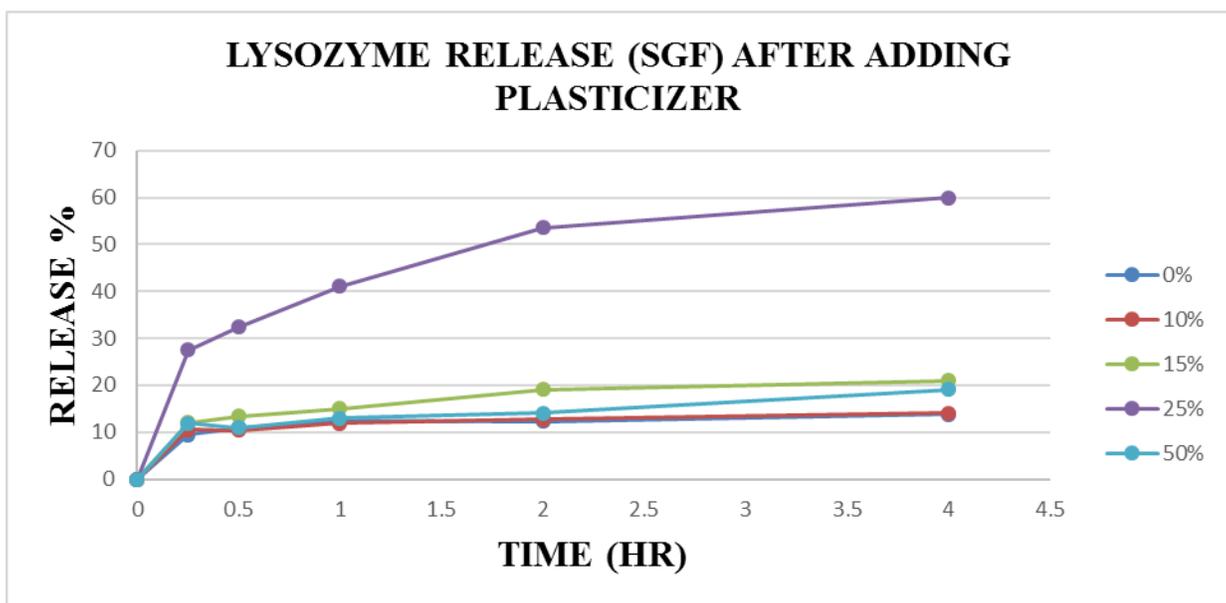


Figure 5.7: Release pattern of lysozyme from polymeric nanocapsule system in SGF after adding polyethylene glycol as a plasticizer at different concentrations.

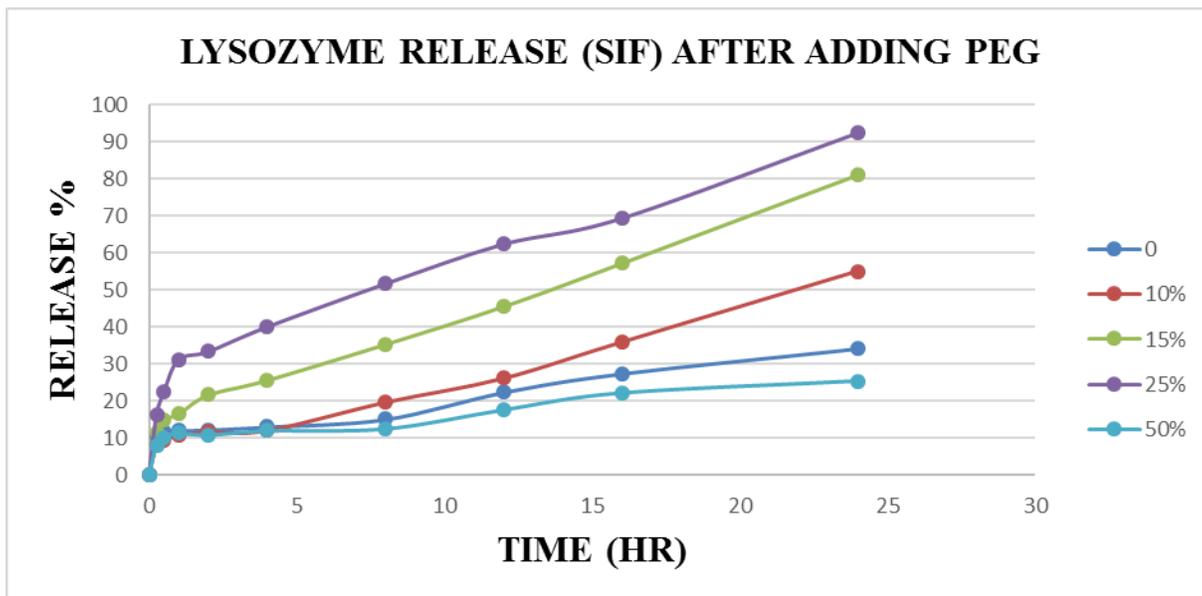


Figure 5.8: Release pattern of lysozyme from polymeric nanocapsule system in SIF after adding polyethylene glycol as a plasticizer at different concentrations.

As illustrated in the figures above (Figure 5.7 and Figure 5.8), the PNC systems have released the protein in SGF and SIF in a biphasic pattern. However, drug release from F4 (with 25% PEG) formulation was observed to be in a triphasic pattern.

Figure 5.8 illustrates the effect of the plasticiser concentration on Total protein release in SIF after 24 hours. PEG interacted with poly (D, L - Lactide- co- caprolactone) via hydrogen bonds interaction with the oxygens on the polymer chain. This interaction has softened the polymer further by increasing the water content around the polymer, since PEG is a hydrophilic polymer, and the polymers containing caprolactone are permeable polymers for small drug molecules with very tiny surface pores [184]. Thus, the release enhancement observed after PEG 8000 addition to the formulations may have been a result of increasing the size of the already exist pores which consequently improved the release profiles by forming a plasticised permeable polymer, as observed by TEM image, Figure 5.9. However, increasing the PEG 8000 concentration to 50% w/w decreased the percentage of drug release to less than that without PEG 8000 addition.

This decrease may be attributed to the relatively high PEG 8000: (D, L - Lactide- co- caprolactone) weight ratio (50%), which may result in forming a shield around the nanocapsule, which might reduce the drug release, Figure 5.10.

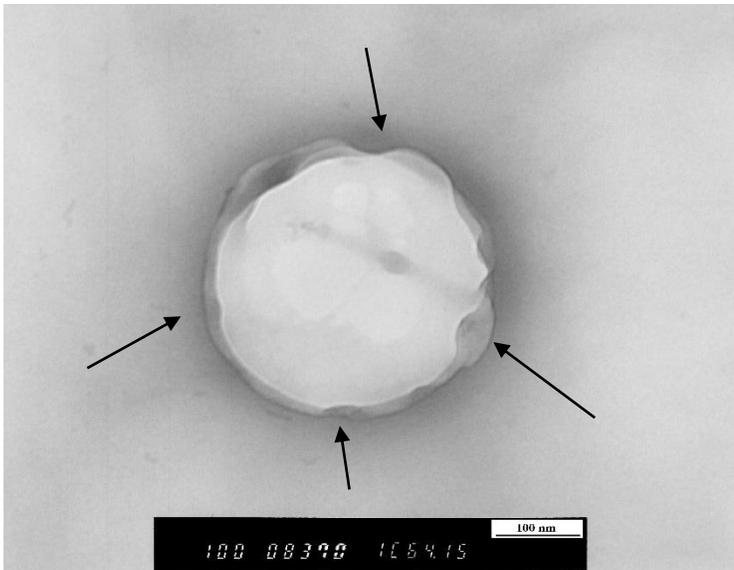


Figure 5.9: Transmission Electron Microscopy image of lysozyme containing polymeric nanocapsule after adding 25% w/w Polyethylene glycol.

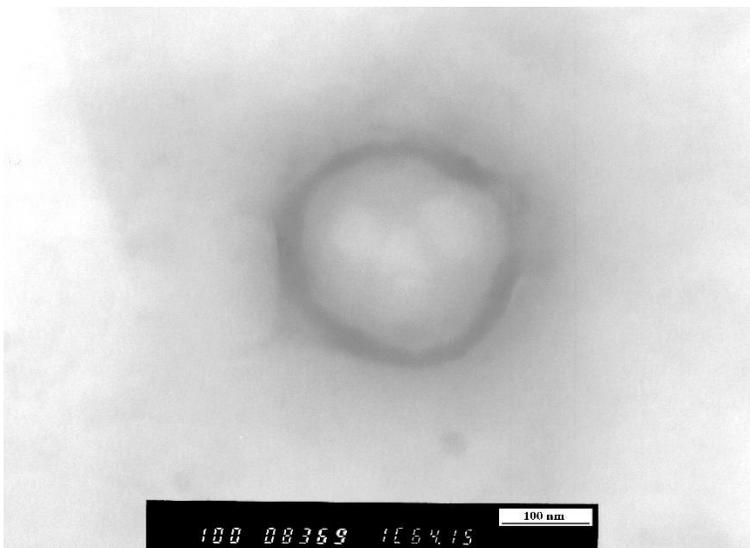


Figure 5.10: Transmission Electron Microscopy image of lysozyme containing polymeric nanocapsule after adding 50% w/w Polyethylene glycol.

Furthermore, the drug release from the 25 % w/w/ PEG 8000 formulation (F4) reached 60% within 4 hours in SGF. However, the protein release from the same formulation after 4 hours in SIF reached up to 40%, as shown in Figure 5.7 and Figure 5.8. This sharp increase in drug release may be attributed to the pH value. PEG is a weak acid acts as a base in the presence of strong acids e.g. HCl which decrease hydrogen dissociation, hence, it makes the positive charge ($-OH^{+2}$) form is the default. Consequently, the electrostatic interaction between the positive charge and the free pair of electrons on the surface of will result in higher drug release.

Accordingly, all the four optimised formulations, mentioned in Table 5.9, were prepared as described in Section 2.4.2. However, 15% PEG w/w was added to each formulation just before lyophilisation. Then, the four formulations were characterised against the desired quality attributes and how good the developed strategy to achieve the CQAs and QTPPs.

5.3.4.2. Characterisation of polymeric nanocapsules containing lysozyme and trypsin at the optimised conditions

The PNC formulations of the both proteins were characterised by their encapsulation efficiency, drug release, and proteins biological activity. All the characterisation results are shown in Table 5.11.

Table 5.11: The optimised formulations conditions and their relevant measured results. For the formulation composition, see Table 5.9.

Formulation	Drug release (SGF)	EE*	BA**	Drug release (SIF)
OL1	16.86	73.66	93.80	80.80
OL2	20.09	75.72	94.13	79.68
OT1	15.71	80.91	91.69	81.23
OT2	17.22	81.16	89.55	80.66

* EE: Encapsulation efficiency.

**BA: Biological activity.

The above results, Table 5.11, show comparable results to the predicted ones, mentioned in Table 5.9, which reflects good and valid design models. The measured characteristics of PNCs demonstrate how the critical quality attributes were achieved by implementation of the QbD approach and applying the DoE and give promises to develop PNCs systems containing proteins intended for oral delivery.

Lysozyme and trypsin were used as the model proteins. However, the developed approach represents strategies to developing future polymeric nanocapsules containing other macromolecules e.g. therapeutic proteins, monoclonal antibodies, and genes.

5.3.5. The validating and applying of the developed strategy for protein loaded

PNCs

The strategy for preparation of PNCs containing stable protein that are suitable for oral protein delivery purposes has been developed as discussed in this chapter. In order to validate the developed strategy, a new therapeutic protein containing PNC formulations should be prepared and characterised according to the suggested optimal conditions. A deoxyribonuclease I (DNase I) is a therapeutically active enzyme which is used to treat cystic fibrosis, has been chosen to be encapsulated into the PNC system intended for oral delivery. Two different formulations have been prepared as described in Section 2.4.5, with the addition of 15% w/w PEG 8000 as a release enhancer. Table 5.12 shows the two DNase I formulations along with their characteristics including: encapsulation efficiency, biological activity, and drug release profiles in SIF, and in SGF.

Table 5.12: The two DNase I containing polymeric nanocapsule formulations as they were prepared according to the developed strategy, along with their characteristics.

Formulation	protein	Polymer blocks ratio ^{***}	Trehalose (mM)	Core physical state	Drug release % in SGF	EE [*]	BA ^{**}	Drug release % in SIF
OD1	DNase I	40:60	9.0	Solid	23.33	79.19	93.82	82.36
OD2	DNase I	40:60	11.0	Solid	16.92	81.09	94.66	84.88

* Encapsulation efficiency.

**Biological Activity.

*** Poly (D, L- Lactide-co- caprolactone) ratio.

As shown in Table 5.12, the encapsulation efficiency, the overall drug release and biological activity of the two DNase I containing PNCs were determined. As clear from the table, both formulations have encapsulation efficiency reached around 80%, with more than 82% drug release in SIF after 24 hours of the dissolution test. Moreover, the biological activity of the encapsulated proteins reached more than 95%, which reflects the ability of DNase I to withstand during the process procedures. Interestingly, the results are comparable to those obtained from encapsulating lysozyme and trypsin into PNCs at the same optimum conditions, Table 5.11.

The morphology of the PNCs containing DNase I was also investigated by using TEM, Figure 5.11.

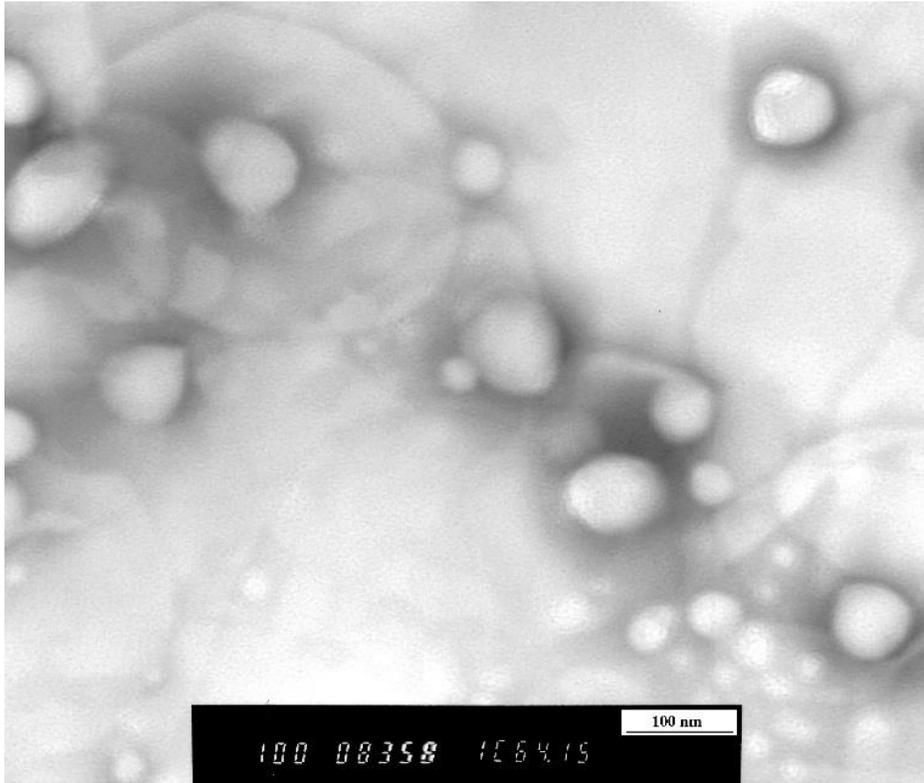


Figure 5.11: Transmission Electron Microscope image showing the morphology of DNase I containing polymeric nanocapsules.

Nanocapsules were spherical in shape and smooth and wrinkled as shown in Figure 5.11, with little distortion on their surface. The distortion may be caused due to the crystalline nature of the used copolymer and also by the accumulated PEG 8000 on the surfaces.

5.3.6. Conclusion

This study has established a strategic approach for the development of polymeric nanocapsules confining intact and active biomolecules those are suitable for oral delivery. Adapting and applying the QbD approach by defining clear QTTPs and CQAs

from the beginning and performing risk assessment saved the time and resources, and provided a practical strategy to confining biomolecules in the polymeric system without significant effect on their biological activity. The polymeric nanocapsules characteristics were investigated by changing the formulation factors according to the design of the experiment. Using two different copolymer ratios helped to monitor the encapsulation efficiency with a controlled release profile in SIF. Formulations material attributes were optimised, and stable formulations with high encapsulation efficiency and controlled release were obtained by adding the so-called release enhancer PEG 8000. Adding 15% (w/w) PEG to the formulations enhanced drug release to up to 80 % in SIF over 24 hours without increasing the risk of proteolytic degradation in the gastric enzyme, and without reducing the encapsulation efficiency. The developed strategy was validated by applying it to prepare and characterise polymeric nanocapsules containing DNase I, and comparable, and promising results were obtained. The results of the current study were totally in agreement with theoretical prediction by the developed approach and models. The method established in this study used was pilot scale offered short time, low cost, more targeted oriented approach, hence this approach could be utilized on a large scale for pharmaceutical and biopharmaceutical nano-development. The following chapter describes the details of preparation and characterisation of the polymeric nanocapsules containing lysozyme and trypsin.

**Chapter Six: Preparation and
characterisation of polymeric
nanocapsules containing
lysozyme and trypsin:
intended for oral route
delivery**

6.1. Introduction

A biodegradable polymeric nanocapsule system (PNC) is a core-shell system, where the core is the drug reservoir, and the shell is composed of the biodegradable polymer [175]. The polymeric nanocapsule delivery system was developed to deliver the therapeutics via several routes of administrations e.g. oral and parenteral. The encapsulation technique protects the medications, controls the drug release, and delivers the therapeutic to the target site [185]. Moreover, the small size of PNCs enhances the formulation drug release [176]. Furthermore, the PNCS are biodegradable and biocompatible with the body tissues and cells[129]. Due to the previously mentioned advantages, the nanoencapsulation technique was employed to deliver the macromolecules e.g. therapeutic proteins. Therapeutic proteins must be provided in a stable, intact and active form in order to play their role in disease treatment. Also, the other product characteristics should be achieved to e.g. high encapsulation efficiency, and drug release. Therefore, the used materials and the preparation conditions and methods should be carefully selected to avoid the formulations failure. Different preparation methods were discussed in the literature, i.e. nanoprecipitation, double emulsion solvent evaporation, emulsion diffusion, and polymer coating. However, the double emulsion is the popular method to prepare protein containing PNCs due to the protein physicochemical properties as a hydrophilic drug.

Different additives can be added to the nanocapsule core containing encapsulated therapeutic protein in order to overcome the stability problems; examples for additives

are sugars, salts, or amino acids [186]. In the current study, the proteins were encapsulated with and without trehalose and encapsulated as solid and liquid proteins.

6.2. Aims and Objectives

Using the solid/oil/water (S/O/W) technique to prepare PNCs was not investigated enough in the literature and no adequate data regarding encapsulation of solid protein inside the nanocapsules system in the literature. Adding additives to the nanocapsule core is still not well studied and not common. Therefore, the aim of this study is to formulate PNCs containing (trypsin or lysozyme) as model proteins with built in quality by applying the QbD aspects from the beginning of the design.

Two biodegradable diblock copolymers of Lactide and ϵ -Caprolactone monomers were used in this to prepare protein loaded nanocapsule. The ratio between the two copolymer blocks is varied to allow for optimisation in terms of the quality attributes e.g. encapsulation efficiency and drug release. Furthermore, the different factors were changed at the same time to investigate the interaction between the factors, like the presence of additives such as trehalose in the core of PNCs by applying DOE under the QbD framework.

6.3. Results and discussion

6.3.1. Polymeric nanocapsules preparation

In the current study, polymeric nanocapsules were prepared by applying the double emulsion method, as described in Section 2.4.2. Based on pre-built Design of Experiments (DOE), eight different lysozyme formulations and eight different trypsin formulations were prepared and characterised as will be discussed in details in the following sections.

6.3.2. Polymeric nanocapsules characterisation

PNCs formulations were characterised in order to evaluate them and optimise the materials attribute and process parameters. And the characterisation methods were adapted from the literature (Section 2.4.3). With a view to quantifying and analysing the encapsulated proteins, the polymeric shells were broken down, and the encapsulated proteins were collected. Because proteins may denature by organic solvents, hence, different organic solvents were used to suspend the proteins and their effect on the proteins structures integrity was examined by size exclusion chromatography (SEC) for the presence of denatured products caused by the used organic solvent. Table 6.1 shows the retention percentage of the both proteins after suspending in three different organic solvents. Nano pure water ($>\Omega$ 18, Milli-Q) was used as a control in all cases.

Table 6.1: Three different organic solvents and their effect on lysozyme and trypsin after preparing suspensions with a final concentration equal to (100 µg/ml).

Organic solvent	Lysozyme content% ± SD	P-value	Trypsin content% ± SD	P-value
Ethyl acetate	99.09% ± 1.91	0.199919	98.67 ± 1.41	0.068845
Acetonitrile	94.38% ± 2.05	0.020822	98.73 ± 2.11	0.196052
Isopropyl alcohol (IPA)	88.36% ± 2.65	0.012845	92.71 ± 2.11	0.025012
Deionised Water (control)	100.45% ± 1.73	-----	101.30% ± 2.27	-----

It is shown in Table 6.1, that using of ethyl acetate and acetonitrile had almost the same impact on trypsin. However, in the case of lysozyme, ethyl acetate gave good recovery when compared to acetonitrile. When isopropyl alcohol (IPA) was used, the overall recovery of both the proteins reduced when compared to ethyl acetate and acetonitrile. It is shown in the table that use of IPA has more impact on lysozyme as compared to trypsin.

The selection of ethyl acetate for trypsin was considered as a choice to use the same solvent for both proteins. This high retention percentage means low denaturation properties of methyl acetate against the proteins. Therefore, it was chosen to break the polymeric shell in order to quantify the encapsulated proteins.

6.3.2.1. Protein encapsulation efficiency

The encapsulation efficiency of drug loading particles is the ability of those particles to entrap the drug molecules, and it is expressed as the percentage of the entrapped drug to the original drug amount used during manufacturing. Encapsulation efficiency of PNCs is one of the critical quality attributes (CQAs) which examined in this work. Therefore, different process parameters and material attributes have been wisely selected, controlled and optimised to enhance the ability of the nanoparticles to encapsulate more drugs, in order to achieve the targeted encapsulation efficiency, which will, consequently, reduce the cost and the bulk volume of the drug dose as QTPPs. The PNC's efficiency of entrapping proteins was determined and quantified as described in Section 2.5.2.

Data from relevant literature showed; that the encapsulation efficiency of hydrophilic drugs including proteins reached up to 10% only [187]. However, when double emulsion method was used to prepare the PNCs containing proteins, the entrapment efficiency reached 65% - 75% on average, depending on the polymer, encapsulated drug, and surfactant used [188].

Experimental design including all the factors, level and the mean encapsulation efficiency of proteins as a response is shown in Table 6.2.

Table 6.2: A total of 16 trypsin and lysozyme PNCs formulations along with the mean values of the encapsulation efficiency. The experiment was performed in triplicate. The standard deviation is provided.

Formulation	ϵ -Caprolactone ratio	Core state	Trehalose mM	Lysozyme EE \pm SD	Trypsin EE \pm SD
F1	14%	Liquid	0	32.1% \pm 2.8	41.2% \pm 6.8
F2	60%	Liquid	0	68.4% \pm 1.5	72.1% \pm 5.2
F3	14%	Solid	0	39.3% \pm 1.5	37.9% \pm 2.0
F4	60%	Solid	0	62.3% \pm 2.6	63.4% \pm 3.3
F5	14%	Liquid	10	31.3% \pm 2.7	34.5% \pm 5.0
F6	60%	Liquid	10	69.2% \pm 5.3	78.6% \pm 2.6
F7	14%	Solid	10	41.5% \pm 1.9	43.1% \pm 3.1
F8	60%	Solid	10	64.1% \pm 1.3	80.7% \pm 2.8

F: formulation.

Table 6.2 illustrates the encapsulation efficiencies of sixteen different polymeric nanocapsules formulations containing trypsin and lysozyme. The encapsulation efficiency of the proteins in this project was recorded between (31.3-80.7%), Table 6.2, and Figure 6.1. Although encapsulation efficiency of trypsin was higher than lysozyme, no significant difference between trypsin and lysozyme encapsulation efficiency was recorded.

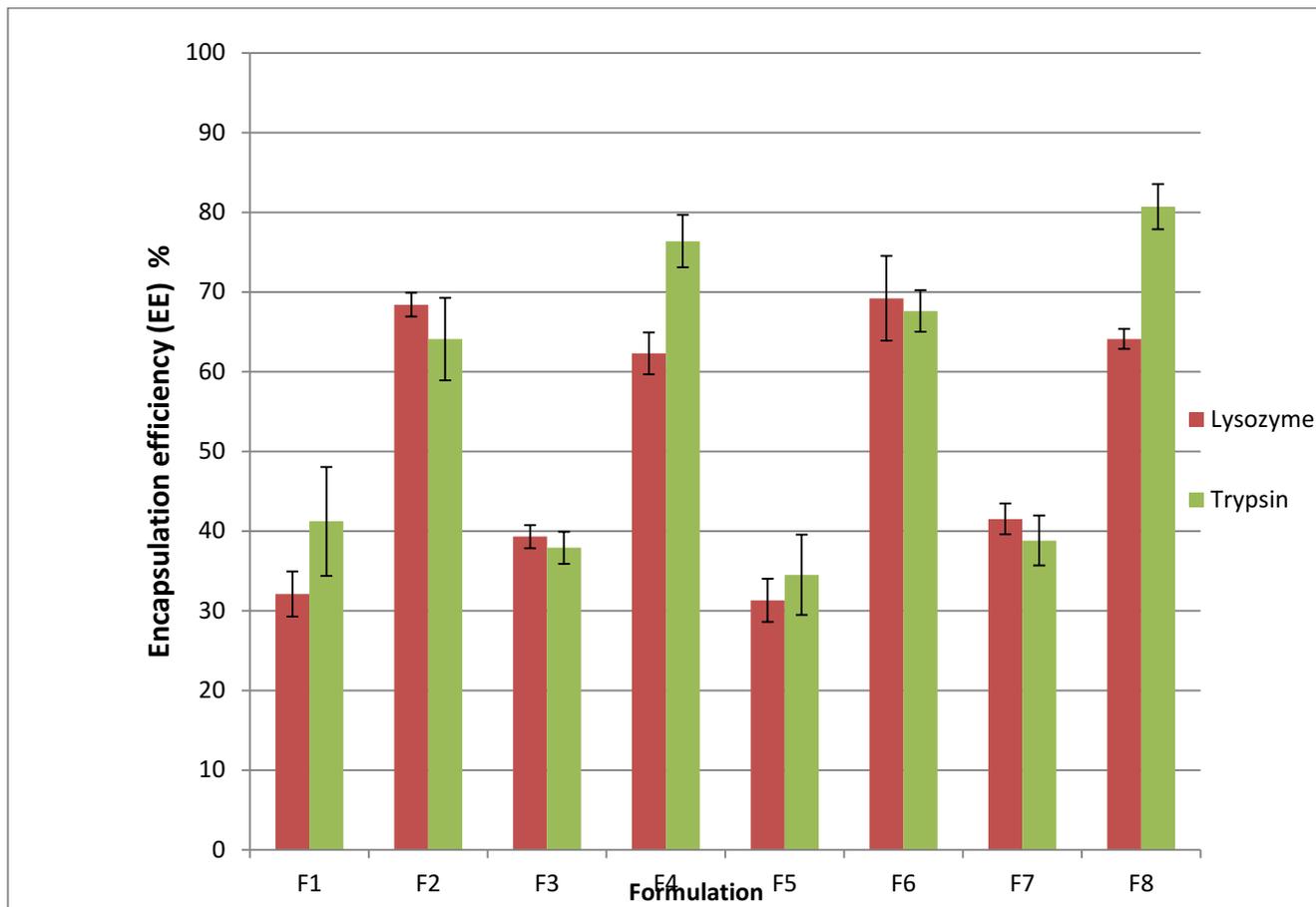


Figure 6.1: Mean encapsulation efficiency of Polymeric nanocapsules containing either trypsin or lysozyme. For Formulations composition refer to Table 6.2.

The highest encapsulation efficiency observed was 80.70% for trypsin formulation number 8 (T8), when the molar ratio of Lactide to Caprolactone of (40:60) poly (D, L- Lactide-co-Caprolactone) was used to encapsulate solid trypsin in the presence of trehalose. In contrary, the lowest value recorded was for lysozyme formulation 5 (L5), that has been prepared by using an (86:14) poly (D, L- Lactide-co-Caprolactone) copolymer. The encapsulation efficiencies ranges were (31.30 – 69.20 %) and (34.50 - 80.70%) for lysozyme and trypsin, respectively.

Protein encapsulation efficiency in PNCs is being affected by different factors such as; method of preparation, type of surfactants, type, nature and concentration of polymers, in addition to the properties of the encapsulated drug [136, 183, 189].

Encapsulation efficiency values were analysed and fitted for both proteins models by Multi Linear Regressions (MLR), and the coefficients were recorded for all factors.

The scaled and centred coefficients were calculated in order to evaluate the effect of different factors on encapsulation efficiency for all formulations. Table 6.3 shows all the coefficients and p-values for the factors and their interactions.

Table 6.3: Factors and their interaction effect on lysozyme and trypsin PNCs encapsulation efficiency as represented by the coefficients, along with p-value for each factor.

Factor	Lysozyme		Trypsin	
	Coefficient	P-value	Coefficient	P-value
ε-Caprolactone	14.975	0.0106	17.2625	0.00571
Trehalose	0.5	0.2952	2.7875	0.051377
Core physical state (liquid)	- 0.774999	0.19865	0.162501	0.447069
ε-Caprolactone * Trehalose	0.149998	0.655961	3.1625	0.0276617
ε-Caprolactone * Core physical state (liquid)	3.575	0.0444464	1.4875	0.0586806
Trehalose * Core physical state (liquid)	- 0.5000	0.295166	- 2.8375	0.0380253

*Interaction between the factors.

As apparent in Table 6.3, the experimental designs analysis concluded that; the type of copolymer or the ratio between the copolymer blocks has significantly (P-value = 0.0106 for lysozyme and 0.0051 for trypsin) affected the efficiency of the nanocapsules

to encapsulate both proteins. Furthermore, the high ratio of ϵ -caprolactone block in the copolymer significantly increased the encapsulation efficiency of the nanocapsule systems.

The coefficients calculation by MLR concluded that; polymer ratio was the only significant factor that affected the proteins entrapment efficiency; the bigger ϵ -Caprolactone ratio, the higher entrapment efficiency. And the molar ratio of ϵ -Caprolactone has a similar effect on both lysozyme and trypsin encapsulation efficiency.

Hydrocarbon chain in D, L- Lactide block is shorter than the ϵ -Caprolactone hydrocarbon chain, see Table 6.3. This fact explains the higher ability of ϵ -caprolactone to confine the protein and protect it from the leakage to the outer aqueous layer during the emulsification phase. Higher drug leakage may occur when the ratio of D, L- Lactide block in the copolymer increases. Since D, L- Lactide is less hydrophobic than ϵ -caprolactone, and it can make the polymer more amorphous and less crystalline.

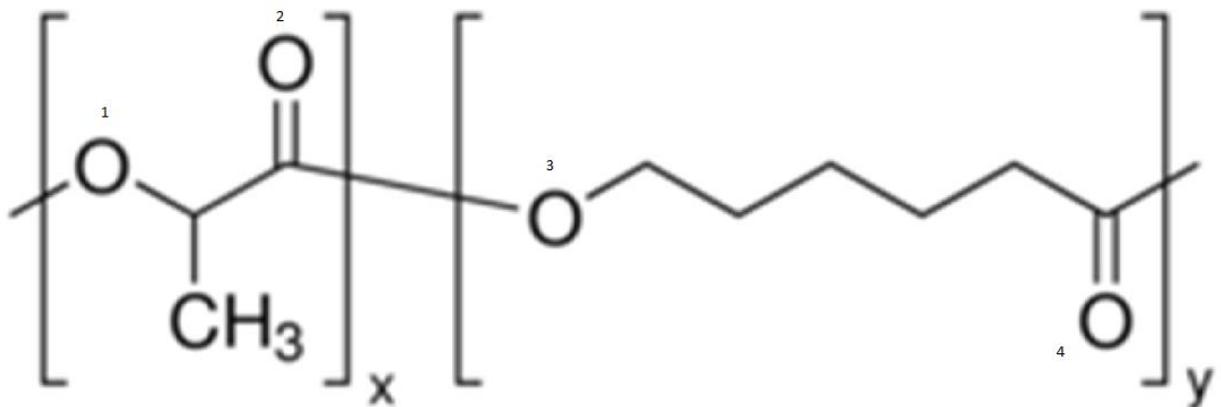


Figure 6.2: Poly (D, L-Lactide-co-Caprolactone) x: Lactide part, while y: Caprolactone. The figure was adapted from [190].

Longer hydrocarbon chain accelerated the solidification of nanocapsule shell during organic phase evaporation. This rapid solidification could increase the polymer ability to confine the protein and reduce the amount of the leaked protein into the outer aqueous medium.

Another reason could have increased the encapsulation efficiency of protein inside high ϵ -caprolactone copolymer nanocapsules is that; proteins normally desire the lower energy state. Lower energy tendency leads the protein molecules to form a hydrophobic interaction with the polymer to reduce the entire energy. Accordingly, the longer hydrophobic chain, the higher protein interaction. Consequently, this leads to increase the interaction between the proteins and ϵ -Caprolactone, thus, it increases the encapsulation efficiency. The drawback of this interaction is; it can increase the chance of the protein unfolding. This is due to an increase in the exposure of the hydrophobic part, which may lead to protein denaturation. However, this will be investigated in details later in this project, in Section 6.3.2.5.

The physical state of encapsulated protein and encapsulation of trehalose with the protein had no significant effect on the encapsulation efficiency. However, the design of experiment (DOE) analysis evident that; the interaction between the physical state and the copolymer factor had a significant effect on lysozyme encapsulation efficiency. Thus, a substantial increase in encapsulation efficiency was recorded when (40:60) poly (D, L- Lactide-co-Caprolactone) was used to encapsulate liquid lysozyme when compared to its ability to encapsulate lysozyme in the solid state.

Moreover, the ability of (40:60) poly (D, L- Lactide-co-Caprolactone) to confine trypsin in the presence of trehalose was higher than its encapsulation ability in the absence of trehalose. Also, encapsulating solid trypsin with trehalose inside the polymeric nanocapsule was significantly greater than the entrapment efficiency of liquid trypsin with trehalose inside the same polymer, regardless of the type of the polymer.

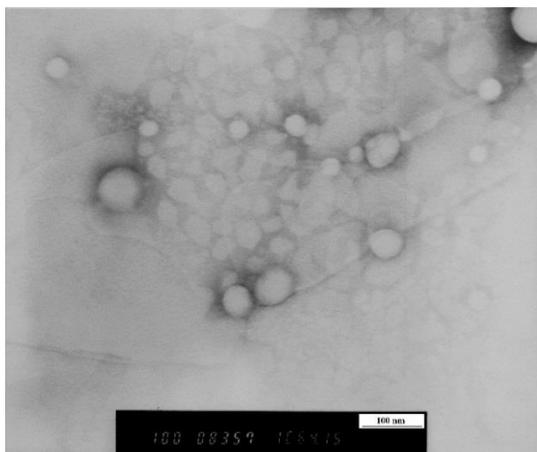
This interaction shows a positive synergistic effect which was found when the two factors tested together at the same time. This interaction effect cannot be observed in the conventional (one factor at the time). Factors interaction is too important to be studied as it cannot be seen in the traditional numerical analysis, one factor a time. Neglecting the interaction between factors can lead to missing critical results and information which may consequently affect the quality of the products especially when working at higher scales [191].

6.3.2.2. Microscopic imaging of polymeric nanocapsules using Negative Staining Transmission Electron Microscopy (TEM)

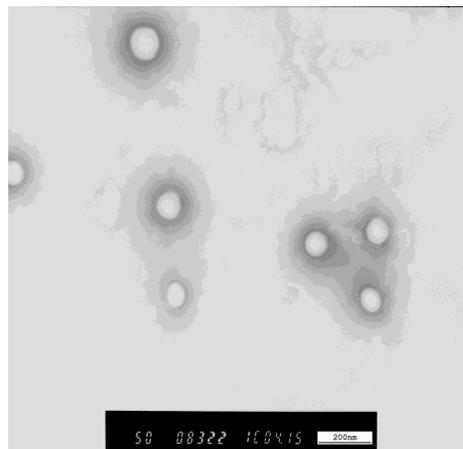
The overall morphology of nanocapsules, as observed by the TEM, has smooth external surfaces and particles of various size. Some of the agglomerates were also shown in the microscopic images, Figure 6.3.

There are three different morphologies observed overall in images, i.e. round with smooth surface, wrinkled surface with spotty images and tiny round smooth surfaces.

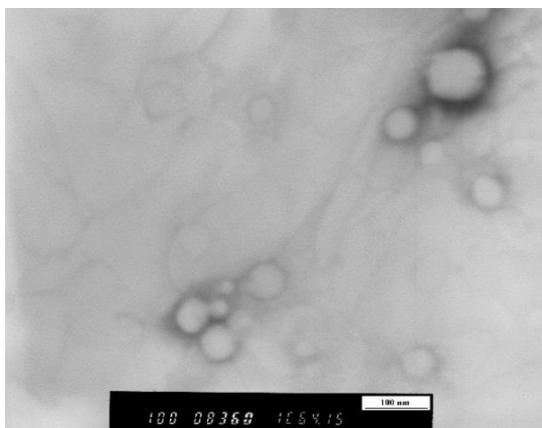
A (Lysozyme 7, L7)



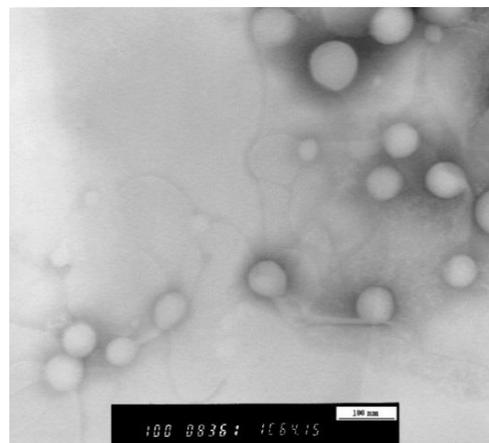
B (Lysozyme 3, L3)



C (Lysozyme 4, L4)



D (Lysozyme 1, L1)



E (Trypsin 3, T3)

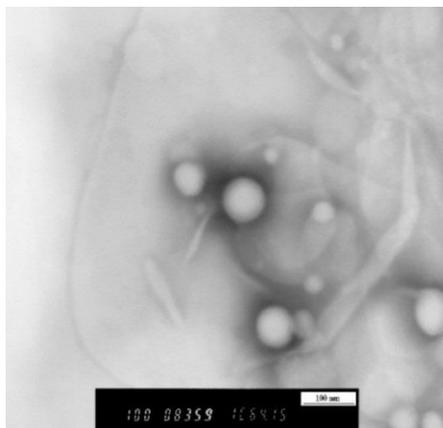


Figure 6.3: Transmission Electron Microscopy image of lysozyme nanocapsules without trehalose. For formulations composition, refer to Table 2.5.

As illustrated in Figure 6.3, no clear differences have been noticed between trypsin and lysozyme polymeric nanocapsule formulations. As the comparison between the same formulation containing either trypsin (T3, E) or lysozyme (L3, B) showed that; both of them had regular and round shape, clear core, and smooth with no distortion surface.

However, the comparison of the same protein formulations showed many differences. Thus, the PNCs prepared by using (86:14) Poly (D, L- Lactide -co- Caprolactone) showed regular, round and clear particles, but, the PNCs prepared by the other copolymer had more distortion and irregularities. These variations have been raised because of the nature of the copolymers used, since the 40:60 Poly (D, L- Lactide -co- Caprolactone) internal structure is dominant, crystalline, while the other copolymer has an amorphous structure. The crystalline structure has less flexibility and more rigid, which may cause distortion and irregularities during the preparation processes. Moreover, the (40:60) Poly (D, L- Lactide -co- Caprolactone) copolymer is more hydrophobic as explained earlier in this project, Section 6.3.2.1. The hydrophobicity may cause more coalescence between the nanoparticles and certain stages during the formation processes which may accordingly lead to some distortion.

Image An (L7) and Image B (L3) represent Lysozyme Polymeric nanocapsule formulations prepared by the same copolymer and have the same core physical state (solid) with and without trehalose, respectively. As concluded from these two images, that the PNC formulations prepared with trehalose had a spotty core when compared to the one without trehalose. This difference may be caused by the increased content of the core of PNCs when trehalose was added, as suggested by the author.

No difference was observed between s/o/w and w/o/w, as reflected by the comparison between Image B (L3) and Image D (L1). Both Images have been prepared by the

same copolymer, and no trehalose was encapsulated with lysozyme. Hence the only difference between them was the core physical state.

6.3.2.3. Particle size of the polymeric nanocapsules containing lysozyme and trypsin

Particles size analysis is one of the key factors and the major determinant of the drug release profiles and the pharmacokinetics properties. Therefore, it must be considered during nanocapsule characterisation [149].

Dynamic light scattering technique was used to determine the mean particle size of nanocapsules as described in Section 2.5.3. Table 6.4 shows that; PNCs particle size varies between the two types of entrapped protein. The sizes of PNCs containing trypsin and lysozyme were in the ranges of 292.5-992.8 nm, and 325.3 – 865.3 nm respectively, as illustrated in Figure 6.4 and Table 6.4. The comparison between two groups showed that the trypsin PNCs and lysozyme PNCs sizes are not significantly different.

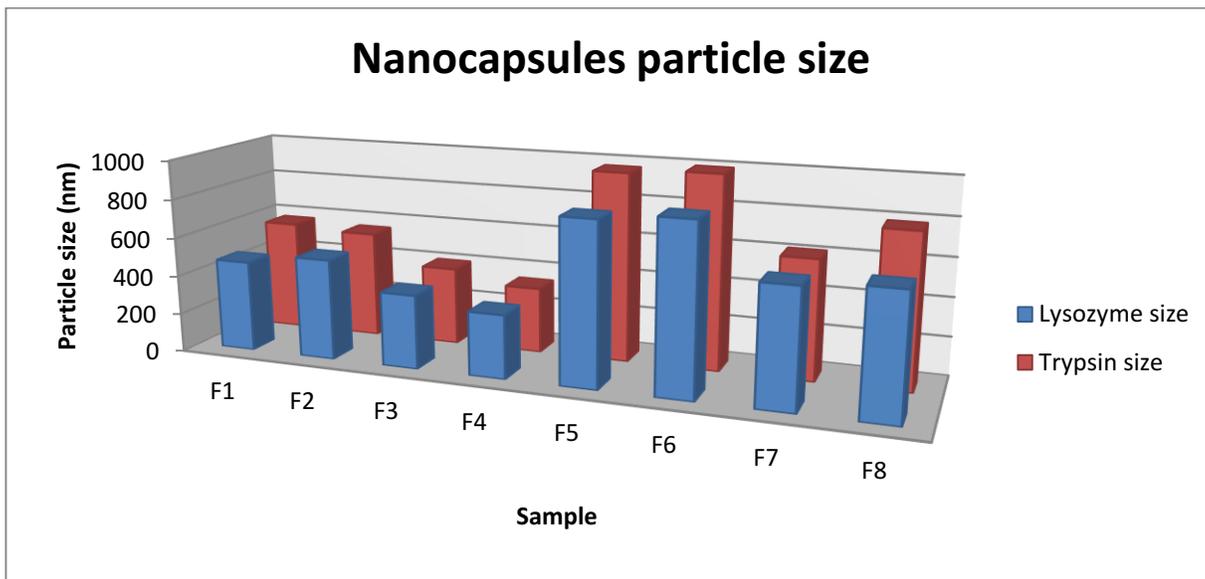


Figure 6.4: Mean particles size of PNCPs containing trypsin and lysozyme. For formulations composition, refer to Table 6.2.

Table 6.4: A total 16 PNCPs containing trypsin and lysozyme samples with their mean particles size and polydispersity. For formulations composition, refer to Table 6.2.

Formulation	Lysozyme nanocapsules size (nm)	Polydispersity	Trypsin nanocapsules size (nm)	Polydispersity
F1	467.1	0.383	571.9	0.183
F2	517.9	0.384	552.3	0.384
F3	378.4	0.335	401.2	0.335
F4	325.3	0.291	337.6	0.291
F5	831.9	0.388	968.6	0.388
F6	865.3	0.382	992.8	0.382
F7	601.1	0.346	713.4	0.346
F8	627.2	0.366	785.2	0.366

Particle size values were fitted and predicted by a 2ⁿ model, which showed that good models were generated. Since R² was (0.828, 0.94), Q² was (0.785, 0.908) and

reproducibility was (0.968, 0.987) for lysozyme and trypsin, respectively. All of these results are considered high, with low pure error, and Q^2 and R^2 were not separated by more than 0.2-0.3 which reflects valid models, as explained earlier in this project.

After design analysis, the presence of trehalose in the formulations was the only significant factor that affected the particle size for both trypsin and lysozyme PNCs formulations. The increase in particles size in the presence of trehalose was obvious in the current study. The exact underlying mechanism is still not clear. However, it could be due to accumulation of both protein and trehalose together in the core which may acquire a larger inner space. This assumption is supported by what has been observed in TEM images (Figure 6.3) when the comparison between the PNCs containing trehalose and the ones do not contain trehalose showed that; trehalose made spotty core due to the accumulation of trehalose.

6.3.2.4. *In vitro* release of trypsin and lysozyme in Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) without enzymes

In order to deliver the proteins orally, different delivery systems have been developed to protect the protein against the various hurdles, and polymeric nanocapsules system is among these techniques. After developing the oral nanocapsules, protein release in the gastrointestinal conditions must be studied. In the current study, protein release from the nanocapsules systems was investigated in the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) (without enzymes) over 4 and 24 hours, respectively.

Drug release from PNCs system depends on different factors and varies when these factors are changed; firstly, the physicochemical characteristics and concentration of

the active ingredients have influences on the release profile. Also, the physicochemical characteristics and solubility of the polymer have a high impact on the release kinetics of the drug [192]. In addition to the previous factors, drug release rate can be affected by PNCs preparation methods [193]. Moreover, particle size and *in vitro* release medium can also alter the drug release pattern [194]. The protein release profiles from PNCs were examined according to the described methodology in Section 2.5.5.

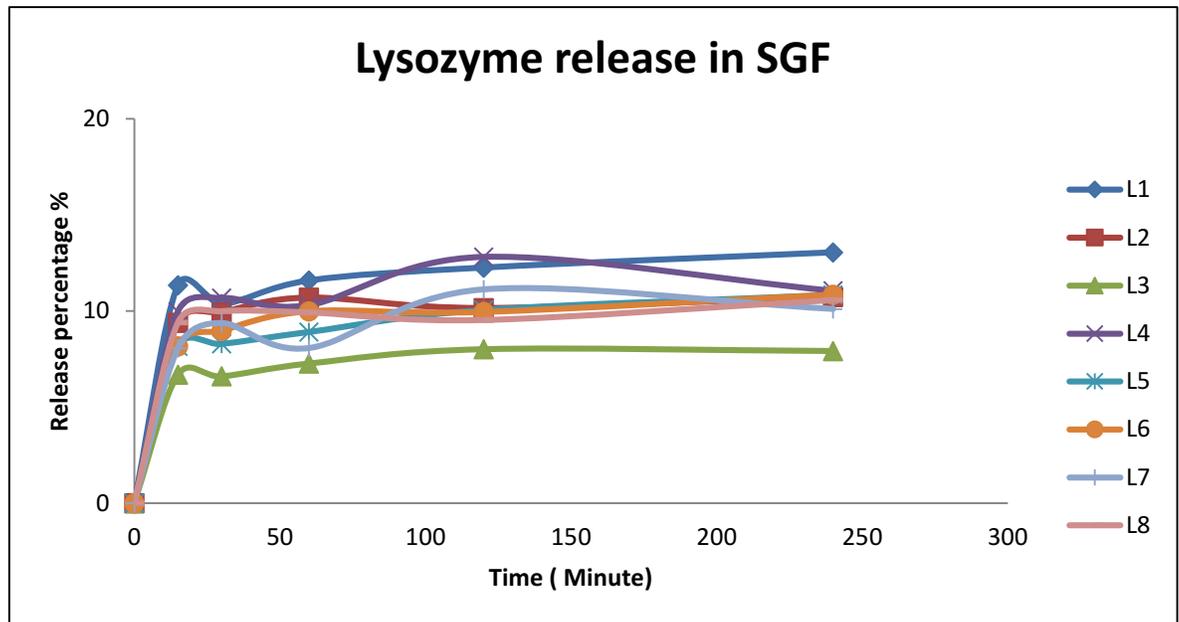
The overall percentage of trypsin and lysozyme released from PNCs in SGF and SIF after four and twenty-four hours, respectively, is shown in Table 6.5.

Table 6.5: Overall percentage of trypsin and lysozyme released from PNCs in SGF and SIF after four and twenty-four hours, respectively. For formulations, composition refer to Table 6.2.

Formulation	Lysozyme in SGF	Trypsin in SGF	Lysozyme in SIF	Trypsin in SIF
F1	13.05	11.12	72.53	62.64
F2	10.73	10.60	32.38	30.75
F3	7.92	11.82	64.36	56.93
F4	11.05	10.22	30.95	29.94
F5	10.84	13.68	64.90	53.98
F6	10.84	11.67	38.52	25.14
F7	10.10	13.30	68.53	64.02
F8	10.57	10.02	35.68	36.60

Trypsin and lysozyme release profile from PNCs in SGF and SIF (without enzymes) are illustrated in Figure 6.5 and Figure 6.6.

A)



B)

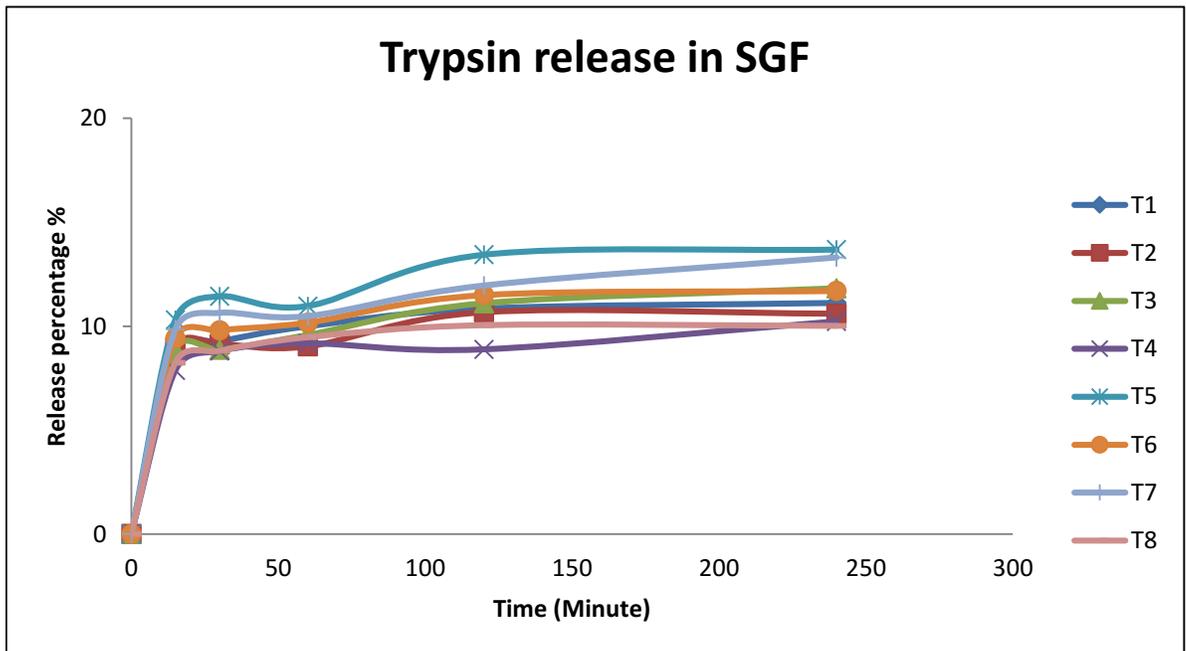
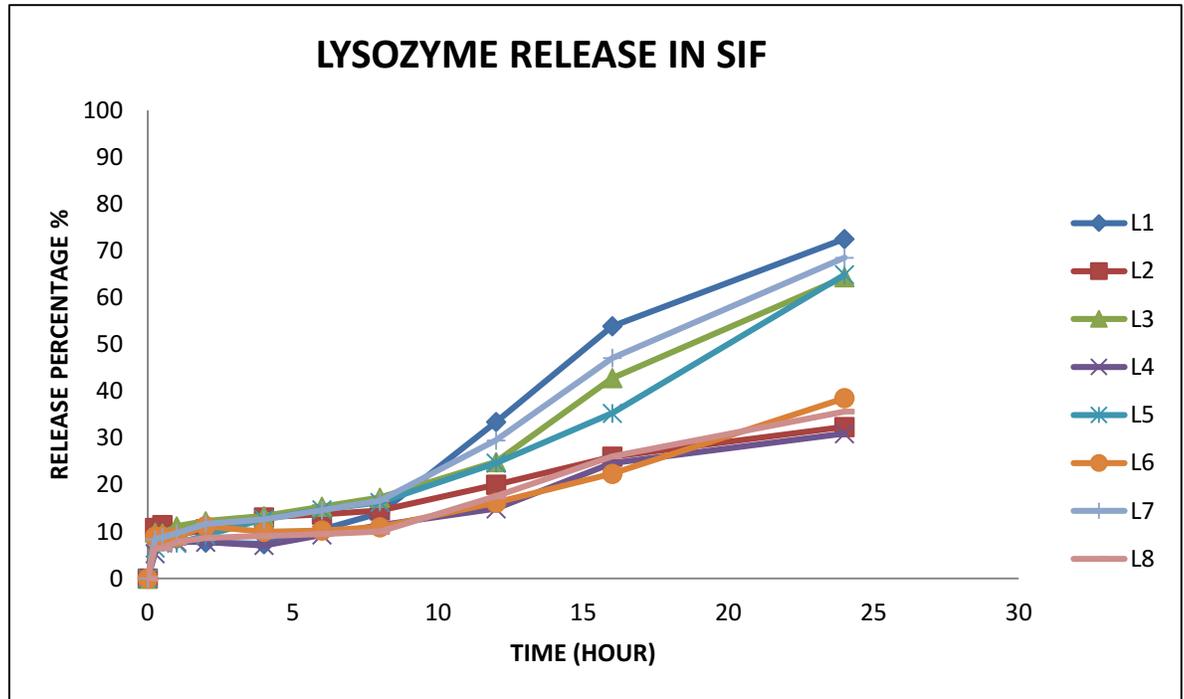


Figure 6.5: Release of proteins from PNCs in SGF (without enzyme) at 37 °C (A) lysozyme containing PNCs and (B) trypsin containing PNCs. For formulations composition, refer to Table 2.5.

A)



B)

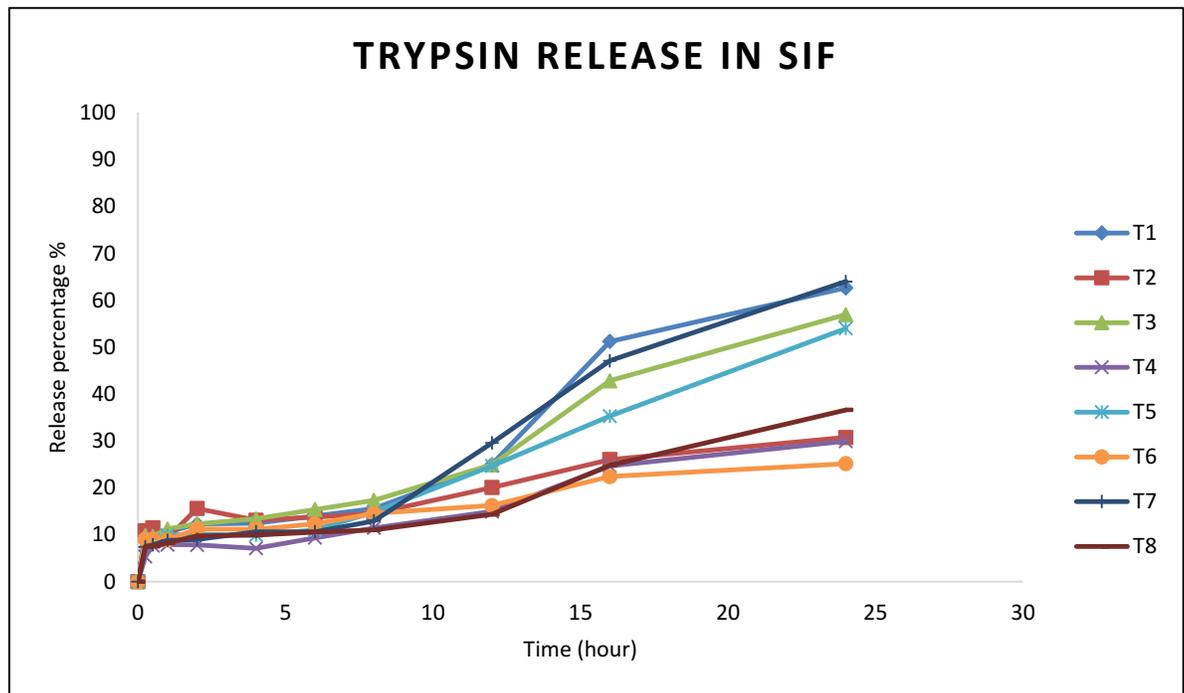


Figure 6.6: Release of proteins from PNCs in SIF (without enzyme) at 37 °C (a) lysozyme containing PNCs and (b) trypsin containing PNCs. For formulations composition, refer to Table 2.5.

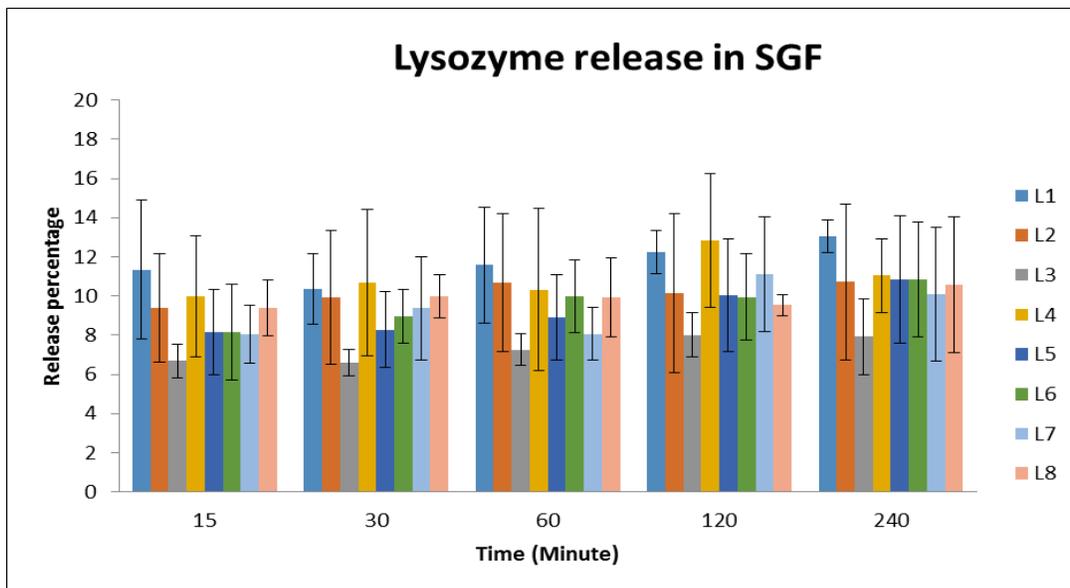
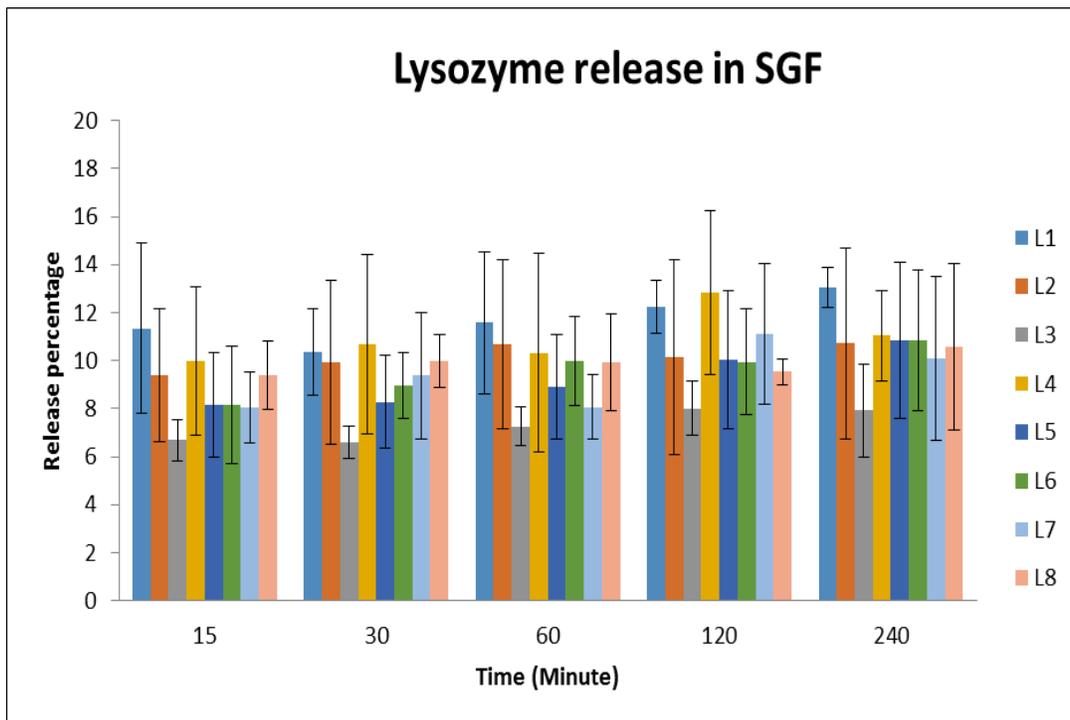
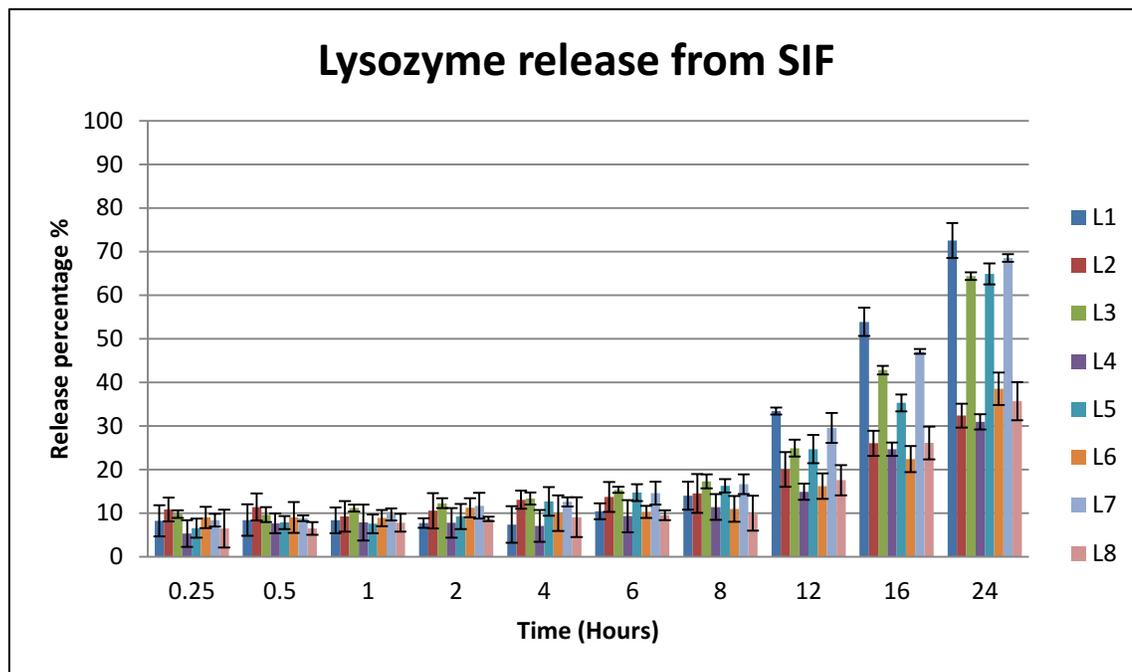


Figure 6.7: Percentage protein released from PNCPs in SGF (without enzyme) at 37 °C (A) Lysozyme containing PNCPs and (B) Trypsin containing PNCPs. For formulations compositions, refer to Table 2.5.

A)



B)

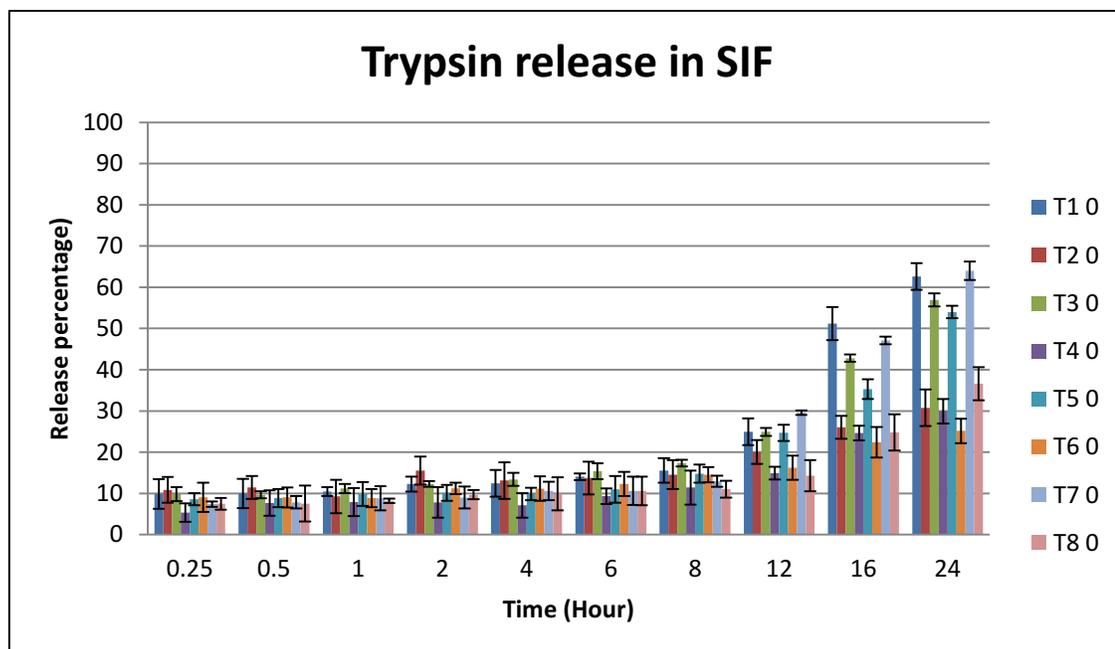


Figure 6.8: Percentage protein released from PNCPs in SIF (without enzyme) at 37 °C (A) Lysozyme containing PNCPs and (B) Trypsin containing PNCPs. For formulations composition, refer to Table 2.5.

As illustrated Figure 6.7 and Figure 6.8, trypsin and lysozyme release from PNCs depends on the medium of dissolution and entire time of the experiment. Proteins efflux from the particles in SGF (within 4 hours of monitoring) was a biphasic pattern. After an initial release phase (8 -8.5 %) of the proteins, this was followed by an equilibrium state or a slower release phase Figure 6.5. The release profile is consistent with what has been observed by [195]. *Perez et al. 2001* concluded that plasmid DNA has been released from Poly (Lactic acid) - poly (Ethylene glycol) nanoparticles prepared by a double emulsion technique in biphasic profile, where there was almost <10 % burst release of DNA content has been burst released from the formulations within < 10 minutes after introduction of the dissolution medium.

The case was different when the release study was performed in SIF for 24 hours; as the proteins were released from the PNC's reservoir in a triphasic process. The triphasic release can be concluded in the following three steps, the first burst phase of the proteins within the first 15 minutes. Then a plateau for 8 – 10 hours reflecting equilibrium or a slow diffusion state in the second phase, following these steps the proteins started to be released in a constant and sustained rate over the rest of 24 hours, Figure 6.6, and Figure 6.8. *Lamprecht et al. 2000* have observed this kind of protein release profile [196]. In *Lamprecht et al. 2000* the Bovine Serum Albumin (BSA) release from poly (D, L-Lactide-co-glycolic acid) (PLGA), and poly (ϵ -Caprolactone) (PCL) nanocapsules has happened in three phase composition, the nanocapsules were prepared by the double emulsion method.

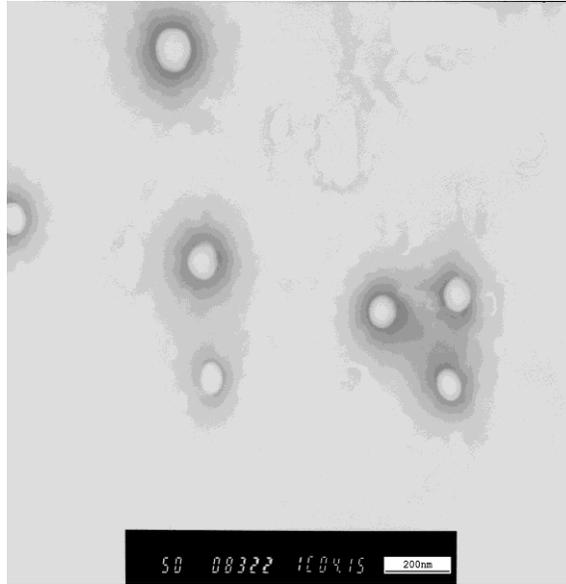
Protein release in SGF over 4 hours was less than 14% in both trypsin, and lysozyme formulations with no significant differences ($p > 0.05$) were noticed inter and intra proteins formulations. Moreover, the standard deviations reported for the protein release in SGF were relatively high (up to 4.37, CV almost 50%), which supports the assumption; that the mechanism of the initial burst releases in SGF was due to the adsorbed protein on the polymer surface.

However, the protein release profiles from PNCs in SIF showed a different pattern, as a triphasic pattern was observed. Initial burst release within the first 15 minutes reached 8 – 8.5% in average for both proteins, which is consistent with the initially recorded release value in SGF. The highest overall release value in SIF after 24 hours detected was 72.5% which was noted for lysozyme formulation, while the lowest was 25.14% in one of trypsin loaded formulations (T6), Table 6.5. The wide range of release records was reported. Different assumptions and hypothesis have been used to explain the drug release from the polymeric nanocapsule systems, and whether the drug release is due to diffusion through the polymer matrix, polymer degradation and matrix erosion, or other mechanisms. The release mechanisms, in different sources of the literature, have been explained based on the published research findings, used materials attributes, and other performed tests to confirm and explain the drug release profile. In the current study, proteins release from the PNCs has occurred via different mechanisms; the initial burst phase may have happened due to the adsorbed proteins at the PNCs surface. This can be confirmed as no significant difference between the different formulations in terms of burst release has been reported. Moreover, the standard deviations of the same sample burst release values are high, which reflects that; burst release has occurred because of a surface adsorption, which does not follow any certain rule. The second release phase or the plateau phase has resulted when

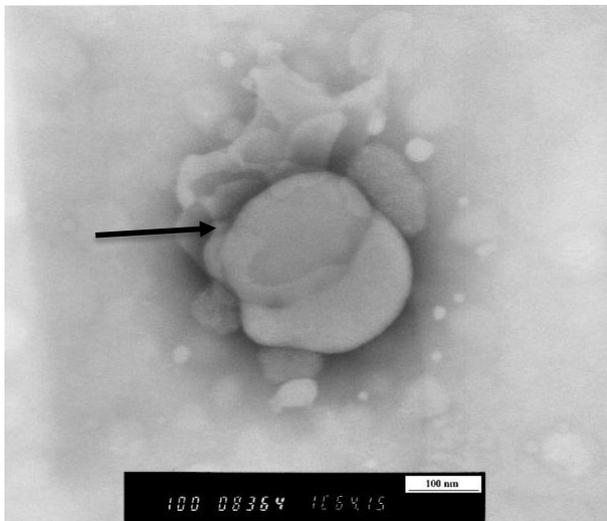
the protein molecules close to the surface has diffused out [196]. Afterwards, the last sustained release phase may have resulted because of different release mechanisms, i.e. weakening of particle structure due to plasticising effect from the dissolution medium, or possibly, polymer degradation and subsequent matrix erosion. Also, diffusion out of the polymer due to various causes, e.g. formation of some pores or water channels within polymer shells as medium penetrated the matrix particles may be the drug release mechanism. The polymer degradation and particle erosion processes usually depend on several factors, e.g. polymer molecular weight and its structure and functional groups. In the current study, the used hydrophobic copolymers with high molecular weight with very long half-lives should have confronted a delay of the polymer erosion rate for a longer time. In order to confirm the release mechanism of the PNCs and whether the erosion was the release precursor or not, additional tests have been performed in the lab. First, dried nanocapsules powder weight has been measured before and after release study has been carried out. No significant difference has been observed between the two times, which may confirm that erosion has not happened to the polymers during the 24 hours' release in SIF. Since erosion can convert the polymer into water soluble monomers and other small molecules or even gases [184] such as, lactic acid and carbon dioxide (CO₂), which causes depletion in overall polymer weight.

The other test was imaging the nanocapsules just after the release by using Transmission Electron Microscope (TEM) to figure out any shape changing in the nanocapsules structure after release. Figure 6.9 illustrates the PNCs forms before and after drug release study.

A



B



C

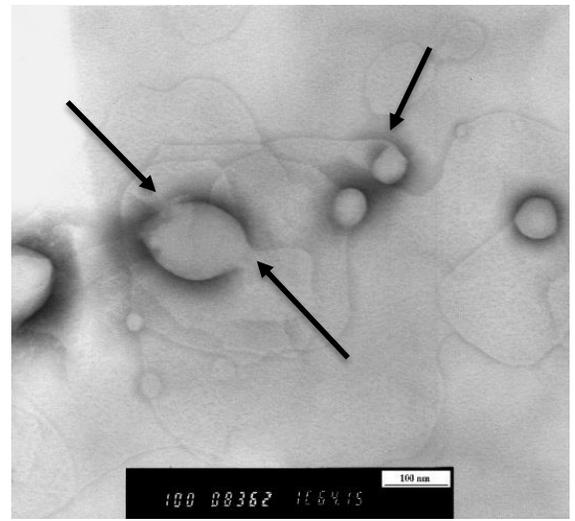


Figure 6.9: Transmission Electron Microscopy (TEM) images of polymeric nanocapsules formulation A) before and B and C) after 24 hours of release in SIF.

As seen in Figure 6.9 that; PNCs have retained their spherical shape after the release study. However, some larger size particles were observed which may confirm that; no erosion has occurred to either polymer, and the release may have happened due to proteins diffusion outside through the polymeric shells. When the PNCs were immersed

in the aqueous medium (SIF), a direct contact between the polymeric outer shell and the aqueous medium, may have led to the plasticization of the polymers chains, [197, 198]. This will allow further fluid influx into the inner core of the PNCs due to their small dimension when compared to the microparticles. Therefore, water influx inside the core dissolved the protein and helped the PNCs to swell and generate some pores and penetrates and increased the permeability due to its weakened and plasticised mechanical properties. Thus, eventually aided the protein to efflux outside and release in sustained release manner over the entire time.

The results of the current study were consistent with the study conducted by *Blasi et al. 2005* [198]. *Blasi et al. 2005* stated that; T_g of PLGA has been dropped by incubating in water at two different temperatures, i.e., 23 °C and 30 °C. Moreover, the same study showed that the decline in T_g was 15 °C after 1 hour of incubation irrespective of temperatures used. This depletion in T_g value has resulted in converting the polymer structure from the glassy state into a rubbery state, thus, increased the polymer fluidity, which consequently, may increase the drug release from polymeric drug delivery systems.

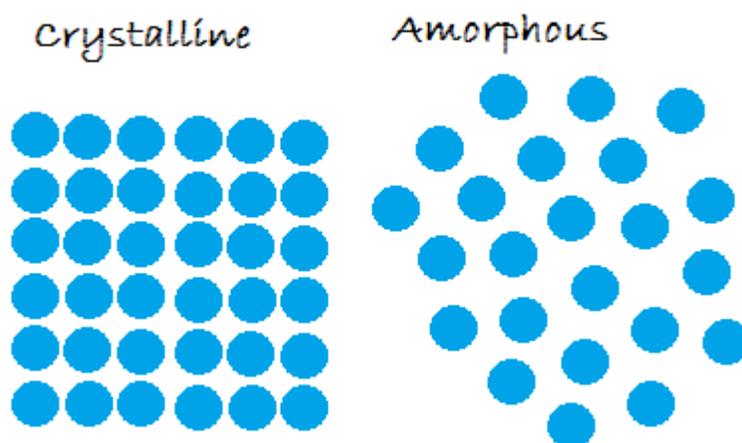


Figure 6.10: comparison between crystalline and amorphous copolymer internal structure. The figure was drawn by the author of this thesis.

After performing experimental designs analysis; it was found that both designs (lysozyme design and trypsin design) have good and valid models and high goodness values, as explained earlier in Section 5.3.3. No significant difference was recorded between the overall drug release of PNCs formulations in SGF. However, the ratio between Lactide and Caprolactone in the copolymers was a significant factor which affected the total percentage of protein release over 24 hours. ϵ -caprolactone: D, L - Lactide ratio is a critical factor and should be optimised in order to achieve the desired CQA (Release >75% over 24 hours). Using a (14:86) poly (D, L -Lactide -co-Caprolactone) ratio assisted to achieve a high drug release percentage over 24 hours release study.

Higher overall percentage of protein release from the dominantly D, L- Lactide systems is expected due to its chemical and mechanical structure. The D, L-Lactide is the racemic form of Lactide moiety due to the presence of chiral methyl group in the Lactide. The molecule backing of racemic Lactide forms changed the mechanical

properties from being crystalline or semi-crystalline to completely amorphous form for the copolymer, which subsequently, decreases the polymer consistency. Figure 6.10 illustrates the difference between the amorphous and crystalline forms. This changing in internal structure can decrease the T_g and make the polymer more plasticized and thus more permeable. The Poly (L- Lactide) form has 35:65 of crystalline: amorphous, while poly (D, L-Lactide) is entirely amorphous. The completely amorphous form poly (D, L- Lactide) has a T_g value of 57 °C in comparison to pure poly (L- Lactide), which has a T_g of 65 °C; this reflects different mechanical properties. Furthermore, forming copolymer can create new molecules with different mechanical properties, which may be able to reduce the rigidity of the polymers as described by [199]. The copolymer used in this work is consisting of 86% D, L- Lactide and 14% Caprolactone moieties and providing a new copolymer with a low T_g value reaches down to 16 °C [200]. This depletion in glass transitional temperature means that the copolymer may be easily plasticized and may become rubbery at a temperature higher than 16 °C (body or release experiment temperature is 37 °C). As provided by the materials supplier [201], the 40:60 (D, L – lactide -co- Caprolactone) copolymer has a melting temperature (T_m) of 31 °C as measured by DSC. Hence, mentioning melting temperature rather than glass transition temperature reflects that the polymer mainly consists of dominantly crystalline or semi-crystalline structure [202]. This means the polymer has more rigid and stable bond backing, thus helps in confining the drug inside the polymeric system for a longer time due to slow release. The slow drug release of (40:60) poly (D, L – lactide -co- Caprolactone) PNCs is caused by its slower softening rate when exposed to the dissolution condition at 37 °C when compared to (86:14) poly (D, L – lactide -co- Caprolactone) PNCs.

Encapsulating trehalose with protein inside PNCs can attract more intestinal and gastric fluids to influx into the particles core due to the difference in osmotic pressure, which may, consequently, increase the drug release percentage. However, this was not the case in this study, which may refer to the lower surface area of the overall PNCs system containing trehalose due to their significantly increased particle size when compared to the sizes of PNCs without trehalose.

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6.3.2.5. Lysozyme stability (from leakage and permeability) after incubation in SIF, and SGF containing digestive enzymes

All orally administered drugs must pass through the gastrointestinal tract (GIT). Passing the (GIT) means; the drug molecules will be exposed to the enzymes of the digestive system, e.g., pepsin, trypsin, and α -chymotrypsin. Therapeutic proteins like lysozyme are degraded by these gastrointestinal enzymes. Formulating oral protein formulations should be able to protect the proteins against assault by the digestive enzymes. This study was performed for lysozyme. However, trypsin was not used because trypsin itself is a proteolytic enzyme. Therefore, there is no point in evaluating the stability of encapsulated trypsin in the presence of the digestive enzymes. Lysozyme stability in simulated GIT conditions was studied, as described in Section 2.5.5, in order to assess the permeability of the nanocapsule shell to allow the digestive enzymes to penetrate the particle core. The samples have been washed and centrifuged; then the supernatants were collected every time and analysed by using size exclusion chromatography (SEC) to ensure a complete removal of the digestive enzymes, see Figure 6.11.

Measuring the diminishing of digestive enzymes was carried out to ensure no lysozyme contact with residue amount of enzymes, which may degrade the protein after shell breaking, and thus, bias the results. After complete washing out of the digestive enzymes from the nanocapsules' surfaces, nanocapsule shells were broken down, and the encapsulated proteins were collected and quantified, as describe in the methodologies, Section 2.5.2.

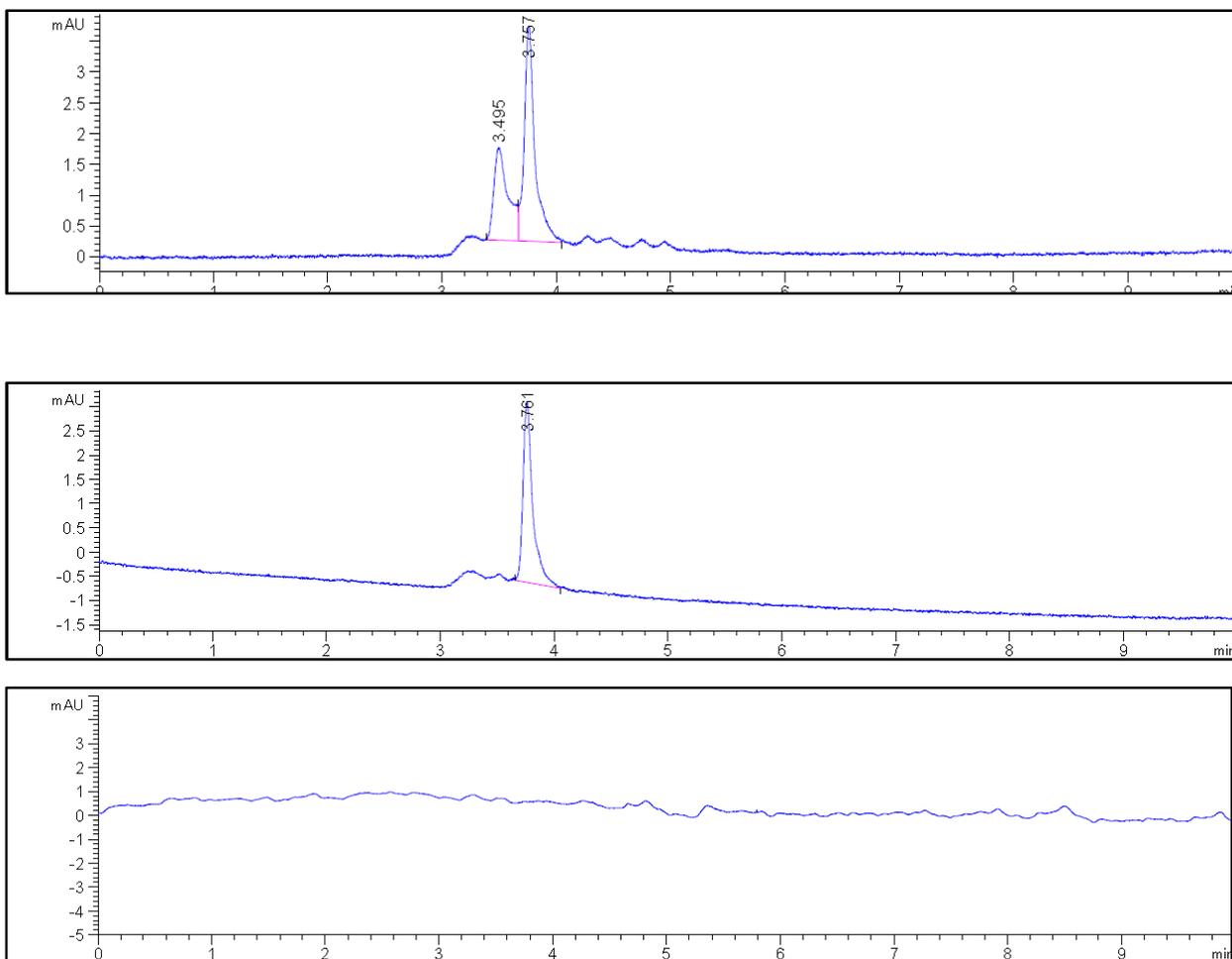


Figure 6.11: Pepsin chromatograms are showing its levels diminishing in the supernatant after nanocapsules washing.

Figure 6.11 illustrates that; the digestive enzymes have been removed completely from the PNCs surface and surrounding medium after three-time of centrifugation and washing. Then, all the formulations were washed three times after the incubation with enzymes to ensure complete removal of the digestive enzymes and no direct contact between lysozyme and the proteolytic enzyme can occur.

The results in Table 6.6 illustrate that; the percentage of non-degraded lysozyme after incubation in pepsin and trypsin containing media for 1 hour and 4 hours, respectively. The nano- encapsulation technique has protected lysozyme against the enzymatic degradation significantly in comparison to free lysozyme. All nanocapsule formulations showed a significant protection for the lysozyme against the proteolytic enzymes as compared to free lysozyme, Table 6.6. However, no significant effect was observed between the different factors in term of lysozyme protection against the degradation in both enzymes. The efficiency of protein protection was expressed as the ratio of the amount of the remaining lysozyme in the nanocapsule after the incubation period to the amount of encapsulated protein. The highest protection in pepsin SGF medium was $97.11 \pm 3.1\%$ while the percentage of non-degraded lysozyme after 4 hours' incubation in trypsin SIF was between $79.80 \pm 5.72\%$ - $9.923 \pm 3.89\%$. The relatively high protection efficiency for lysozyme is attributed to the encapsulation of the protein that slows down the efflux of the protein from the polymeric shell, as well as a low permeability of fluid throughout the polymeric layer. Thus, it prevents the digestive enzymes to influx to the capsules.

Table 6.6: Percentage of the remaining lysozyme from different formulations after incubation in SGF and SIF for 1 hour and 4 hours (in the presence of the digestive enzymes), respectively. For formulations composition, refer to Table 6.4.

Formulation	SGF	SIF
Control	—	—
F1	86.062	79.796
F2	95.005	85.865
F3	93.235	83.921
F4	89.213	87.802
F5	92.988	82.869
F6	93.732	83.256
F7	91.450	88.057
F8	97.112	91.923

As seen in Table 6.6, no provided values for the control samples, which represents the non-processed lysozyme. Since no clear and well-separated peak has been noticed due to the digestive enzymes proteolytic effect, which destroyed the native lysozyme structure.

Figure 6.12 illustrates the chromatograms obtained from lysozyme control analysis by HPLC after incubation in SIF and SGF for 1 hour and 4 hours, respectively.

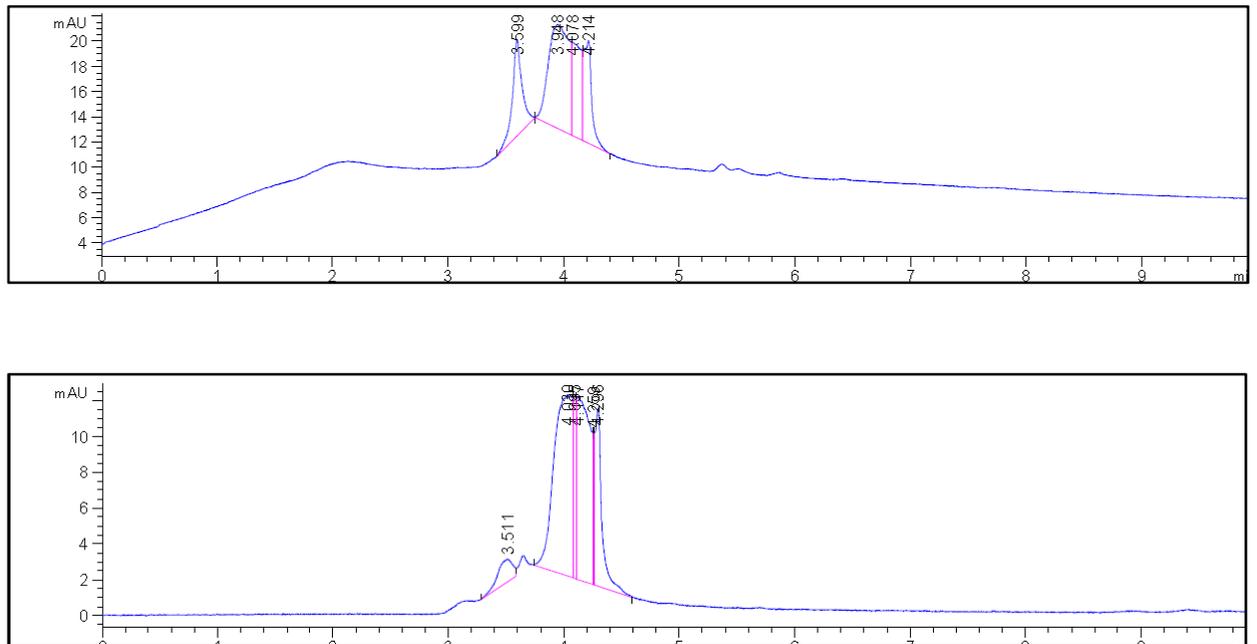


Figure 6.12: control lysozyme chromatogram after 1) 1 hour in Simulated Gastric Fluid (SGF) and 2) 4 hours in Simulated Intestinal Fluid (SIF).

As clear from Figure 6.12, lysozyme was destroyed in the presence of digestive enzymes, and no well-defined peak has been observed for lysozyme, which even made lysozyme quantification difficult.

The above results confirmed that nanoencapsulation technique had formed a strong shield against gastric and intestinal enzymes degradation which can protect the encapsulated proteins and make the formulation suitable and convenient to be taken orally.

6.3.2.6. Effect of polymers and process on the protein structure (biological activity)

Therapeutic proteins must be structurally intact and remain active all the times and especially when the administration is taking place. However, delivery of a stable protein formulation is still challenging. Protein integrity can be determined by several methods

e.g. chemical analytical techniques and/or enzymatic assays which are usually applied to measure the biological activity of the proteins, which reflect their ability to perform the desired therapeutic action. Therefore, the biological activity was examined for both the proteins after incorporation into the biodegradable nanocapsules, as they were prone to denaturation by the stressful processes and when exposed to different potentially destabilising materials such as hydrophobic polymers and organic solvents. The procedures of biological activity determination were described in Section 2.2.2.2, and Section 2.2.2.3. Lysozyme biological activity was investigated by measuring the rate of hydrolysis of β -1,4-glycosidic linkages between N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) in bacterial cell walls by lysozyme. The enzymatic activity of trypsin can be determined by measuring the rate of ester link cleavage in N-benzoyl-L-arginine ethyl ester (BAEE).

Table 6.7 and Figure 6.13 show the biological activity values for the encapsulated lysozyme and trypsin in different formulations. The results of biological activities of the proteins have been statistically analysed in order to figure out the significance of the various factors.

Table 6.7: The Mean biological activity of lysozyme and trypsin biological activity after encapsulation in eight different polymeric nanocapsule formulations. The biological activity was performed in Triplicate. The coefficient of Variance is provided. For formulations composition, refer to Table 6.4.

Proteins formulations	Lysozyme BA* \pm CV**	Trypsin BA* \pm CV
F1	43.56	21.32
F2	39.70	18.69
F3	56.53	44.32
F4	58.96	41.56
F5	79.24	59.01
F6	77.24	61.89
F7	95.63	81.47
F8	97.42	84.65

* Biological Activity.

**Coefficient of variance.

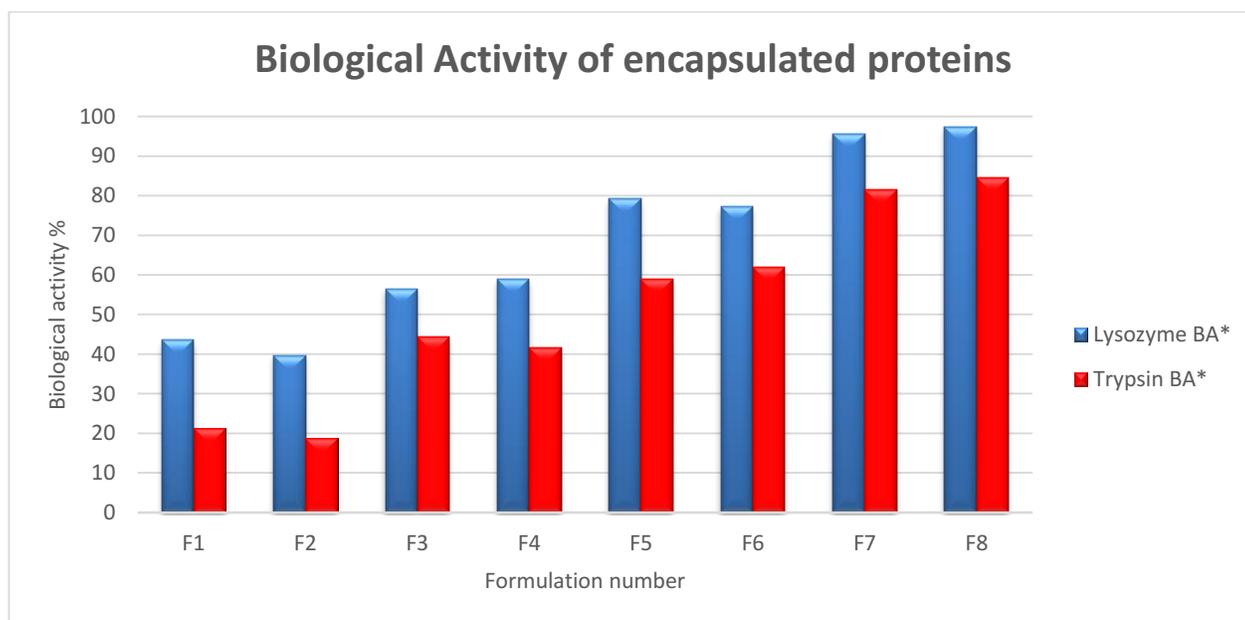


Figure 6.13: Biological activity of lysozyme and trypsin encapsulated in a total of sixteen different formulations. For formulations composition, refer to Table 6.4.

As observed in Table 6.7, lysozyme formulations restored 39.69 – 97.42% of their original activities after processing, whilst trypsin preserved up to 84.65% of the initial activity.

The percentage of intact and active moieties for both proteins have been auto fitted and predicted by their different models, and the outcome suggested an accurate model fitting as R^2 (0.828, 0.94), Q^2 (0.785, 0.908), as detailed in Section 5.3.3. Table 6.8 shows the coefficient of significant factors affected both proteins. After the models' analysis, it was observed that trehalose presence as an additive has a significant stabilising effect on both of trypsin and lysozyme.

Table 6.8: Factors and their interaction effect on the encapsulated lysozyme and trypsin biological activity as represented by the coefficients, along with p-value for each factor.

Factor	Lysozyme		Trypsin	
	Coefficient	P-value	Coefficient	P-value
Polymer ratio	-0.205	0.631	0.084	0.363
Trehalose	18.846	0.011	20.141	0.002
Core physical state (liquid)	8.602	0.023	11.386	0.003
Polymer ratio * Trehalose	0.153	0.710	1.431	0.023
Polymer ratio * Core physical state (liquid)	1.261	0.155	0.021	0.760
Trehalose * Core physical state (liquid)	0.544	0.333	-0.081	0.372

*Interaction between the factors.

Moreover, the physical state of encapsulated proteins also significantly affected their biological activities. Biological activities were higher when preparing the PNCs by an S/O/W technique (formulations 3, 4, 7, and 8) than the preparations made by a W/O/W method (formulations 1, 2, 5, and 6) with p-value 0.003 and 0.023 for lysozyme and trypsin, respectively.

Figure 6.14 shows the contour plots for the relationship between trehalose concentration, core physical state, and polymer type from one side, and proteins biological activity from another side. The blue region indicates the lowest biological activity whilst the red region represents the highest biological activity.

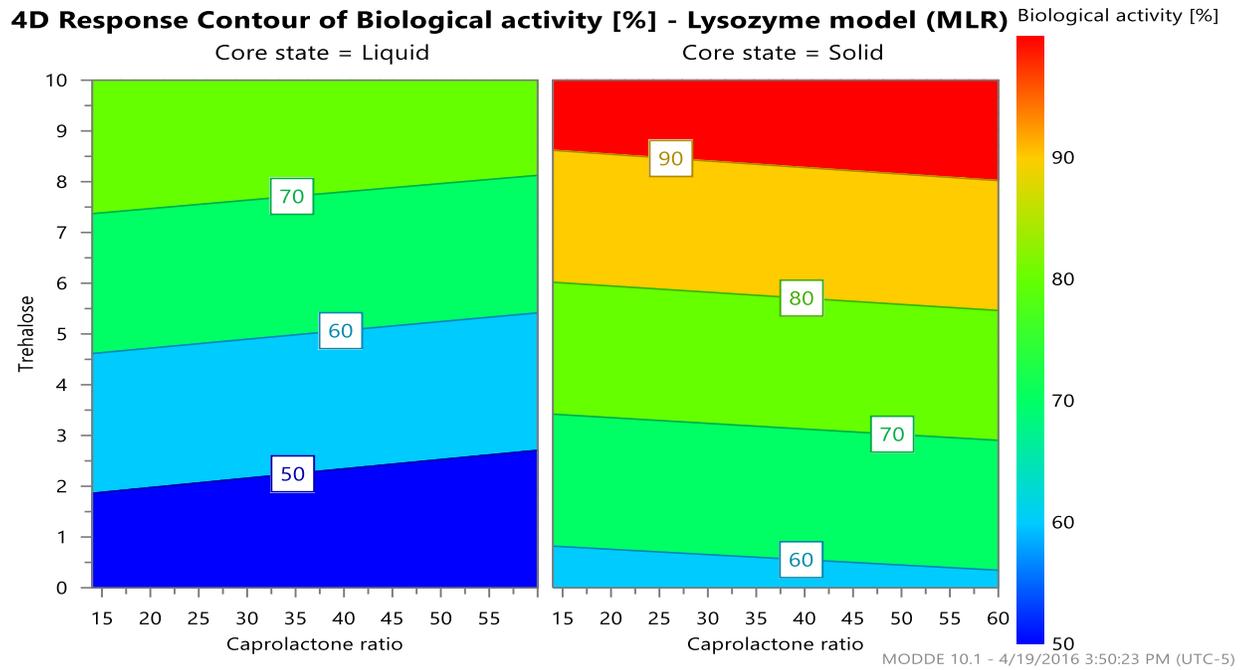
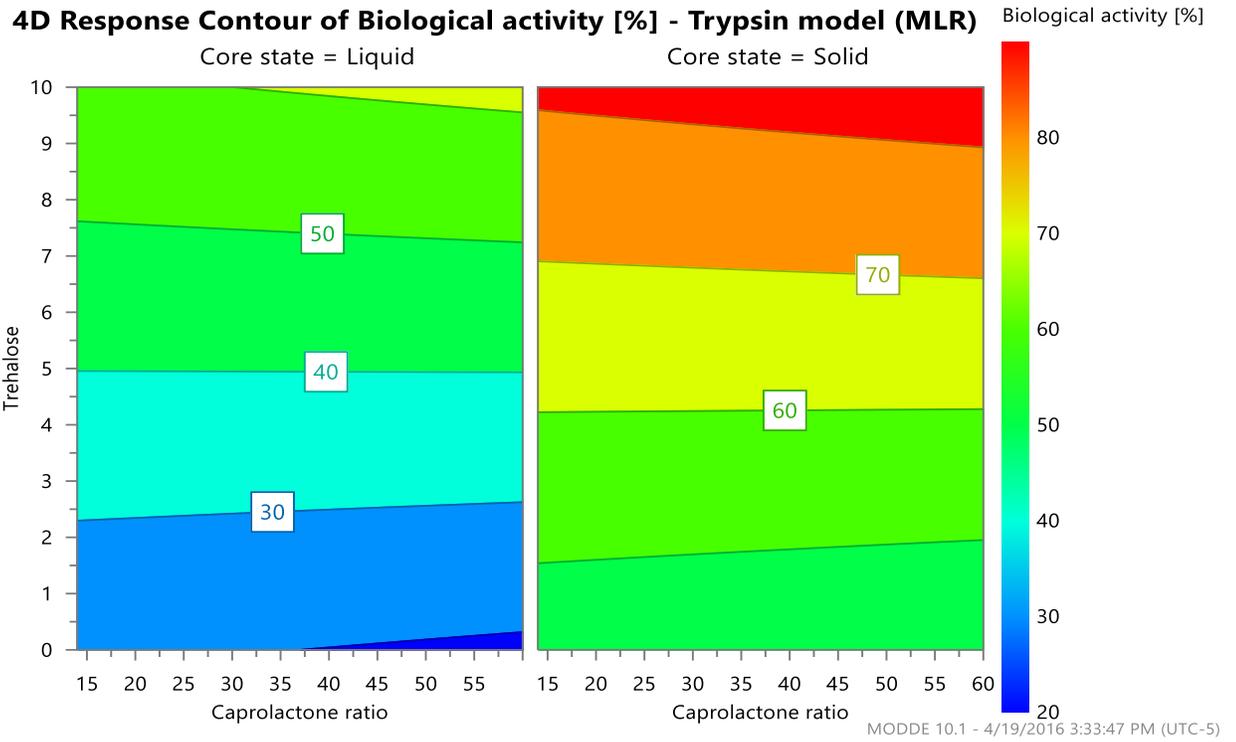


Figure 6.14: 4D response contour plot showing the relationship of trehalose, and polymer on trypsin activity in the solid and liquid state.

As illustrated in the contour plots above, trehalose has increased the both proteins activity, and no effect of changing the polymer type in either two physical states was observed. The plots on the right side represent the formulations prepared by S/O/W, and it is evident that they retained higher biological activity with and without trehalose when compared to the ones developed by W/O/W.

In order to explain the influence of the core physical state of encapsulated proteins on the proteins activity, several pieces of evidence were found supporting the results. The proteins used in this study were dissolved in water at a pH value below their isoelectric points; hence, they were positively charged which created the electrostatic interaction between proteins and polymers, as the polymers have many nucleophilic oxygens with a free pair of electrons in their structure, Figure 6.2. This could possibly cause protein adsorption due to hydrophobic interaction and ultimately leads to unfolding of the protein.

From a chemistry point of view, proteins in solution are also prone to hydrolysis due to their contact with water, which can result in amide bond cleavage [203]. This deamidation reaction results in denaturation and degradation forms e.g. protein fragmentation and unfolding, especially in lysozyme and trypsin. These types of degradation are still detectable in the solid proteins, however, less than the in solution due to water absence [204].

Moreover, proteins in solution form have high internal energy when compared to solid proteins, which has energy nearly zero, which decreases the rate of chemical reactions, and thus preventing the protein physical and chemical degradations during the encapsulation processes [11].

The proteins in the formulations in the current study retained their biological activity, as shown in the results when trehalose was added to the formulations. The role of the disaccharides (i.e. sucrose and trehalose) in stabilising proteins has been widely investigated by the researchers. The most reasonable explanation could be that; trehalose stabilises the proteins in solution by preferential hydration mechanism [114]. Preferential hydration is a phenomenon where there is an increase in the water content around the protein molecules, which favours proteins to keep themselves in the folded states, by excluding direct binding of trehalose to the protein structure. In this case, trehalose is called preferentially excluded [11].

On the other hand, and as concluded by [205]; that trehalose can stabilise proteins by playing a “water substitutes” role during the drying state of the lyophilisation process. Freeze drying of the protein formulations removes the hydrogen bonds from the protein hydration shell which increases the protein unfolding rate. However, sugars’ e.g. trehalose existence in the formulations can maintain hydrogen bonding with the protein by linking the hydroxyl groups of sugar molecules to the protein, which eventually reduces the unfolding and protein deactivation.

Moreover, trehalose affects the surrounding environment in the protein solution by increasing hydration around the protein molecules and reducing the molecules mobility, which stabilises the protein indirectly [206].

In addition to the ability of trehalose to stabilise the proteins in a liquid state, *Chang et al. 2005* concluded that; sugar can stabilise proteins in solid or in the lyophilized state [205]. Encapsulated proteins in the solid state also experienced less stress during freeze drying process, as freeze drying the proteins makes them prone to various stresses which may denature and deactivate their structure [207, 208].

Furthermore, several researchers have discussed and explained the role of trehalose in protein stabilisation in the solid state. As mentioned by *Chang et al. 2009*, trehalose stabilises solid proteins by so-called “Glass Dynamic Hypothesis”. The glass dynamic hypothesis states that trehalose forms a rigid, inert solid filler around the proteins, which separates the protein molecules and inhibits any chance of protein motion and collision with other protein molecules. This eventually restricts the proteins unfolding and denaturation [209].

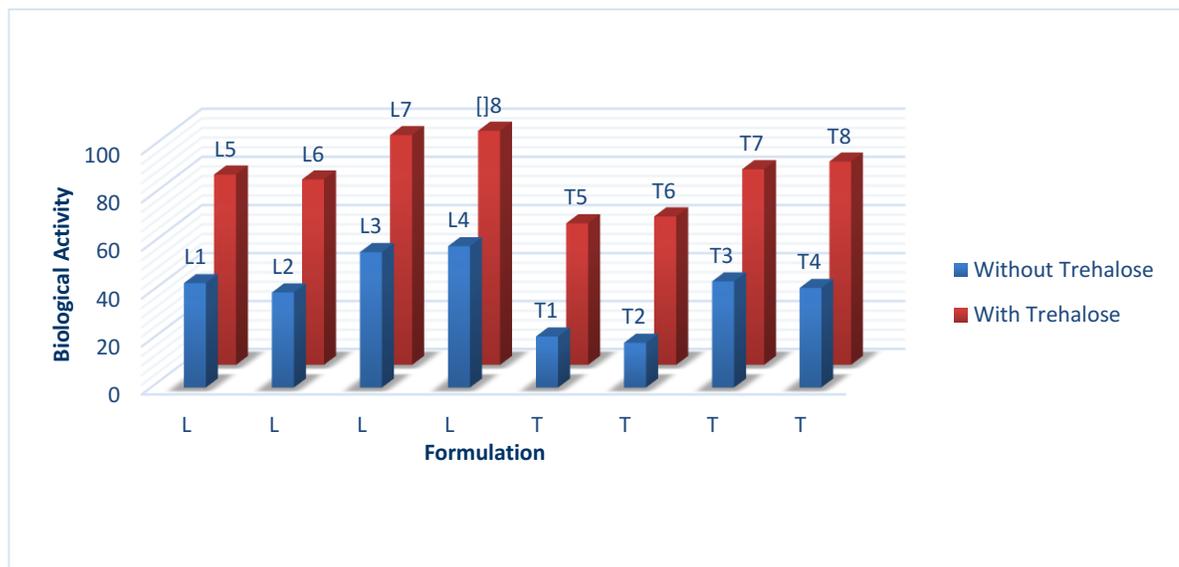
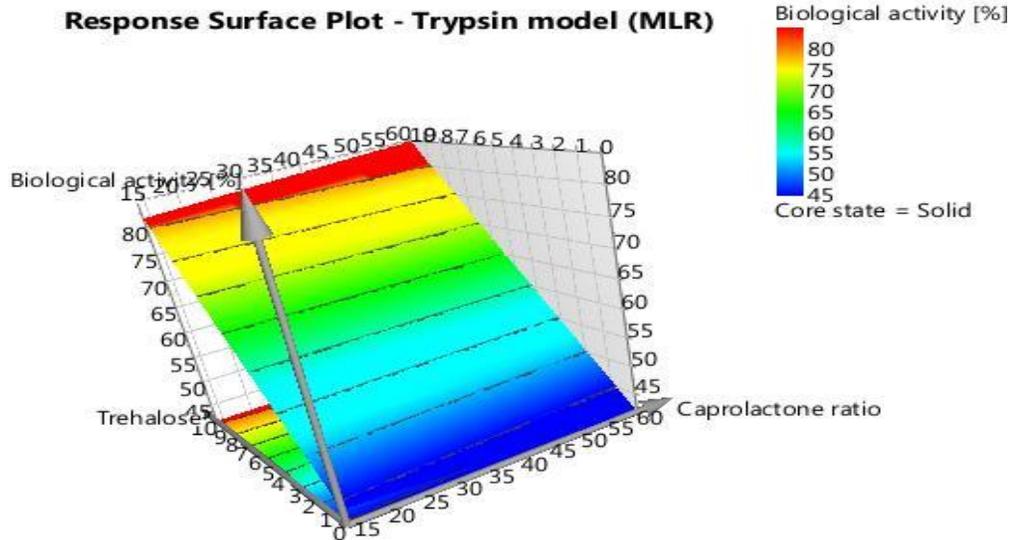


Figure 6.15: Comparison between the biological activity of both proteins with and without trehalose.

Table 6.9: The list of factors which had significant effects on lysozyme and trypsin biological activity along with their coefficients.

Protein	Factor	coefficient	P-value
Trypsin	Trehalose	20.1413	0.00170
	Core physical state (Solid)	11.3862	0.00300
	Polymer*Trehalose	1.43125	0.02389
Lysozyme	Trehalose	18.8462	0.01057
	Core physical state (Solid)	8.602	0.02315

Although the different diblock ratio as a factor has not affected the protein biological activity, the interaction between the proportion of copolymer block as a factor from one side and trehalose from the other side was found to have a significant effect on trypsin activity. There is a synergistic effect exists between the two factors. Since the addition of trehalose has better-retained trypsin biological activity in a non-additive manner when the (caprolactone) block portion in the copolymer increased from 14% - 60%. This interaction for trypsin especially rather than for both proteins attributed to the high degree of denaturation of trypsin when it is prone to any stress such as; high hydrophobic polymer. This denaturation can be prevented by trehalose existence which has even greater impact when the denaturation opportunities increased.



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Figure 6.16: Response surface plot represents the interaction between the used polymer and trehalose on trypsin biological activity.

As clear from the figure above (Figure 6.16), the width of the red area at high caprolactone portion (near 60%) is greater than the width of the lower part (14%), which emphasise the increased trehalose influence with growing the caprolactone block percentage. Observing this details is one of the advantages obtained by applying QbD and employing mathematical DOE, which cannot be achieved by the traditional experimental method.

6.3.2.7. Storage stability of Polymeric nanocapsules

In therapeutic protein formulations, the proteins should remain biologically active, intact, and folded in their native form during the entire period of storage in order to obtain the desired therapeutic effect. Thereby, accelerated and conservative stability studies have to go through by using the appropriate stability indicating assays. In this

project, the accelerated stability study was carried out to measure the ability of lysozyme and trypsin to maintain their native folded and active structures within the polymeric system during the storage period, six months, at 5 °C, 21 °C, and 40 °C. Proteins biological activity, physical and chemical stability, and formulations' moisture content were obtained by applying enzymatic assay, SEC, and KFT, respectively, as explained in Section 2.5.8.

Performing the accelerated stability study at 40 °C was not a good idea as the polymers did not withstand due to low T_m and T_g value (31 °C and 16 °C) exhibited by the copolymers. Thus a coalescence between the nanocapsules has observed.

All the accelerated stability results held by enzymatic assay and SEC are shown in Table 6.10 and Table 6.11, respectively.

Table 6.10: Biological activity of lysozyme and trypsin in polymeric nanocapsules post-storage for 6 months (in a desiccator at 5 °C and 21 °C at 76%RH,), (n=3). For formulations composition, refer to Table 2.5.

Proteins Formulation	% Biological activity	
	Stored in desiccator at 5 °C ± CV*	Stored in 76% RH at 21 °C ± CV
L1	85.23% ± 5.88%	37.23% ± 5.09%
L2	87.11% ± 9.65%	68.09% ± 9.61%
L3	76.07% ± 9.09%	49.81% ± 3.02%
L4	80.12% ± 7.21%	77.66% ± 10.77%
L5	99.09% ± 2.48%	82.10% ± 12.11%
L6	97.23% ± 4.95%	94.23% ± 10.26%
L7	103.23% ± 3.96%	83.84% ± 8.14%
L8	101.11% ± 3.54%	99.03% ± 3.91%
T1	74.69 ± 4.56%	26.98 ± 7.28%
T2	71.98 ± 9.34%	66.11 ± 14.76%
T3	74.66 ± 7.06%	18.86 ± 13.38%
T4	72.08 ± 10.22%	69.96 ± 12.03%
T5	94.09 ± 8.62%	85.69 ± 13.65%
T6	102.63 ± 4.33%	94.11 ± 8.89%
T7	96.02 ± 4.66%	82.63 ± 6.47%
T8	97.56 ± 6.26%	95.23 ± 4.50%

* coefficient of variance.

Table 6.11: Physical stability of lysozyme and trypsin in polymeric nanocapsules post-storage for 6 months (in desiccator at 5 °C and 21 °C at 76%RH,), (n=3). For formulations composition, refer to Table 2.5.

Proteins Formulation	% Recovered Intact Protein	
	Stored in desiccator at 5 °C ± SD	Stored in 76% RH at 21 °C ± SD
L1	101.02% ± 1.73	42.56% ± 2.52
L2	96.32% ± 2.03	71.69% ± 2.82
L3	97.01% ± 1.87	51.96% ± 3.61
L4	99.56% ± 0.51	79.63% ± 3.13
L5	102.63% ± 1.08	84.53% ± 2.34
L6	93.88% ± 1.42	101.23% ± 3.38
L7	96.76% ± 1.71	78.56% ± 1.94
L8	100.09% ± 2.10	98.64% ± 3.89
T1	95.63 ± 2.59	26.53 ± 1.76
T2	99.23 ± 1.50	77.88 ± 3.41
T3	101.23 ± 1.46	36.69 ± 1.13
T4	98.63 ± 0.64	70.66 ± 4.82
T5	93.65 ± 1.60	72.66 ± 3.85
T6	92.56 ± 2.12	97.86 ± 3.30
T7	97.88 ± 0.89	81.10 ± 1.10
T8	96.11 ± 1.75	97.53 ± 1.49

The results of the accelerated stability study suggest that; lysozyme retained more biological and structural shelf stability than trypsin formulations, as lysozyme was able to withstand during the entire period with retained biological activity.

At 5°C both proteins retained more than 92% of their original structure over 6 months of storage, Table 6.11. However, the biological activity of lysozyme formulations was

higher when compared to trypsin. For lysozyme, the biological activity was over a range of 76% and up to almost 100%, while for trypsin, it was from 71% onward to 100%, Table 6.11. While, at 21 ± 3 °C, the physical of both proteins was dramatically different, since some formulations retained only almost 25% of their initial physical stability (as indicated by SEC). Nevertheless, lysozyme was able to withstand higher than trypsin, as reflected by their biological activity retained over the period of storage.

The difference between biological activity and physical stability of the proteins was observed, which may reflect that there are some types of chemical degradation which did not lead to physical degradation and vice versa. Proteins may be physically intact, however not active, due to chemical changing in the active site without altering in molecular weight, which is attributed to that; chemical changes did not lead to aggregation due to the proteins' physical state, as solid protein is less dynamic, therefore, less collision and less chance of aggregation.

Statistical models analysis suggests that; trehalose was able to protect both proteins' biological activity during the entire time of storage under both conditions, this attributes to the properties of trehalose as a unique stabiliser for biologics, which helps them to resist the harsh and destabilising conditions as mentioned earlier in Section 6.3.2.6.

No significant difference (p value > 0.05) was observed between the two different physical states of encapsulated proteins, S/O/W and W/O/W, on the storage stability of both proteins at both conditions.

For stability study held at room temperature (21 °C ± 3 °C) and 76% RH, biological activity and physical stability of the nanocapsules prepared by using (60:40) poly (D, L-

Lactide-co-Caprolactone) copolymer were higher than the formulations developed by (14:86) poly (D, L- Lactide-co-Caprolactone). This difference was due to the higher hygroscopic characteristics of 14:86 in comparison to 60:40. This result is evident by higher moisture content in (14:86) poly (D, L- Lactide-co-Caprolactone) formulations as measured by Karl Fisher Titration (KFT) as shown in Table 6.12. Increasing water content around the polymers in the formulations increased the chance of the proteins to prone to higher stress factors due to the adsorption at the solid (polymer) liquid (moisture) interface, which consequently leads to physical structure changes and aggregation, according to [210]. The percentage of physically intact and biologically active proteins was affected because the moisture content altered the chemical properties of the proteins and increased the dynamic properties of the proteins which led to unfolding that subsequently caused aggregation, which has been reflected by a change in molecular weight, as obtained from SEC analysis.

In addition to the difference in chemical natures of both copolymers, the difference in thermal stability between them played a crucial role in protein stability as well. As mentioned earlier, T_g value of (14:86) poly (D, L- Lactide-co-Caprolactone) is 16 °C, which is below the storage temperature. The relatively high storage temperature (25 °C) has not affected the visual characteristics of the formulations (appearance) compared to ones stored at 40 °C. However, it increased the exposure of the encapsulated protein to the surrounding moisture (74% RH). Consequently, it reduced the protein stability.

At 5 °C, no significant difference was observed between different copolymers in term of SEC, and biological activity as both copolymers have been kept below their transition temperature and proteins encapsulated in polymers were protected from moisture.

Table 6.12: Moisture contents (%) of polymeric nanocapsules containing lysozyme and trypsin post-storage for 6 months (in desiccator at 5 °C and 21 °C at 76%RH), (n=3). For formulations composition, refer to Table 6.4.

Proteins Formulation	% Moisture content	
	Stored in desiccator at 5 °C ± SD	Stored in 76% RH at 21 °C ± SD
L1	2.36 % ± 0.96	21.35 % ± 4.85
L2	4.96 % ± 1.02	8.01 % ± 2.01
L3	6.32 % ± 3.20	16.52 % ± 2.65
L4	3.45 % ± 1.22	10.12 % ± 3.88
L5	4.09 % ± 1.52	19.23 % ± 6.85
L6	1.99 % ± 0.76	6.36 % ± 1.25
L7	2.19 % ± 0.86	18.55 % ± 6.53
L8	4.36 % ± 1.33	8.56 % ± 2.01
T1	4.32 % ± 1.86	19.86 % ± 4.85
T2	0.89 % ± 0.46	10.23 % ± 1.55
T3	2.56 % ± 0.79	23.52 % ± 3.23
T4	2.13 % ± 1.02	6.33 % ± 1.12
T5	1.78 % ± 1.06	14.56 % ± 5.23
T6	5.22 % ± 2.71	7.25 % ± 1.56
T7	3.66 % ± 1.14	19.40 % ± 4.96
T8	2.87 % ± 1.45	10.23 % ± 2.86

6.4. Conclusion

The current study revealed the impacts of the different formulation attributes which affected the quality characteristics of the polymeric nanocapsule formulations. The polymeric nanocapsules were prepared by the double emulsion solvent evaporation method. Using two different copolymer ratios, (86:14) poly (D, L- Lactide-co-Caprolactone) and (40:60) poly (D, L- Lactide-co-Caprolactone), increased the understanding of the role of the chemical nature of the used copolymers in monitoring the encapsulation efficiency and drug release. Encapsulation efficiency has reached up to 80% in some formulations prepared by high proportion of the Caprolactone block. While the increase in the Caprolactone: Lactide blocks ratio kept the proteins confined inside the nanocapsule for a longer time. High biological activity (97%) was observed when trehalose had been added to the formulation especially when they were prepared by *S/O/W*. Accelerated stability study suggested that; the proteins had retained their activity and physical stability over the entire storage time when (40:60) poly (D, L- Lactide-co-Caprolactone) copolymer and trehalose were used. This study represents the characterisation of the developed formulations according to the strategy established in the chapter.

Next chapter (Chapter Eight:) reveals the results of storage stability of liquid formulations containing lysozyme and trypsin under three different conditions.

**Chapter Seven: Liquid
formulations containing
lysozyme and trypsin
stability study**

7.1. Introduction

Stability of pharmaceutical formulations is the competence of the medicinal product to preserve their inherent physical, chemical, and biological properties during not only the time of processing but also throughout time of storage.

Several regulatory agencies have defined the stability and release standards to identify the requirements of stability studies, the conditions of the storage, the required essential analytical assays, data needed for shelf life calculation, and the duration of the storage. International Conference on Harmonisation (ICH) of Technical Requirements for Pharmaceuticals for Human Use was announced in 1990 as an initiative to independently evaluate the pharmaceutical products under a unified regulations between the three major drug developers in the world; Japan, Europe, and USA [211].

According to ICH Guidelines, stability study of a new pharmaceutical product should be performed by the producer with the aim to fulfil the safety and efficiency requirements and eventually to get registered [212]. Different approaches were identified by ICH to investigate the stability of the pharmaceutical products including accelerated, intermediate, and long-term stability studies. Accelerated stability studies are usually performed under the aggressive storage conditions. However, long-term stability, in general, is investigated under the conservative conditions. Sometimes, the intermediate stability study is carried out at storage conditions between the aggressive and the conservative conditions over a period of storage between the former two

approaches. Accelerated and an intermediate stability are often applied to find out the shelf life and the propensity of the drug products towards degradations as a cost and time-efficient approaches. Data obtained from the aggressive and intermediate approaches are usually a good indicator or even accurate tool to calculate the long-term stability of small molecules by applying the Arrhenius equation and extrapolating the obtained results. However, in the stability study of biological products, Arrhenius equation does not apply, and the long term stability study should be performed in order to inspect the degraded products [48]. Biologics are a category of pharmaceuticals that are composed of active ingredients which are generated from a biological source e.g. proteins and vaccines [213]. Biologics usually are macro-biomolecules with sensitive physicochemical characteristics, and they can be degraded by different and unpredictable pathways depending on the formulations and storage conditions, e.g. pH and temperature. Therefore, the biologics containing products' long-term stability should be carried out to in order to assure safety and efficacy of the products. Protein degradation products should be identified accurately and precisely, and the QC analytical assays should frequently be performed over the duration of storage. The feasibility of applying the aggressive and intermediate approaches in protein stability studies is still a point of researchers' interest.

7.2. Aims and Objectives

The current study was carried out to evaluate the effect of studied factors, with well-known effects on the conformational stability, on long-term stability of liquid formulations containing lysozyme and trypsin. Therefore, the investigated factors were selected based on the primary screening performed on both proteins' conformational

integrities, as explained in Chapter Three. The selection was for the factors from the two interval ends (factors with higher stabilising effects and the ones demonstrated the least protection effects on the protein structure). The stability study was carried out by three different approaches, conservative (long-term), intermediate, and aggressive (short-term), to critically evaluate the feasibility of the accelerated stability studies to predict the long-time stability.

7.3. Results and discussion

7.3.1. Stability study of liquid formulations containing lysozyme and trypsin by conservative approach

Long term stability study has to be performed in order to determine the accurate shelf lives of biologics containing pharmaceuticals. The therapeutic proteins efficacy and safety must be retained over the entire period of storage over a long time [214]. The protein and polypeptide formulations stability usually do not follow Arrhenius model [48]. Therefore, the prediction of long-term stability of liquid protein medicines still a challenging task which has been investigated by different researchers.

In the current study, the conservative approach in the determination of the stability of lysozyme and trypsin liquid formulations was applied by storing the proteins formulations in the refrigerator under chilled conditions ($5 \pm 3^{\circ}\text{C}$), as detailed in Section 2.6.4, as recommended by [215].

Enzymatic assay, Size Exclusion Chromatography (SEC), and turbidimetry methodologies were applied to investigate the biological activity, soluble aggregates and protein fragments, and non-soluble aggregates, respectively.

Figure 7.1 and Figure 7.2 illustrate the biological activities and the physical stabilities of a total forty different liquid protein formulations containing lysozyme and trypsin.

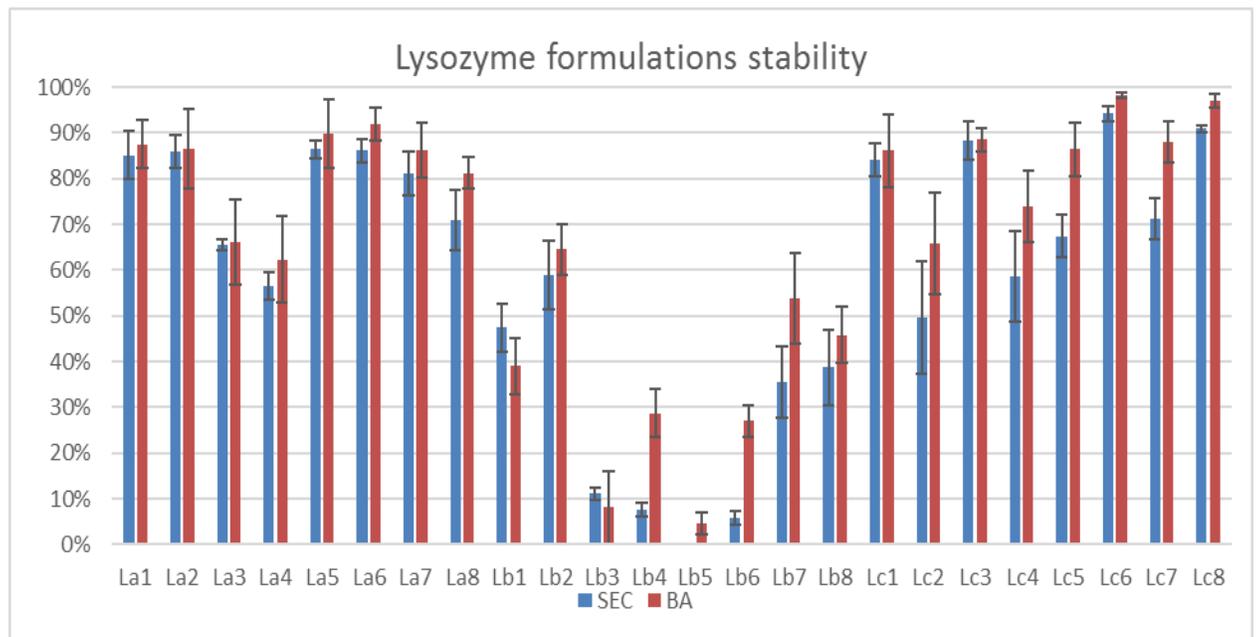


Figure 7.1: The biological activities and physical stabilities of different 24 liquid lysozyme formulations after the storage period of 18 months at $5 \pm 3^\circ\text{C}$. The tests were performed in triplicate SD is provided. For formulation compositions, refer to Table 2.8. BA: biological activity, SEC: size exclusion chromatography.

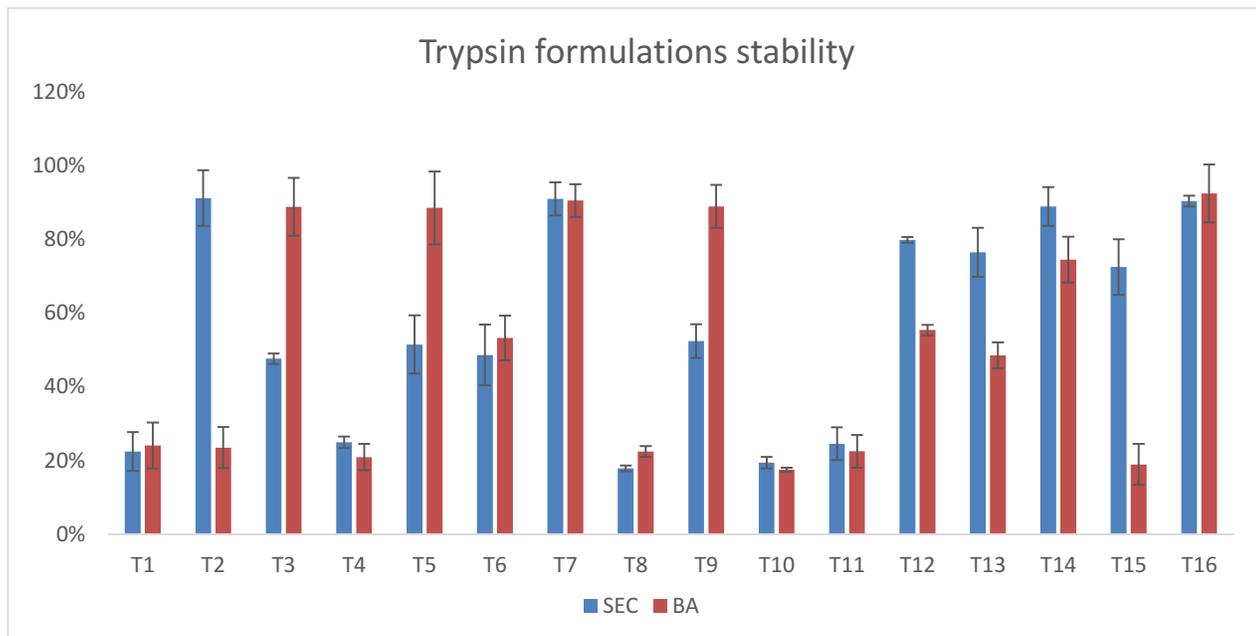


Figure 7.2: The biological activities and physical stabilities of different 16 liquid trypsin formulations after the storage period of 12 months at 5 C. The tests were performed in triplicate SD is provided. For formulation compositions, refer to Table 2.8. BA: biological activity, SEC: size exclusion chromatography.

As illustrated in Figure 7.1, large variations were observed between the lysozyme formulations stability under the experiment conditions. The inter-groups stability results after 18 months of storage show that; the eight formulations involved in DOEb (Lb1- Lb8) have the lowest physical and biological stability among all 24 formulations. The low stability of DOEb formulations may refer to the absent of trehalose protection, which may have played a crucial role in stabilising the other sixteen formulations (La1- La8, and Lc1 –Lc8). Also, the destabilising effect may be due to different factors i.e.; high salts (ions) content caused by high ascorbate and high buffer salt concentration, as per formulations number Lb5, and Lb 6. The strong link between the stability of the protein and the amount salts was demonstrated as a result of a long time of research on stability of proteins accomplished by other researchers.

High salt or ionic content in the liquid protein formulations may affect the conformational stability of the proteins as confirmed by Hofmeister series. Hofmeister series classifies the ions according to their ability to stabilise or degrade the proteins. In Hofmeister series, Na^+ occupies the third position in order of the cations, while HPO_4^{2-} is considered the third among the anions series [216]. According to *Yang Z. 2009*, [216], Na^+ and HPO_4^{2-} are called Kosmotropic ions, the effect of the Kosmotropic depends on the ion charge, since the Kosmotropic cations have a destabilising effect on the protein structure, while the Kosmotropic anions stabilise the proteins. When both the destabilising cations (Na^+) and the stabilising anions (HPO_4^{2-}) exist in the solution, strong ion-pairs between the Kosmotropic anions and cations are formed, which reduces the stabilisation effect of HPO_4^{2-} . Therefore, the increase in the sodium ion concentration may lead eventually to the protein destabilising effect. The interaction nature has been investigated intensively in the literature. However, it is still unclear [217].

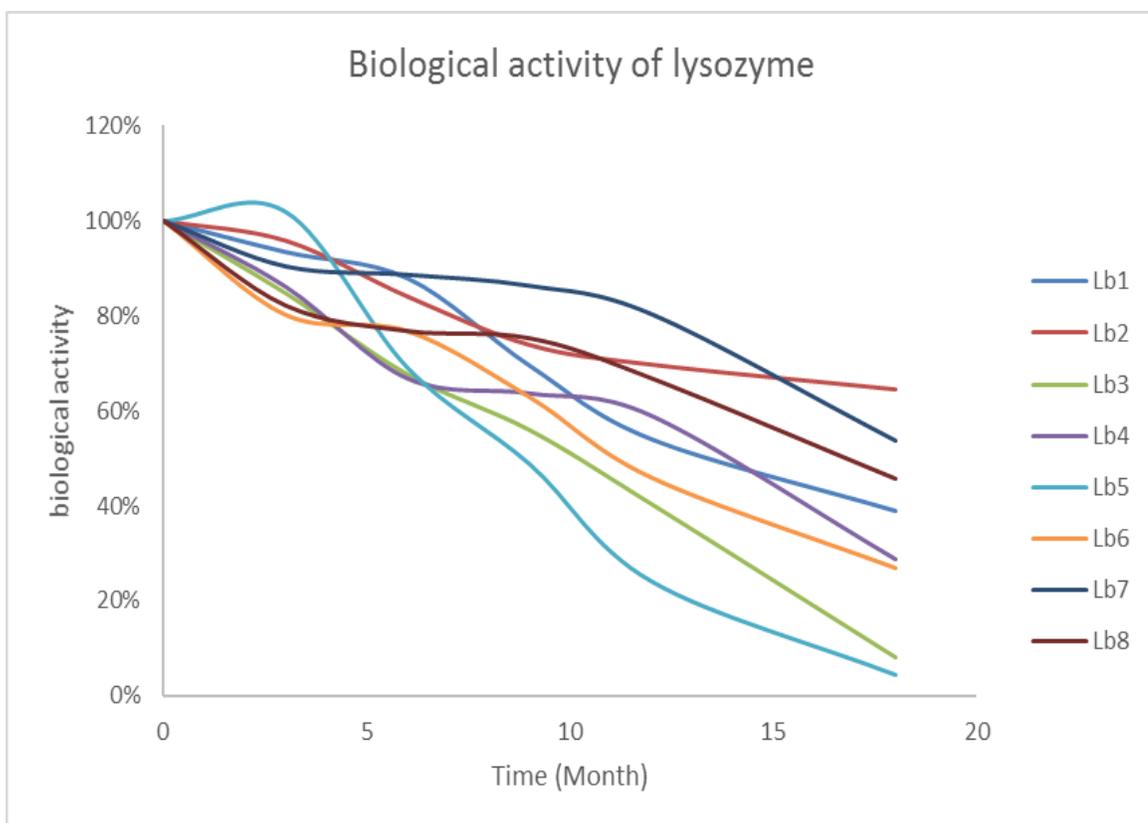
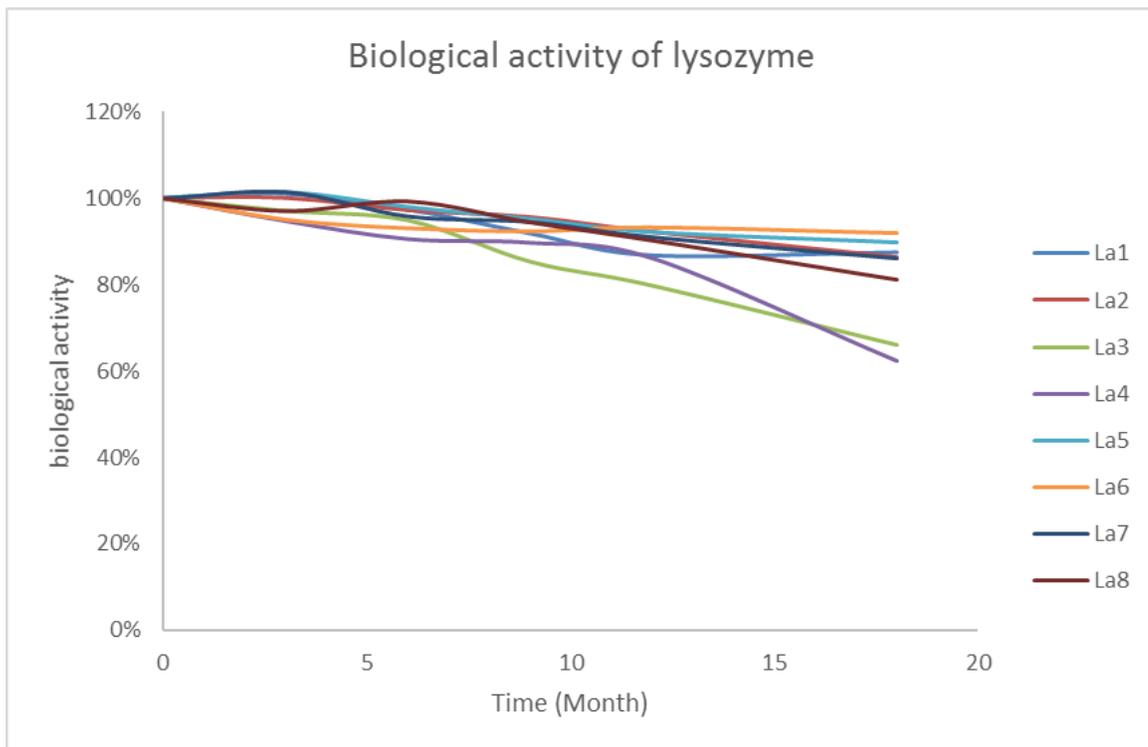
Furthermore, Sodium ascorbate, as a conjugate base for the weak acid (ascorbic acid), increases the solution pH significantly, as the pH of the formulation based on DOEb (Lb1, Lb2, Lb3, and Lb4) was measured after the storage and was more than pH 7. As concluded in chapter three of this research, the lysozyme conformational stability could be decreased significantly by increasing the surrounding pH value. The negative impact of the high pH value caused by the ascorbate on lysozyme stability was observed in formulations (Lb3, and Lb4).

The models' statistical analysis revealed that; trehalose played a significant role in stabilising the lysozyme formulations of DOEa and DOEc (p-value: 0.026 and 0.041, respectively). In DOEa, ascorbate has a significant destabilising effect (p-value: 0.02),

when it was concluded that; there is an inverse relationship between the ascorbate concentration and the lysozyme biological activity and physical stability.

In spite of the trehalose stabilising effect, the physical stabilities of some the formulations in DOEc (Lc2, Lc4, Lc5, and Lc7) prepared at pH 8 were low. Nevertheless, the biological activities of the non-physically stable formulations were relatively high. The high biological activity of the highly aggregated lysozyme may be explained in the light of the ability of trehalose to preventing the irreversible aggregation. SEC analysis is being performed for the original concentration of the protein solutions, however, in the enzymatic assay, the solutions are being diluted more than hundred times. This dilution decreases the closeness of the particles together, which gives a chance for the reversibly aggregated protein to go back to the native monomer structure.

Moreover, the rate of decreasing the biological activity and physical stability over the entire period of storage was also studied, Figure 7.3 and Figure 7.4.



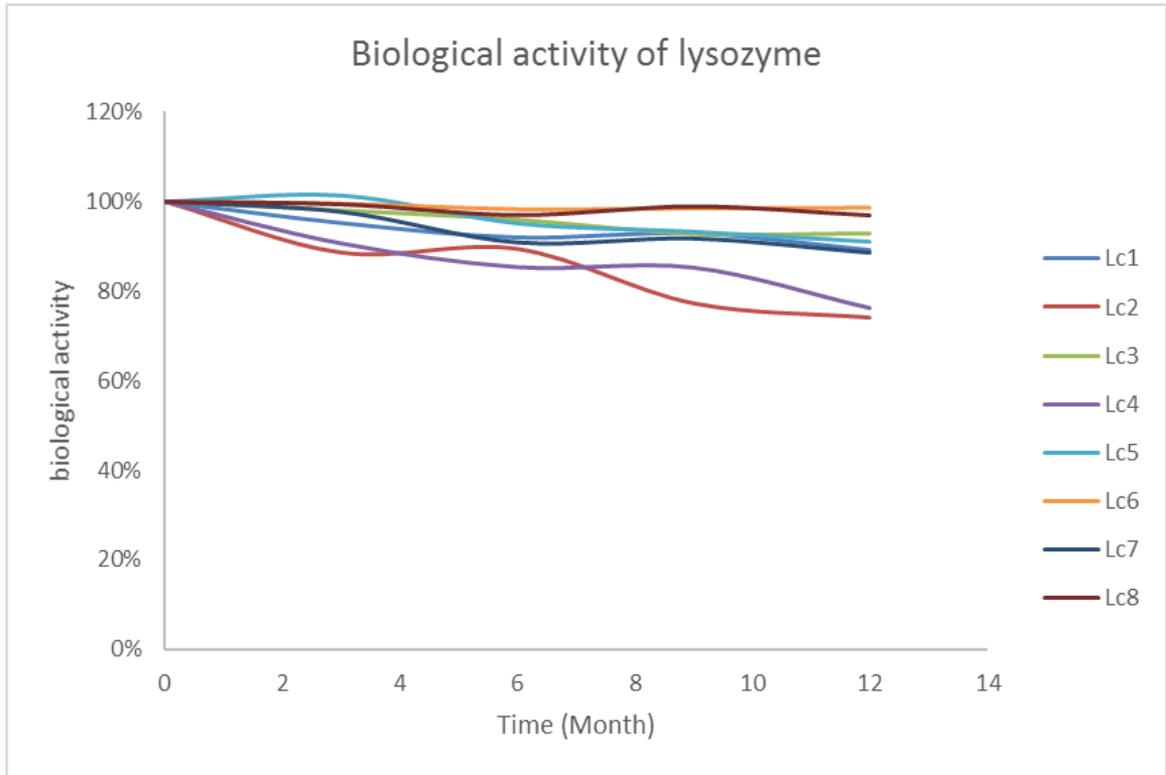
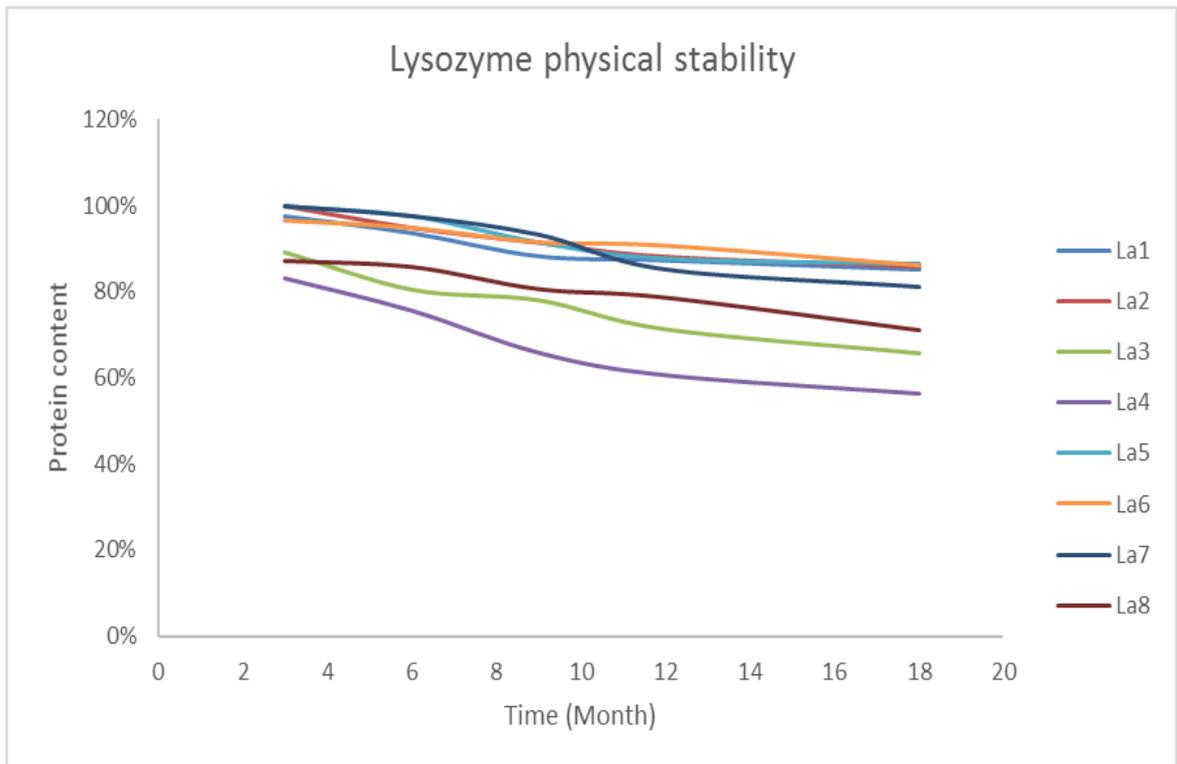


Figure 7.3: The decrease of biological activity of lysozyme samples over the storage period (18 months) at 5 °C.



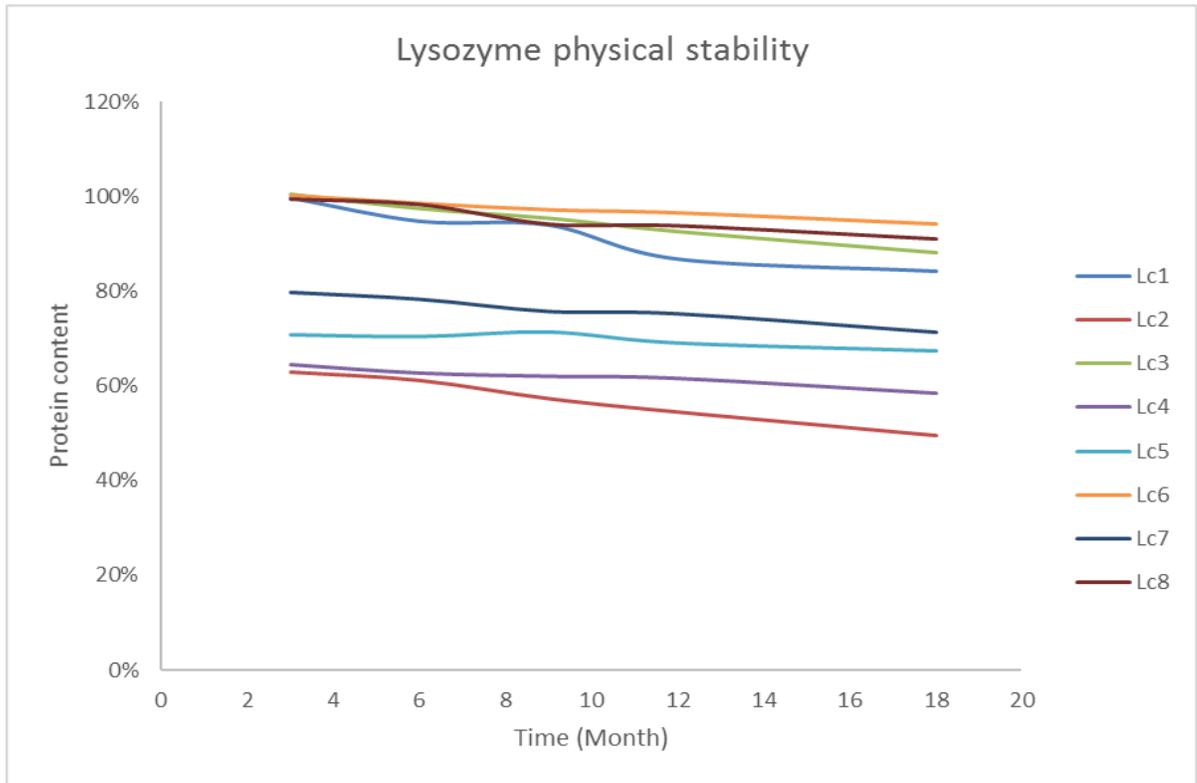
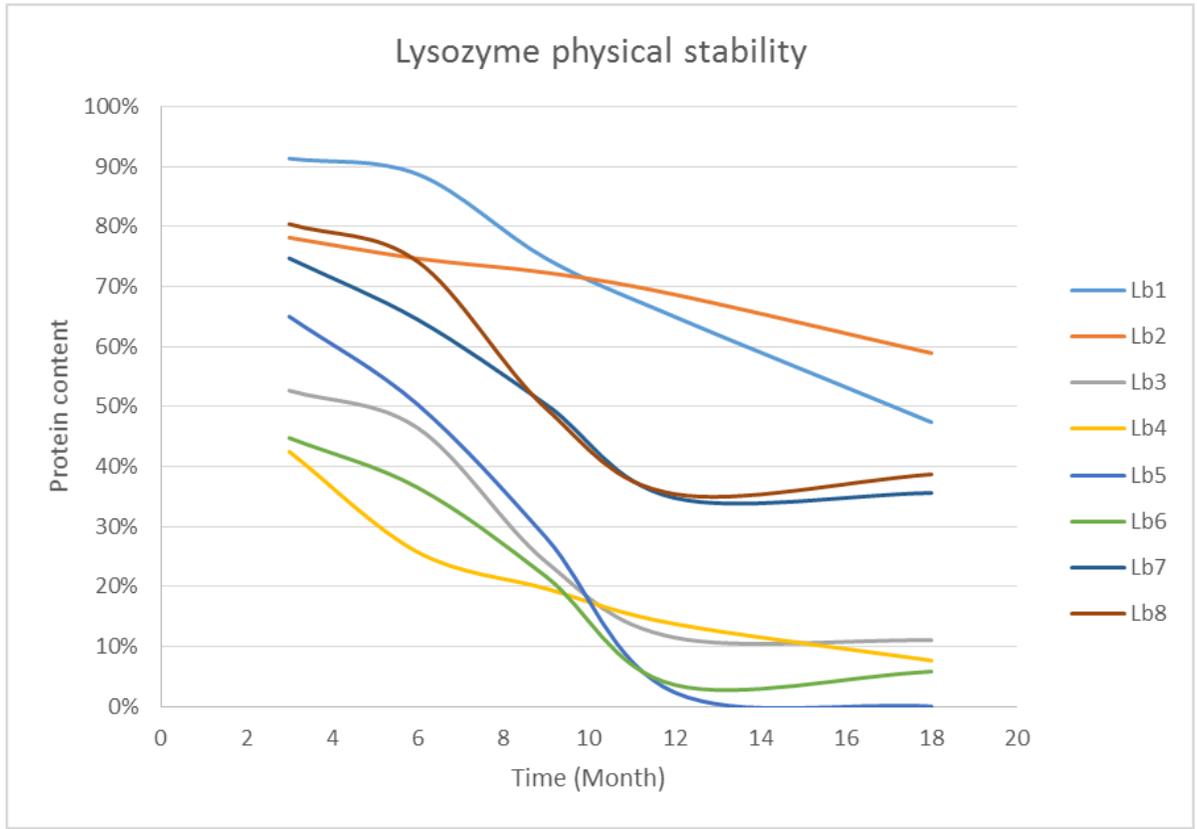


Figure 7.4: The decrease of physical stability of lysozyme formulations over the storage period (18 months) at 5 °C.

The results revealed that; trehalose was able to protect lysozyme under the harsh conditions e.g. alkaline pH. Although the biological activity and physical stability of these formulations were relatively low, the rate of degradation was low as reflected by the calculated slopes of the curves generated by plotting the time points versus the protected content of protein, as shown in Figure 7.3 and Figure 7.4. This conclusion was proven by the statistical analysis, as the interaction between the pH and trehalose has a significant stabilising effect on both of biological activity and physical stability of lysozyme (p-value: 0.031, and 0.038, respectively) in the group (DOEc).

Furthermore, the formulation number 2 in DOEb (Lb2) has relatively high physical stability and biological activity in comparison to the other formulations in the same group. This high stability results in Lb2 may be explained in the light of the ascorbate stability. As detailed earlier in Section 2.6.3, all the excipients in all formulations were analysed and quantified by High Performance Liquid Chromatography by using Hydrophilic Interaction Liquid Chromatography (HILIC) column. Excipients analysis revealed that the ascorbate concentration in Lb2 after the period of storage was very small and less than the other formulations. The analysis of ascorbate in Lb2 showed that; two peaks have come up for the ascorbate with different retention times. Two peaks are representing two molecules, native ascorbate structure and the oxidised form. The high oxidation of ascorbate reflected its ability as an antioxidant to decrease the oxidation degradation pathway, which consequently, retain the lysozyme biological activity by the scarifying mechanism.

Excipients analysis revealed that; trehalose retained more than 90% of the original concentration after the period of the storage. This high percentage of the retention of trehalose may have been the cause behind the stabilising effect of trehalose, as the novel structure of trehalose stabilises the proteins by the preferential hydration mechanism, as explained in details in Chapter three and Chapter six.

Size exclusion chromatography is the method of choice to analyse the proteins and able to separate the molecules based on their molecular weight. However, the non-soluble aggregates cannot be quantified or detected by SEC, as they remain in the filter and do not pass through with the samples. Therefore, the turbidimetry was employed in this study to detect the non-soluble aggregates. The turbidimetry results are semi-quantitative and are useful for the comparison purposes between the formulations. Detection of non-soluble aggregate is crucial regardless the percentage of the overall protein concentration. In the current study, for example; Formulations La1, and La5 retained a high percentage of native lysozyme as measured by SEC. However, up to 8% of non-native lysozyme was insoluble, which restricts the delivery of the protein to the patients due to the probability of developing immunogenicity caused by the aggregation [47].

On the other hand, trypsin formulations stability after 12 months of storage is illustrated in Figure 7.2. Among the sixteen formulations, the formulation number 7, and 16 have the highest biological activity. However, formulations T2, T7, and T16 have the highest physical stability, as obtained from SEC results.

The physical stability of trypsin in the current study was performed by SEC and Turbidimetry. However, for the formulations containing Pluronic F127 at a concentration 0.2% (w/v), SEC was unable to quantify trypsin, as most of the protein content was incorporated inside micelles, as illustrated in the chromatogram obtained from SEC analysis, Figure 7.5.

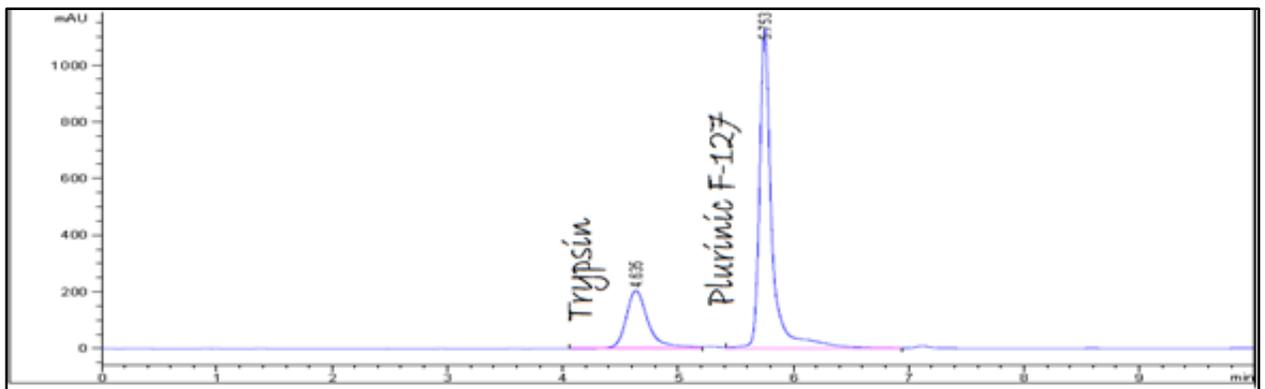


Figure 7.5: Size Exclusion Chromatogram is showing the peak of Pluronic F127 micelles at a concentration above the CMC.

As shown in the chromatogram, Pluronic F127 at a concentration above the CMC has formed micelles with a particle size less than the trypsin molecular size, as reflected by the late peak. It is clear that; trypsin peak is so small, which can confirm that most of the trypsin molecules are surrounded by Pluronic F127 and incorporated into the micelles, which prevented the protein quantification. In order to overcome the quantification hurdles, the formulations were diluted four times (0.05% w/v, Pluronic F127 CMC is 0.1% w/v), well-shacked, and then analysed by SEC. The obtained trypsin concentration was multiplied by four to get the right trypsin concentration.

After performing the statistical analysis for the formulations, it was concluded that; the high biological activity was affected significantly by trehalose (p-value 0.021), citrate concentration (p-value 0.024), and Pluronic F127 concentration (p-value 0.047). While Pluronic F127 concentration, and citrate concentration, had significantly affected the

physical stability of trypsin as measured by the SEC with p-values are <0.001 , and 0.028 , respectively. Therefore, a Pluronic F127 concentration above CMC protected the physical stability of trypsin significantly. The observed high physical stability may be caused by the isolation of trypsin particles by micelles formation, which decreases the chance of trypsin aggregation by reducing the overall system energy [218]. However, the biological activity was observed low for these formulations. The low biological activity imitates the chemical degradation in the active site of trypsin or the protein unfolding which may lead eventually to a decrease in the biological activity. The chemical degradation of trypsin may be caused by the adsorption of the protein on the surface of the surfactant, [219, 220]. *Kishore et al., 2011* concluded that; adding polysorbate 80 (the non-ionic surfactant) to the liquid protein formulation increases the chance of the protein to be prone to the chemical degradation [219].

Furthermore, the observed chemical degradation of trypsin formulations caused by the Pluronic F127 micelles was minimised by adding the trehalose to the formulations. As concluded from the statistical analysis, trehalose has a significant stabilising effect on the trypsin biological activity. The impact of trehalose and its mechanism in stabilising the proteins in liquid formulations was explained in details earlier in this study.

Moreover, citrate buffer has a significant negative impact on both physical stability and biological activity of trypsin dissolved inside it. Preparing of trypsin liquid formulations in 100 mM citrate buffer had the lowest stability among the other formulations after the storage at $5\text{ }^{\circ}\text{C}$. However, 0.2% w/v Pluronic F127 and 10 mM trehalose had countered the destabilising effects caused by the citrate buffer. Hence, the physical stability (48.65%) and the biological activity (53.25%) of the formulation number T6 (100 mM citrate, 0.2% Pluronic F127, and 10 mM trehalose) were higher than the observed

physical stability (19.45%) and the biological activity (17.52%) of the formulation number T10 (100 mM citrate, 0.02% Pluronic F127, and without trehalose).

As shown in Figure 7.2, formulation T6 had low physical stability after the storage period, despite the presence of 0.2% Pluronic F127. The effect of Pluronic F127 concentration above CMC on the physical stability of the protein was proven and detailed early. Analysing the excipients by HILIC increased the awareness about the additives role in stabilising the protein. The results of the HILIC revealed that; the Pluronic F127 in formulation T6 retained only 23.65% of its initial used concentration. The high percentage of the Pluronic F127 degradation may have decreased the Pluronic F127 function as a non-ionic surfactant, especially, the retained intact concentration is less than the CMC, which reduced the Pluronic F127 micelles protective effect.

The previous result is also supported by the turbidity measurement results. Hence, the observed turbidity readings of the formulations containing Pluronic F127 above the CMC were relatively low. The high turbidity reflects that; great non-soluble aggregation of the protein has happened.

Excipients analysis showed that; intact trehalose has been obtained for all the formulations contain trehalose.

To sum up, the formulations had more than 85% biological activity and physical stability were listed in Table 7.1.

Table 7.1: A list of formulations retained more than 85% biological activity and physical stability after the period of storage at 5 °C ± 2 °C. Biological activity, native protein content (SEC), non-soluble aggregates, and excipients stability.

Formulation ID	Biological activity	Physical stability (SEC)	Non-soluble aggregates	Turbidity	Trehalose stability	Ascorbate/Pluronic
La1	87.44	85.02	7.6%	High	>90%	78.52%
La2	86.50	85.86	1.76%	Low	>90%	86.25%
La5	89.77	86.36	7.90%	High	>90%	73.23
La6	91.89	86.10	2.03%	Low	>90%	63.25
Lc3	88.49	88.22	<1%	Low	>90%	-----
Lc6	98.19	94.19	<1%	Low	>90%	-----
Lc8	96.97	90.97	2.63%	Low	>90%	-----
T7	90.56	91.02	3.04%	Low	>90%	>85%
T16	92.52	90.41	1.63%	Low	>90%	>85%

The formulations shown above (Table 7.1) are the formulations retained more 85% biological activity and physical stability, consequently, it is worth performing further stability investigations and considering them for further preclinical studies. However, formulations La1 and La5 had higher non-soluble aggregates, as calculated indirectly

by SEC. The high non-soluble aggregates content must be further studies to control the factors had driven to non-soluble and reversible aggregation. Although these formulations retained high percentage of biological activity and physical stability, such formulations cannot be provided to the patient due to the probability of immunogenicity reactions. Moreover, the quality of the other formulations is high, and the excipients are stable. However, ascorbate retained relatively low stability which may have participated in lysozyme protection against the oxidation. Ascorbate degradants should be investigated by more detailing analytical methods such as liquid chromatography- mass spectroscopy (LC-MS) to make sure of the safety of its byproducts.

7.3.2. Stability study of liquid formulations containing lysozyme and trypsin by intermediate approach

Long term stability study is the essential and acceptable requirement to determining the biologics stability. However, different studies were published in the literature discussing and investigating the role of intermediate and accelerated long-term stability in predicting the long-term stability of the protein formulations [221].

The intermediate approach was employed to assess the stability of the lysozyme and trypsin formulations after nine months of storage under accelerated conditions $25^{\circ}\text{C} \pm 2^{\circ}\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$, as recommended by [212]. Biological activity and physical stability over the storage period, nine months, were evaluated, Figure 7.6.

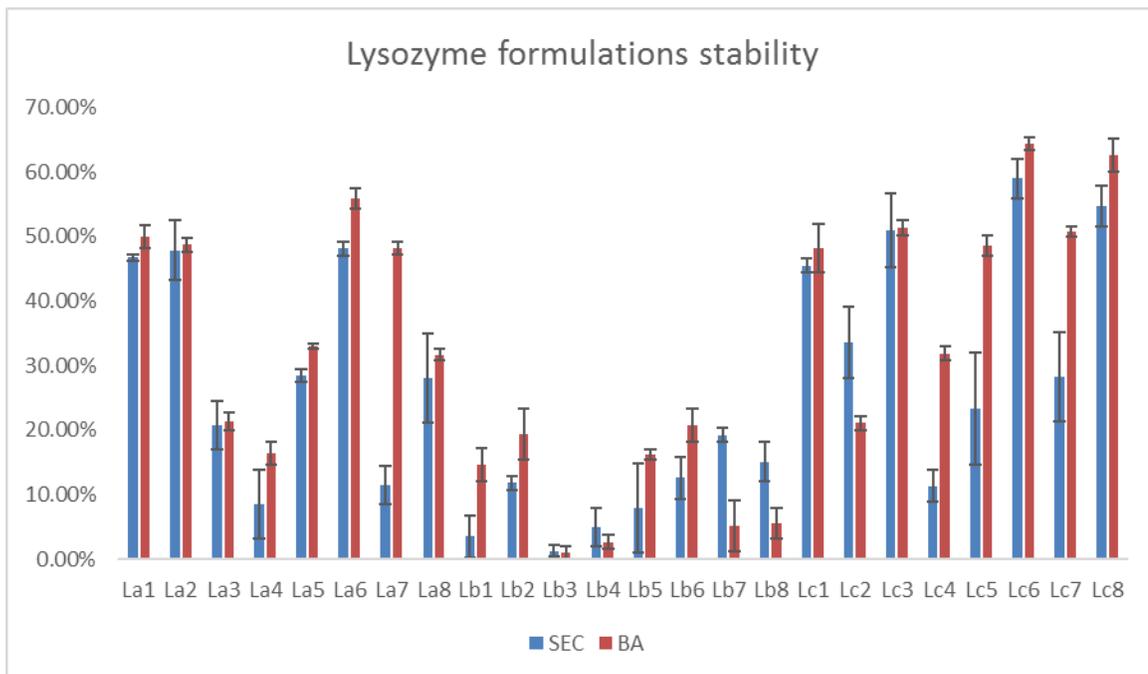


Figure 7.6: The biological activities and physical stabilities of different 24 liquid lysozyme formulations after the storage period of 9 months at 25°C ± 2°C/60% RH ± 5% RH. The assays were performed in triplicate, SD provided. For formulation compositions, refer to Table 2.8. BA: biological activity, SEC: size exclusion chromatography.

As shown in the figures above (Figure 7.6), not all stability values were obtainable. The initial physical assessment of the samples revealed that the liquid protein formulations at the intermediate stability conditions were highly prone to the microbial growth. Hence, most of the formulations were contaminated and spoiled, in spite of using the sterile autoclaved glass vials. The degree of microbial growth is different from sample to sample and depends on the formulation compositions. Microbial contamination was higher in low containing trehalose formulations rather the higher containing ones. Most of the trypsin formulations retained less than 30% biological activity and physical stability due to the bacterial growth. Therefore, robust and accurate measuring of the stability was not possible, as more variations in results occurred due to the technical, analytical issues e.g. filtration. Furthermore, Turbidity measurement provided results reflecting the microbial growth rather than providing results about the aggregation. In

conclusion, the intermediate approach was not a good idea due to; time-consuming, less reality reflecting, non-stability of the formulations, and difficulty of analysis.

7.3.3. Stability study of liquid formulations containing lysozyme and trypsin by the aggressive approach

The aggressive approach to studying the stability of trypsin and lysozyme liquid formulation was conducted by performing an accelerated stability study under $40\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $75\% \text{ RH} \pm 5\% \text{ RH}$, as recommended by ICH Q1A R2 guidelines, [212]. The tool box used for assessing the stability for this approach was the one used for both the conservative and intermediate approach. The results of the stability under the current approach was dramatically different from the previously discussed ones, Figure 7.7 and Figure 7.8.

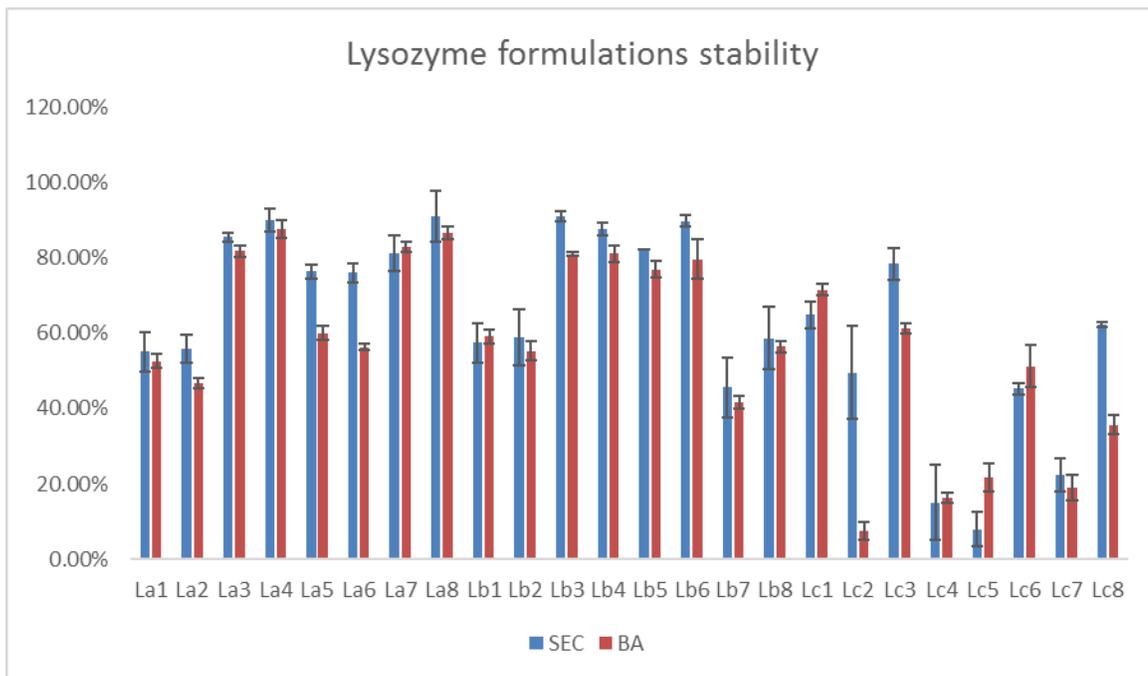


Figure 7.7: The biological activities and physical stabilities of different 24 liquid lysozyme formulations after the storage period of 6 months at 40 °C ± 2°C and 75% RH ± 5% RH. The assays were performed in triplicate, SD provided. For formulation compositions, refer to Table 2.8. BA: biological activity, SEC: size exclusion chromatography.

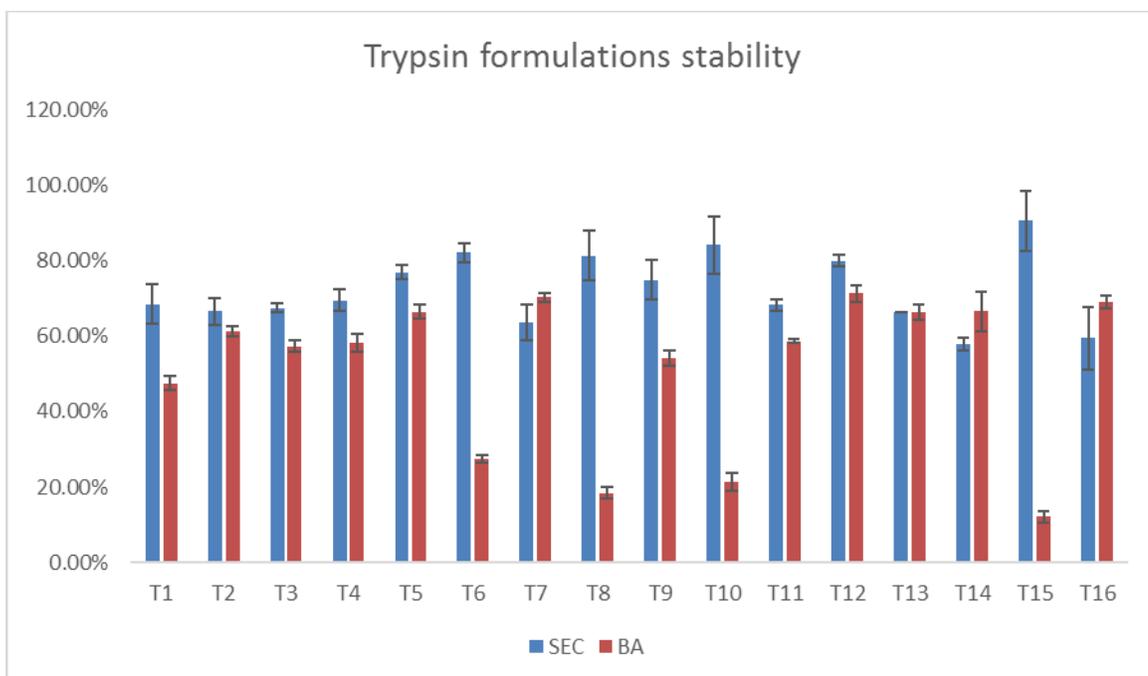


Figure 7.8: The biological activities and physical stabilities of different 16 liquid trypsin formulations after the storage period of 6 months at 40 °C ± 2°C and 75% RH ± 5% RH. The assays were performed in triplicate, SD provided. For formulation compositions, refer to Table 2.8. BA: biological activity, SEC: size exclusion chromatography.

As illustrated in Figure 7.7 and Figure 7.8, the accelerated biological activity and physical stability have a different trend from the ones discussed previously in this chapter, which reflects different degradation pathways had occurred when the conditions were changed.

Unlike under the chilled conditions, no significant difference was observed for the effect of trehalose towards the protection of lysozyme against the degradation at high pH and high temperature. Both biological activity and physical stability were recorded low, regardless the composition of the formulations. This may be explained in light of the fact saying that; the high pH and high temperature are stressful conditions which may induce and accelerate the rate of protein deamidation and oxidation [25]. Deamidation usually happens at Aspartate (Asp) amino acid residue and changes the amino acid sequence composition. In lysozyme, Aspartate is located in the active site of lysozyme. Therefore, any change in Asp structure may end up with lysozyme deactivation, and it may result in changing the amino acid backbone due to Asp isomerization and forming isoAspartate (isoAsp) which adds a methyl group to the amino acid.

Ascorbate played a stabilising role in the lysozyme formulations. The protection effect of sodium ascorbate is clear from comparing the groups containing ascorbate to the formulations with no ascorbate. The presence of ascorbate and trehalose together in the formulations even increased the stability further as in group DOEa. The stabilising effect of ascorbate may be a result of its antioxidation mechanism. Oxidation usually triggered by high temperature, and the proteins at high temperature are prone to the oxidation reaction. Ascorbate has protected the protein by working as an antioxidant scarifies [34].

On the other hand, citrate played a significant destabilising role in term of BA and turbidity in trypsin formulations. However, SEC results were very high, especially when Pluronic F 127 presented at a concentration above the CMC. Since high citrate concentration made the solution very viscous and with almost gel consistency (as observed visually by the naked eye), that reduced the particles motion and consequently, reduced the collision between the proteins particles and reduced the opportunities of aggregation even between the chemically degraded molecules. The previouslyly observed effect of citrate buffer on the stability of a a protein is in agreement with what was concluded by *Esue et al. 2010* about the destabilising effect of multivariate carboxylic buffers on monoclonal antibodies [222].

7.3.4. Critical comparison between the approaches

The feasibility of performing accelerated and intermediate stability as an indicator of the long-term stability of protein formulations is still under researchers' investigation. In the current study, liquid lysozyme and trypsin formulations accelerated, intermediate, and long-term stability was studied. Thereafter, data obtained from each approach was critically evaluated and compared to other approaches.

Intermediate stability study was not a good idea as concluded by the stability results for lysozyme and trypsin due to high microbial growth content at the intermediate storage conditions.

Data obtained from the accelerated and long-term approaches were, therefore, correlated with each other. All formulations were ranked according to their stability reading and the correlation coefficients (Pearson Coefficient R^2) between the

formulations ranking were calculated. Pearson coefficient values have been computed between the accelerated and long-term biological activity and accelerated and long-term physical stability for both lysozyme and trypsin.

The correlation results were promising for trypsin stability, with a correlation coefficient value recorded more than 0.4. However, the highest correlation coefficient calculated for the lysozyme was less than 0.2. Both numbers are still low and cannot be relied on to predict the long-term stability study. However, the accelerated degradation pathways for trypsin formulations had 40% with the pathways of decomposition after a long time of time. Therefore, it may be worth it if the accelerated stability study is performed for trypsin formulations to predict some of the expected degradation or to screen the formulations factors within a short time and with less cost.

7.3.5. Conclusion

The current study investigated the role of different excipient, trehalose, sodium ascorbate, and Pluronic F 127, and different buffer conditions on the stability of liquid lysozyme and trypsin formulations. Three different approaches were applied to assess the stability of the formulation. Conservative stability approach was the one could provide the real results about the stability. However, it was cost, effort, and time-consuming. From the conservative approach, it was concluded that; trehalose was a good stabiliser for both of lysozyme and trypsin over the period of storage. Moreover, although some formulations retained very high stability, they will not be a good candidate to be provided to the patient due to a small percentage of non-soluble

aggregate which may induce immunogenicity for the patient. Furthermore, the aggressive approach may be reliable for evaluation of the impact of the formulation factors on storage stability, however, in the short term.

Chapter Eight: General Conclusion

8.1. General conclusion

The genetic revolution and DNA-technology made the protein synthesis easier than before and generated the recombinant proteins in specific host cells e.g. bacteria, yeast, or mammalian. Therapeutic proteins are the recombinant proteins engineered in the lab for pharmaceutical and therapeutic uses, such as vaccines and hormonal replacement therapy. A wide range of serious diseases is clinically treated by the therapeutic proteins. The extensive uses of therapeutic proteins have arisen from the unique physiological functions of the protein inside the living systems.

Therefore, therapeutic protein formulations have emerged strongly in the pharmaceutical development and according to the FDA, 50% of recently registered drugs are proteins or protein related medications.

Formulation and delivery of proteins are very challenging. Chemical and physical instability of proteins are the major challenges. Different approaches, for example, protein encapsulation, drying, and adding excipients, are currently in use to overcome these challenges. Therefore, developing bioassays and bioanalytical methods is a crucial part in determination protein formulations stability.

Before investigation of protein formulation instabilities, one must know the protein structure levels, and the potential degradation pathways should be known. Proteins in general, have four main structural levels: primary, secondary, tertiary, and quaternary. All levels of protein structure play a critical role in protein stability, especially, higher levels which are strongly connected to the biological activity of proteins. The complexity of the protein structure as a diverse group of the biomolecules restricted the choice of

dosage forms, for example; the majority of proteins cannot be delivered orally due to the degradation by the digestive enzyme along the GIT.

The aim of this research was to investigate the main suggest contributory factors on the stability and activity of lysozyme and trypsin (as model proteins), consequently, to control and optimise the factors to obtain protein formulations intended for oral or injectable drug use administration route. Excipients and buffer conditions were studied and optimised, and two types of formulations were developed: liquid, and polymeric nanocapsule formulations. The current research was accomplished by applying quantitatively based design of experiments through adopting the quality by design (QbD) framework to achieve the main objectives of the project (to develop stable and active protein formulations within reasonable time and resources).

The primary emphasis was on the screening of the formulation factors on the conformational stability of lysozyme and trypsin. The screening was carried out by adopting a systematic approach starts with identifying the quality target product profiles (QTTPs) and critical quality attributes (CQAs). Then as a part of the screening process, a risk assessment was performed by identifying most of the potential risk factors affecting the liquid protein formulations and analysing the listed risk factors, based on the relevant literature and previous knowledge, to clarify their effect on liquid lysozyme and trypsin formulations. Risk analysis revealed that pH, types of buffer, buffer concentrations, and the excipients are critical factors that should be further investigated, monitored and optimised before the development of the liquid formulations. Then, the experiment was designed, to evaluate the risk effect, by formulations preparation through using three types of buffers (citrate, acetate, and phosphate) at three different concentrations (10, 50, and 100 mM), and two excipients

for each protein (trehalose and sodium ascorbate for lysozyme, and trehalose and Pluronic F127 for trypsin).

The conformational stability of the prepared formulations was investigated by utilising high sensitivity differential scanning calorimetry (HSDSC), and the denaturation temperature (T_m) was determined for the proteins in all formulations. When reviewing the results of DOE analysis, the formulation conditions were optimised, and the optimised formulations were prepared, and their conformational integrity and biological activity were assessed after the storage for six months under the accelerated conditions. The main findings of Chapter Three: were; combining trehalose and phosphate buffer at the optimised levels, (Table 8.1), provided promising results, in terms of the conformational stability of lysozyme and trypsin, more than using them individually.

Table 8.1: The optimised buffer and excipient conditions as obtained from analysing and fitting the excipient models, generated by Design of Experiment (DoE) methodology.

Protein	pH	Buffer	Concentration mM	Excipient	Concentration mM
Lysozyme	4.2	Phosphate	5	Trehalose	50
	4.2	Phosphate	27.5	Trehalose	5
Trypsin	3	Phosphate	69	Trehalose	40
	3	Phosphate	65	Trehalose	10

Based on the results of the investigated factors in 113Chapter Three:, the most stabilising and destabilising factors on the conformational stability of lysozyme and trypsin were collected together in DOEs to prepare a total of twenty-four lysozyme formulations and sixteen trypsin formulations. The rationale behind these formulations

was to; assess the effect of the selected factors on the long-term stability of the proteins as the degradations of proteins are not predictable, and stabilising factors before storage are, indeed, not stabilising factors for proteins after storage. Moreover, the stability study was carried out according to three different approaches: conservative, intermediate, and accelerated for (eighteen months for lysozyme, twelve months for trypsin), nine months, and six months, respectively. The correlation between the three approaches was performed to assess the ability of the aggressive and intermediate approach in the prediction of long-term stability. A toolbox of quality control analytical methods was utilised in this stability study including size exclusion chromatography, turbidimetry, and enzymatic assay, in addition to hydrophilic liquid interaction chromatography (HILIC) to assess excipients degradation and ensure the quality of the stable formulations. Size exclusion chromatography (SEC) and HILIC methods were developed and validated in this research. The analytical methods were confirmed to be specific, accurate, precise, robust, and linear at the experiment concentrations range.

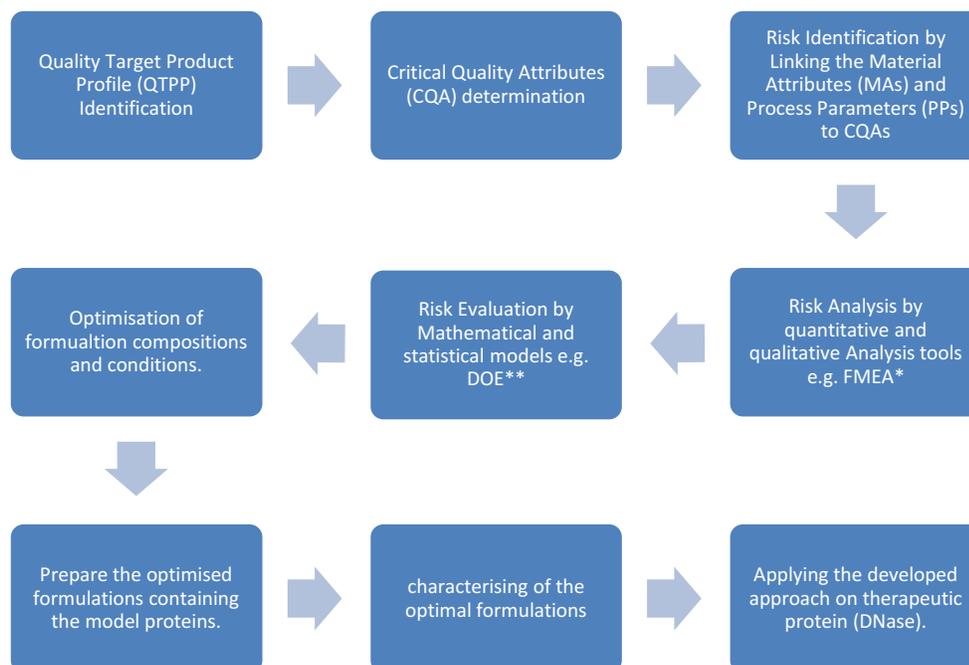
Stability study (Chapter Eight) revealed the role of trehalose in stabilising the protein over the time of storage even for the formulations at pH 8. Moreover, ascorbate 10 mM had a stabilising effect on lysozyme structure, especially when the ascorbate was oxidised, which emphasise its scarifying mechanism of protecting proteins against the oxidation degradation. Pluronic F127 at a concentration above CMC (0.2% w/v) protected the protein to retain their inherent physical stability by surrounding the trypsin molecules via forming micelles, accordingly, the aggregation was reduced. Citrate buffer at (100 mM) has accelerated the chemical degradation of trypsin. However, trehalose protected trypsin from the chemical degradation induced by high

concentration of citrate buffer. Seven lysozyme formulations retained more than 85% of their physical stability and native biological activities. However, the quality of two of them was not acceptable due to developing non-soluble aggregations which increased the formulations turbidity and hence, may cause immunogenicity for the patients.

The quality of the stored formulations under the intermediate approach was not acceptable due to microbial growth.

Storing the proteins under the harsh condition ($40\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$) accelerated deamidation of the lysozyme formulations with high pH values and the deamidation degradation could not be overcome by adding trehalose.

Furthermore, a strategic approach to develop oral administered proteins was established in this study to deliver the protein carried by using polymeric nanocapsules (Chapter Five). The establishment of the approach was commenced by adopting QbD throughout the project by using model proteins (lysozyme and trypsin). QbD was implemented by identifying the QTPPs and, accordingly, determination of the CQAs. Then the risk assessment was performed by risk identification, risk analysis, and evaluation of the risk factors. The assessment of the risk factor was performed by preparation the polymeric nanocapsules according to the design of experiments and characterising them against the desired CQAs. Accordingly, the optimisation of the formulation conditions and compositions has also carried out to prepare the optimal formulations capable of delivering the oral protein efficiently and safely. Finally, the optimal formulations were prepared to encapsulate a therapeutic protein (deoxy ribonuclease I) as an application of the developed approach, Figure 8.1.



*FMEA: Failure Mode and Effects Analysis.

**DOE: Design of Experiment.

Figure 8.1: A schematic diagram comprises the development of the approach, step by step, starting with QbD implementation and ending with applying the approach on therapeutic protein.

A total of sixteen polymeric nanocapsule formulations containing model proteins (lysozyme and trypsin) were prepared by the double emulsion method, and characterised for the following characterisation: encapsulation efficiency, protein release in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), particle size, morphology, biological activity, the protection of encapsulated lysozyme from digestive enzyme, and storage stability for six months (Chapter Six).

The characterisation test results revealed the role of the used copolymer in controlling the drug release and encapsulation efficiency. Using 40:60 Poly (D, L – Lactide-co-caprolactone) entrapped more protein with an encapsulation efficiency value reached up to 80%, but the percentage of drug release lower than 40% during 24 hours of study

dissolution period. However, the other side was when the percentage of Lactide part was increased to reach 86:14 Poly (D, L – Lactide-co-caprolactone), thus higher release (up to 70%) with low encapsulation efficiency (30% - 43%) was obtained. In order to increase the release percentage hydrophilic polymer usually blended with the used hydrophobic polymer. However, the blending of hydrophilic polymer reduces the encapsulation efficiency. Therefore, adding polyethylene glycol (PEG 8000) to the formulations after the nanocapsules preparation and immediately before the freeze drying enhanced the ability of 40:60 Poly (D, L – Lactide-co-caprolactone) to release proteins from 35% on average to more than 80% without observing any changes in the entrapment efficiency (Chapter Five). Adding trehalose to the core of the polymeric nanocapsules protected the biological activity of the encapsulated proteins, and the value of proteins biological activity has increased from almost 40% to 98%.

The current study has showed promise for the protein delivery via oral and injectable route. Trehalose played an exceptional stabilising effect for both of lysozyme and trypsin in liquid and nanocapsule formulations. QbD approach predicted the formulation factors and variables that have a potential influence on the products quality. Therefore, the prediction reduces the early development applied features, hence, reduces the necessary time, labour, raw materials, storage process and overall operation cost. QbD base studies are required especially in biopharmaceutical formulations when the materials are very expensive and limited with more complicated techniques and skills required due to their target orientation properties.

8.2. Suggestions for future work

The impact of the used excipients (Trehalose and sodium ascorbate for lysozyme, and trehalose and Pluronic F127 for trypsin) and the used buffer (acetate, citrate, and phosphate) on conformational stability and biological activity of the proteins in liquid forms at different pH values was critically investigated under the quality by design (QbD) framework.

This study has demonstrated a close link between the stability and the activity of the protein in liquid form from one side and the type of excipients and the buffer conditions from the other side. Moreover, the study optimised the formulation factors to obtain active and stable protein liquid formulations. Therefore, it would be beneficial to evaluate the effect of the same conditions and same development processes on other proteins stability.

Trehalose revealed a protection effect on the bioactivity of both proteins against the destabilising conditions, consequently; it is worth to try other kinds of sugar and extremolytes on preserving the proteins activity.

A promising toolbox with developed and validated analytical assays was utilised in this study, it would be useful for the researchers if they apply the developed methods to analyse more protein formulations without the need to going through the full validation process.

The author established a strategic approach for the development of polymeric nanocapsule intended for the oral delivery route. Promising data was collected, and all the formulation factors were optimised with excluding the risk factors and applied to encapsulate therapeutic proteins. The approach is recommended to be adopted by the

researchers to develop other biomolecules within polymeric nanocapsules such as genes or insulin.

Polymeric nanocapsules demonstrated high release percentage in the intestine during 24 hours, in addition to their ability to protect the proteins from degradation by digestive enzymes. Therefore, combining the polymeric nanocapsule with permeability enhancer would be a good idea to deliver the nanocapsules to the circulation through the intestinal tissues and allowing them to degrade inside the blood stream.

Moreover, formulating the polymeric nanocapsules into mucoadhesive tablet may allow the nanocapsules to remain longer in the intestine, thus, releasing more proteins.

Chapter Nine: Bibliography

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