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"NOVEL ORAL PEGylated POLYMERIC NANOPARTICLE FOR THE DELIVERY OF TRYPSIN TARGETED TO THE SMALL INTESTINE"

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Keywords: Trypsin, Polymeric Nanocapsule, Shelf Stability, Targeted Delivery, physicochemical characterisation

Abstract

The lack of trypsin in the intestines may end up with malnutrition; thus, trypsin replacement therapy is required in such cases. The objective of this study was to formulate and evaluate polymeric nanocapsule (PNC) systems able to deliver trypsin to the small intestine with the minimal release in the stomach with the maximum biological activity. Four nanocapsule formulations were prepared by double emulsion/evaporation method as w/o/w and s/o/w. Particle size, encapsulation efficiencies, drug release in simulated gastric fluids (SGF) and simulated intestinal fluids (SIF), morphology, the biological activity of encapsulated trypsin and shelf-life stability were investigated for all formulations. All formulations had a spherical shape with submicron size, and encapsulation efficiency more than 80%. The biological activity of encapsulated trypsin was significantly affected by the amount of trehalose and whether the formulations were prepared as s/o/w or w/o/w ($P<0.05$). Most of the encapsulated protein was sustained released at the target site (SIF) over 24 hours with minimum amount release in the gastric fluids. Also, more than 90% of physical integrity trypsin encapsulated in all formulations was retained after storage under chilled conditions for six months. However, the enzymatic assay results show that with low trehalose content, the biological activity was low, while increasing the trehalose amount increased the shelf stability to reach around 100% after six months of the study. The results obtained in this research work clearly indicated a promising potential of controlled release polymeric nanocapsules containing trypsin to target the small intestine and protect trypsin from the harsh condition facing the proteins during the process of preparation or the period of storage.

1. Introduction

Trypsin is a proteolytic enzyme produced by the pancreas and normally exists in the small intestine; its main function is breaking down proteins and continuing the digestion after stomach emptying [1]. The lack of trypsin may cause a chronic clinical case called malabsorption which consequently leads to malnutrition due to the deficit of essential protein absorption. In such cases, the patients are provided oral trypsin to replace the shortage of endogenous trypsin [2]. Trypsin should be delivered to the small intestine in its active form to perform its biological activity successfully. The conventional dosage forms deliver trypsin to the GIT. However, sufficient amounts of trypsin may be released in the stomach, and the frequency of pills intake is very high; thus, it should be taken many times a day. Therefore, the development of sustained-release formulation able to deliver trypsin all over the day to be taken only once a day is considered necessary to increase patient compliance.

The advances of nanocarriers, e.g. liposomes, niosomes, and polymeric nanoparticles, provided solutions to many therapeutic protein delivery problems, e.g. oral protein and sustained release [3-5]. Polymeric nanoparticles are carrier drug delivery systems have particle diameters up to 1000 nm with a structure composed mainly of biodegradable polymers [6]. Polymeric nanocapsules is a nanotechnology-based drug delivery system consists of a core-shell design able to encapsulate a wide range of drugs in its internal cavity, e.g. protein, gene, and other small molecules [7, 8]. Different polymer categories are used in the formulation of polymeric nanoparticle carriers. The commonly used polymers are usually characterised by being biodegradable and biocompatible to avoid any potential accompanied toxicity [9]. Polymeric nanoparticle drug delivery systems were developed and widely investigated by researchers in the past couple of decades [10, 11]. 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 [12]. Each polymer has its advantages and disadvantage and its specific applications in drug delivery systems.

Poly (ε-Caprolactone) (PCL) is a highly hydrophobic aliphatic polyester polymer with a very long half-life and requires years in order to be fully biodegraded [13]. Poly (ε-caprolactone) is being used in drug delivery systems as a homopolymer, copolymer or blended with other polymers to accommodate its properties to be suitable for formulation purposes [14-16]. PCL-based nanoparticulate systems have been described for the delivery of proteins when prepared by single and double emulsification solvent evaporation method [16]. The high crystalline and hydrophobic properties of PCL micro- and nanoparticles contributed significantly to enhancing their abilities to confine as much as possible of the used active ingredients. However, the same features limit their release and may slow drug liberation rate, which may be not suitable for many drug delivery systems. Therefore, the need for modifying PCL by, e.g. adding or copolymerisation with hydrophilic polymers to make it more releasable is considered crucial to making up a successful drug delivery system compatible with the intended route of administration.

In this study, a novel polymeric nanocapsule system was developed to deliver trypsin to the small intestine in prolonged, localised and active form around the clock. All factors that may be affecting protein delivery were considered to reduce the degradation of trypsin and deliver it to the small intestines with the least cost possible. Many challenges face therapeutic protein delivery by the polymeric systems due to the harsh conditions of preparation, e.g. hydrophobic surface interaction, sonication, temperature and protein overhydration [17-19]. Several studies addressed many approaches to overcome these challenges, e.g. encapsulating the proteins with sugars, optimising the type and amount of polymer and surfactants, and

choosing the suitable preparation method [16, 20, 21]. To our knowledge, this is the first report that addresses the development of PEGylated PCL-based nanocapsules for targeted delivery of trypsin to the small intestines to attain sustained-release replacement therapy.

2. Material and methods

2.1. Materials

Trypsin (Serine protease, lyophilised powder, 13,000-20,000 BAEE units/mg protein) obtained from porcine pancreas, was supplied by Sigma-Aldrich. Ethyl acetate, polyvinyl alcohol PVA, Span$^{60®}$, poly (D, L-Lactide-co-Caprolactone) (40:60) (PLC 90.000 40:60), hydrochloric acid and polyethylene glycol 8000 (PEG 8000) were purchased from Sigma–Aldrich Co. (Poole, Dorset, UK). Electrochemical analysis grade acetonitrile was obtained from Fischer Scientific (Loughborough, UK). Nα-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE). Trehalose dihydrate was provided from VWR Co., (Radnor, PA, USA).

2.2. Polymeric Nanocapsules preparation

PNC formulations were prepared using the double emulsion method reported previously [22, 23]. The aqueous phase comprised of trypsin (6.25mg/ml), trehalose (1mM or 10mM) and water was emulsified in the organic phase (ethyl acetate, polymer and Span60) by probe sonicator for 60 seconds at 65 watts to form w/o. Then, w/o/w was formed by sonication at 65 watts for 90 seconds in pulsing mode (3 seconds on and 1 second off) after adding the emulsion to a concentrated solution of PVA. However, some formulations were prepared by s/o/w method, where trypsin and trehalose were suspended in the organic solvent and dispersed by sonication to prepare a fine s/o suspension. The organic solvent was evaporated by overnight magnetic stirring at room temperature. The remaining organic solvent and free proteins were removed by centrifuging and washing the nanocapsules twice for 30 minutes at 4 °C and 15000 rpm. Then, PEG 8000 was added to nanocapsules suspension and distributed uniformly before the lyophilisation by VirTis Benchtop Freeze Dryer (Biopharma) under 25-36 m Torr and at a freezing temperature of – 105 °C for 48 hours. The composition and characteristics of all formulations were clarified in Table 1.

2.3. Encapsulation efficiency of proteins

The encapsulation efficiency of nanocapsules was quantified by applying the previously developed method [24]. Briefly, the polymeric shells were dissolved in ethyl acetate by magnetic stirring for two hours under fume cupboard. The resulted system was centrifuged, using Mikro 220R centrifuge (Hettich centrifuges, Germany), at 10000 rpm for ten minutes and washed twice by nanopure water (>Ω 18, Milli-Q). The pellets containing trypsin was completely dried from any organic solvent residues to be dispersed in water to

dissolve the protein. The suspension was centrifuged twice, and the aliquot was taken to quantify the protein content by using Size Exclusion Chromatography (SEC). 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 isocratic separation system was used with a mobile phase constituent of 150 mM sodium phosphate buffer pH 7 at a flow rate of 1 ml/minute for a total chromatographic run time 10 minutes. This method was developed and validated in our lab. Specificity, accuracy, precision, linearity, and robustness of the method were investigated. The value of encapsulation efficiency was calculated by the following equation:

$$\mathbf{EE\%_{(w/w)}} = \frac{\boldsymbol{Amount\ of\ encapsulated\ drug}}{\boldsymbol{Amount\ of\ initial\ drug\ used\ in\ the\ formulation}} \times \mathbf{100}\%$$

2.4. The particle size of Trypsin-PEGylated polymeric nanocapsules

The particle size and Poly Dispersity Index (PDI) of the polymeric nanocapsules were determined by utilising Zeta PALS® dynamic light scattering (Zeta PALS, Brookhaven Instruments Corporation, USA). Ten mg of dried nanocapsule powder was suspended in 5 ml water and vortexed for one minute, then sonicated in a water bath for five minutes. The hydrodynamic diameters were measured three times at 25 °C.

2.5. Morphology of polymeric nanocapsules using Transmission Electron Microscopy (TEM)

TEM (Hitachi H7000 transmission electron microscope, Japan) was used for the investigation of nanocapsules morphology by using negative staining technique 1% (w/v) of sodium silicotungstate solution. One drop of PEGylated-NC suspension was introduced to a 400 mesh Formvar copper grid on paraffin, and the sample has adhered on the Formvar at room temperature for 15 minutes. Then, a drop of 1% (w/v) of sodium silicotungstate solution was applied for 5 minutes. The obtained specimen was later investigated by the TEM.

2.6. *In vitro protein* release in Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF)

As a formulation intended to be delivered orally, simulated gastric fluids (SGF) and simulated intestinal fluid (SIF) were used as drug release media for 4 and 24 hours, respectively. SGF and SIF were prepared according to the British Pharmacopeia 2014. Briefly, the SGF is an acidic solution (pH 1.2) comprised of 80 mM HCl and 0.2% NaCl. The SIF was prepared by mixing 0.2 M sodium hydroxide (NaOH) with Potassium Dihydrogen Phosphate (KH2PO4) to obtain a solution of KH_2PO_4 with a final concentration of 0.78% and pH 6.8. No digestive enzymes were added to any of the SIF or the SGF. Powder nanocapsules containing trypsin were suspended in both media and incubated in a shaker water bath at 37 °C with 50 cycles/minute for the entire time of the experiment. One ml sample was withdrawn at each time, and the protein content was determined by SEC. All release tests were performed in triplicate.

2.7. Measurement of biological activity of the encapsulated trypsin

The biological activity of trypsin was determined by measuring the rate of ester link cleavage in *N*-benzoyl-l-arginine ethyl ester (BAEE), as described by [25, 26]. Briefly, the polymeric shell was broken by adding ethyl acetate to the nanocapsules powder. Then, trypsin was dissolved in HCl and added to (BAEE) solution to initiate the enzymatic kinetic reaction, and the increase in A253 was recorded for 5 minutes by M501 Single Beam Scanning UV/Visible spectrophotometer Camspec (Biochrom, UK). 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°. Fresh trypsin solution was prepared, and its activity was considered as control. The biological activity trypsin was expressed as a percentage relative to the fresh trypsin solution. The biological activity of fresh trypsin was 100%.

2.8. The storage stability of the Polymeric Nanocapsule formulations containing trypsin

The encapsulated proteins in the PNCs and the overall formulation stability studies were carried out to examine the withstanding of protein and the PNCs over the shelf life. Thus, all formulations were stored at two different storage conditions: 5 ± 2 °C in dissector, and 22 ± 2 °C at 76% relative humidity. The stored formulations stability was studied to investigate the biological activity 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.

Moreover, the water content of the PNCs powder formulations was determined by Karl Fisher Titration (KFT) (701 KF Titrino 67 with 703 Ti stand, Metrohm, Switzerland), as described by [27]. The min principle of KFT is based on two successive reactions, and the amount of water required to perform the second reaction provides the estimation of the moisture in the dry powder sample. In this method, dry

samples were introduced into the 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 typically ranges from 15-35 mg. In this study, the moisture of PNCs containing trypsin after storage was determined. 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.

2.9. Statistical analyses

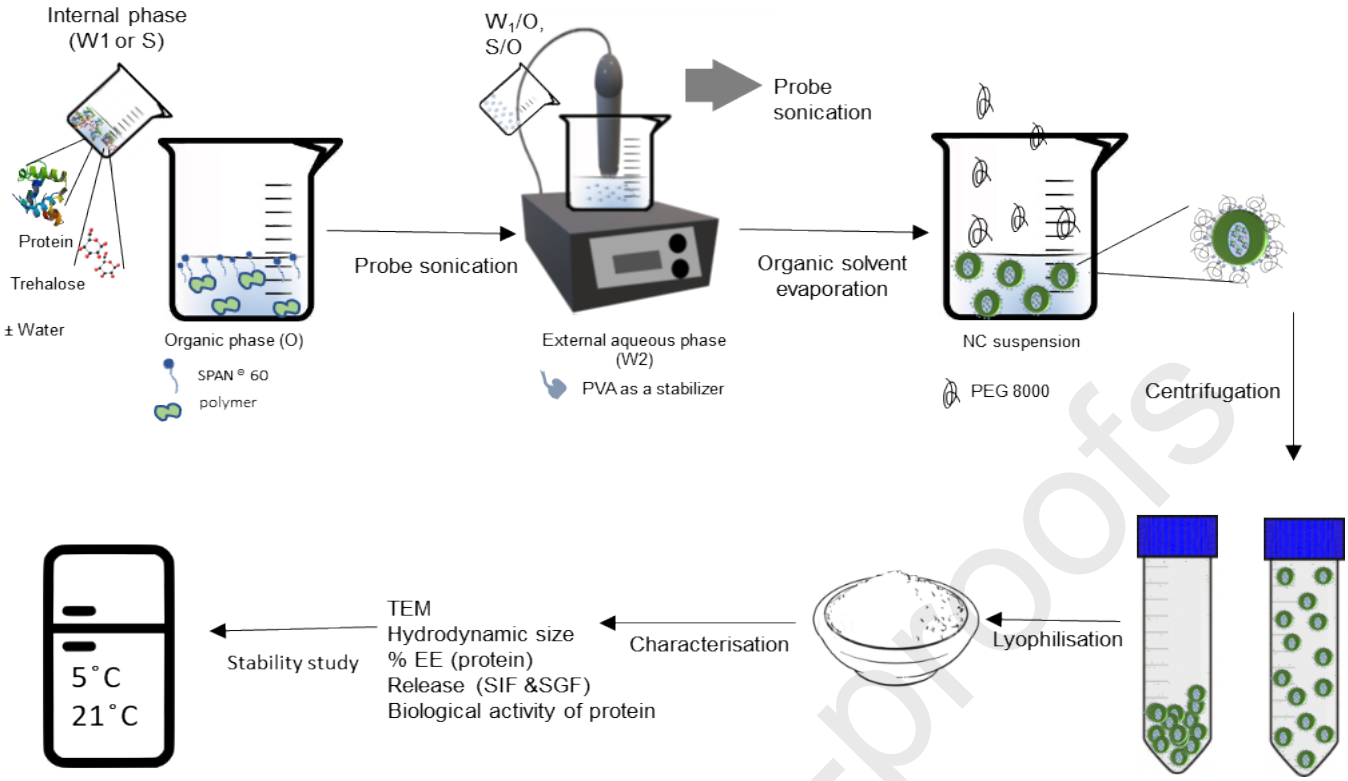
For all experiments, data were presented as the mean of triplicate ± SD. Statistical significance of the data was evaluated using Student's t-test $p< 0.05$.

3. Results

3.1. Formulation and physicochemical characterisation of polymeric nanocapsules

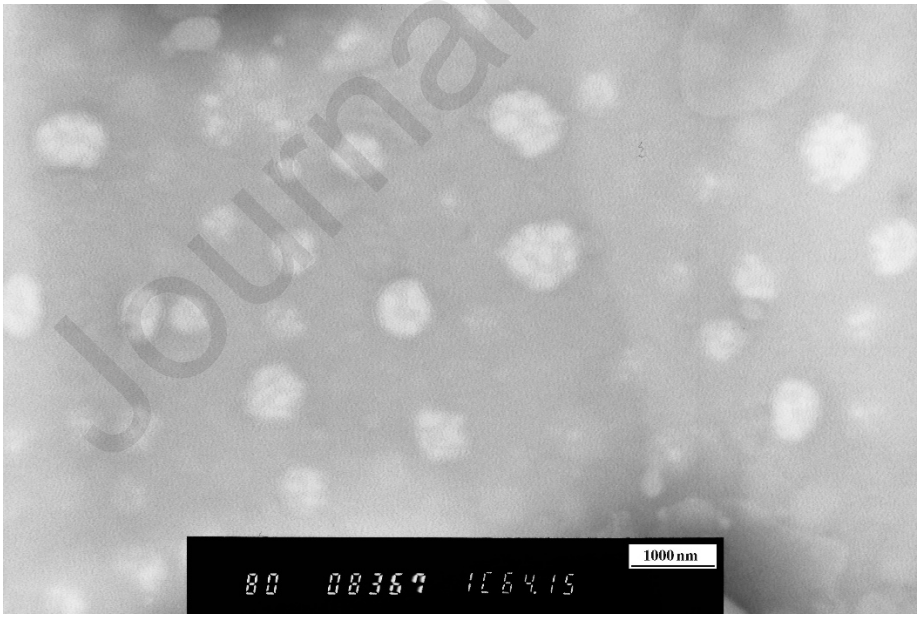
Trypsin containing PNCs were formulated using (40:60) poly (D, L-Lactide-co-Caprolactone) (PLC) to encapsulate trypsin in liquid or solid state with different concentrations of trehalose (1 mM and 10 mM). All formulations were prepared either by w/o/w or s/o/w methods, Scheme.1.

The morphology of the developed polymeric nanoparticle was investigated by TEM and revealed that all formulations were in round shape and comprised of core-shell capsule structure, Figure 1. Also, the morphology test confirmed that all nanocapsules have smooth surfaces without any distortion or wrinkles. However, various particle sizes were observed. Thus, the hydrodynamic particle size of the developed vesicles was measured by dynamic light scattering; it was concluded that the obtained particles were in the submicron range (337.2-912.3) nm. The statistical analysis revealed that the particle size was significantly affected by the amount of trehalose used; the higher trehalose added the larger particles size obtained.



Scheme 1. Double emulsion (w/o/w or s/o/w)/solvent evaporation method for the preparation of PEGylated PNCs. Schematic diagram illustrating the method of fabrication of trypsin-loaded PEGylated-PNCs. The Polymeric systems were prepared by emulsifying the internal aqueous phase (trypsin and trehalose in water) or suspending the internal solid protein and trehalose into organic phase (ethyl acetate) containing polymers, and Span® 60. Afterwards, w/o/w or s/o/w was prepared by adding the internal emulsion to the external aqueous phase using probe sonication. Then, the solvent was removed, forming PEGylated-NCs aqueous dispersion. Finally, the formed suspension was freeze-dried over 48 hours.

A



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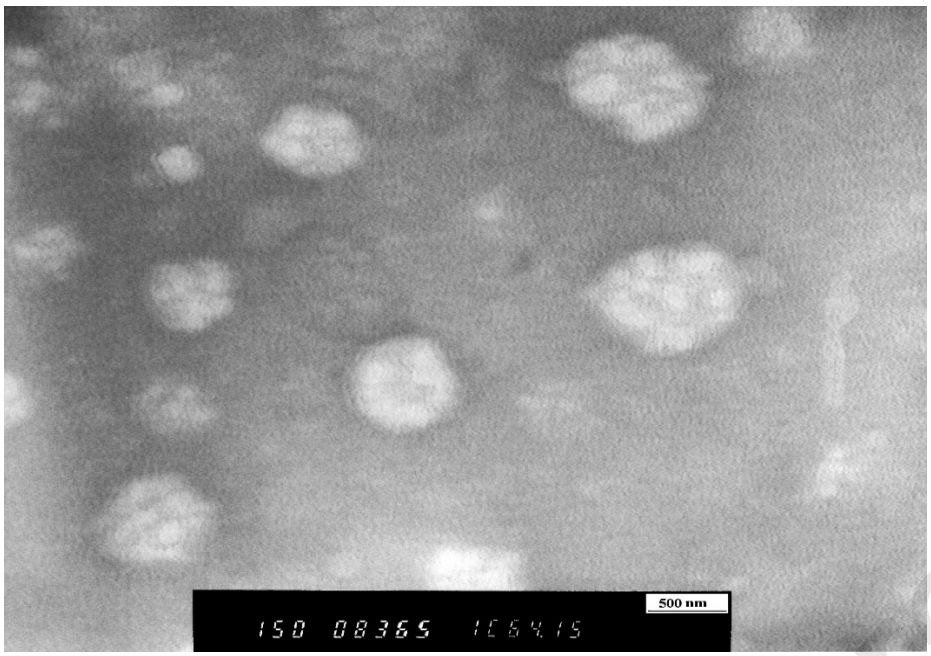

Figure 1. Morphological examination of polymeric PNCs by TEM. PEGylated-NCs were formulated by double emulsification/solvent evaporation. The images were taken by using negative staining technique.

Furthermore, the encapsulation efficiency (EE%) of the nanocapsules was examined and quantified by size exclusion chromatography, Table 1. The values of EE% were found to be between (76%-84%), which is considered high readings. The high values of EE% are attributed to the use of a copolymer with much of its structure is Ɛ-Caprolactone, which contains a long chain of the hydrocarbon backbone.

Table 1. Optimisation of the trypsin-loaded NC formulations and their physicochemical properties. (**Size (nm) not match with TEM images Need explanation in the discussion**)

ID	Trehalose (mg)	Core physical	Size (nm) [a], [b]	PDI [a]	%EE [c],[b]	Drug release (SGF)[d],[b]	Drug release (SIF)[e],[b]	BA[f]	LE%[g]
NC1	1	Liquid	552.3±61.1	0.264	83.4%±2.5	17.0%±2.1	78.2%±4.8	52.70%	3.34%±0.02
NC2	1	Solid	337.2±43.5	0.296	80.9%±4.2	16.5%±1.0	80.6%±6.8	78.96%	3.24%±0.10
NC3	10	Liquid	912.3±62.1	0.198	76.2%±3.6	12.4%±1.6	83.2%±4.3	83.02%	3.05%±0.21

NC4	10	Solid	821.4±16.7	0.288	81.8%±5.1	14.2%±1.2	82.8%±6.4	93.12%	3.27%±0.05

[a] Size and Poly Dispersity Index (PDI) were measured by dynamic light scattering in deionised water.
[b] Results are expressed as mean ± SD (n=3).
[c] % Encapsulation efficiency (%EE) of protein was determined by measuring absorbance at ƛ 21 nm in a size exclusion liquid chromatography. %EE was determined using the following equation:
%EE= (amount of encapsulated protein/ initially used protein) *100%.
[d] % of the total protein release in SGF after 4 hours.
[e] % of the total protein release in SIF after 24 hours.
[f] Biological activity was assessed by measuring the ability of encapsulated trypsin to cleave ester link in BAEE.
[g] % Loading efficiency (%LE) of nanocapsules was calculated by using the following equation:
%LE= (amount of encapsulated protein/ amount of polymer used) *100%.

3.2. *In vitro* release of trypsin under simulated oral conditions

The pattern and whole released trypsin in SGF and SIF were studied over 4 and 24 hours, respectively, Figure 2 and Table 1. The release of trypsin in SGF was less than 18% for all formulations, which is consistent with the aim of these formulations as a targeted delivery system to the small intestine. More than 80% of the entrapped protein was retained to be released in the intestines. Thus, trypsin release in SIF was also studied to investigate whether the trypsin can be released in enough amount in the small intestine or not. Drug release experiment in SIF revealed that all formulations released around 80% of encapsulated trypsin in SIF with no significant difference between the different formulations.

A biphasic release pattern of trypsin from the core of nanocapsules was reported where a dumping dose (10%-15%) was released within the first 15- 30 minutes of the study. Then, the rest of the protein was dissoluted and detected as a free protein in the surrounding medium in a sustained release way.

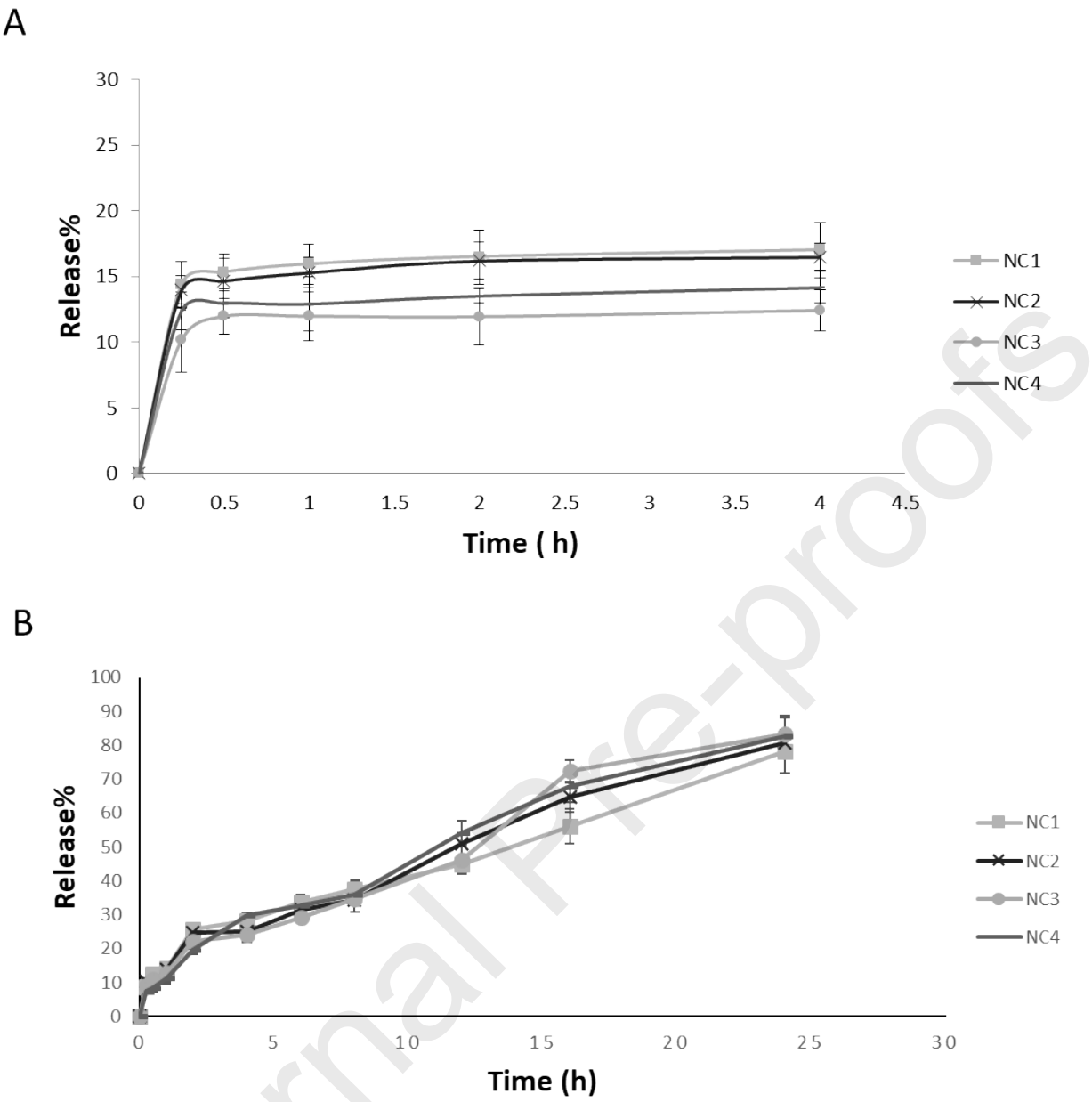

Figure 2. ***In vitro*** **release profile of trypsin from PEG-PNCs in simulated gastrointestinal fluids (without enzyme) at 37 °C.** Trypsin release from the PEGylated PNCs was determined in SGF and SIF without enzymes for 4 and 24 hours, respectively. Protein release was determined by the quantification of protein released by using SEC. All results were an average of three runs, SD is provided. **a.** The release profile of trypsin in SGF over four h. **b.** The release profile of trypsin in SIF over 24 h.

3.3. Biological activity of encapsulated trypsin

There is no doubt that exposing the protein to harsh conditions, including organic solvents, or sonication may change in its conformational stability and consequently changing in its biological activity. Thus, the biological activity of the encapsulated proteins should be investigated. Encapsulated trypsin retained up to 93% of its biological activity in some formulations, Table 1. Increasing the amount of trehalose

significantly enhanced biological activity (p-value<0.05). Also, trypsin encapsulated by s/o/w was found to be substantially more stable than the ones encapsulated by w/o/w p-value<0.05.

3.4. Storage stability of Polymeric nanocapsules

Accelerated stability study was carried out to measure the ability of trypsin to maintain its native folded and active structure within the polymeric system during the storage period; six months, at 5 °C, and 21 °C. Proteins biological activity, physical and chemical stability, and formulations' moisture content were obtained by applying enzymatic assay, SEC, and KFT, respectively. At 5°C, trypsin retained more than 92% of its original structure over six months of storage. While the biological activity was different; a wide variation between the formulations was recorded (71%- 100%), Figure 3. However, both physical and biological stability results of trypsin stored at 21 ± 3 °C RH 76% were significantly less than the ones investigated under chilled conditions, namely in formulations with less trehalose content. 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 at both conditions. Trehalose was able to protect the biological activity of trypsin 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. 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 deterioration or aggregation and vice versa. The moisture content of the dry formulation was investigated after the period of storage to understand the mechanism of degradation and protection of trypsin throughout storage. Moisture content performed by Karl Fisher titration revealed a strong correlation between the moisture content and the spoiling of the formulations, which explains the high degradation of trypsin stored under 21 °C, Table 2.

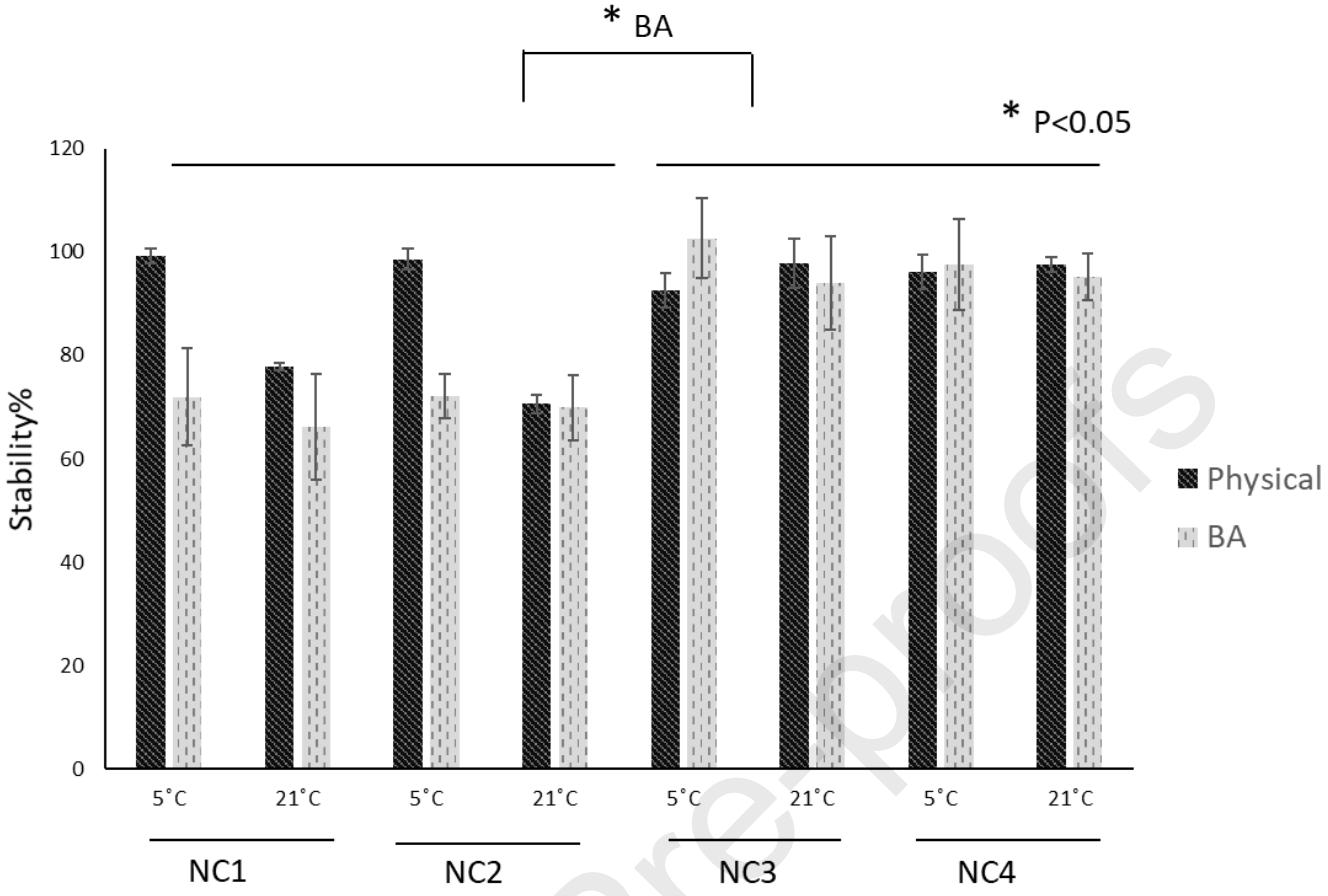

Figure 3. Six-months stability study of four trypsin-PNCs at two different storage conditions. An accelerated stability study was carried out after the storage of four formulations at 21 ˚C at 76%RH and five ˚C in a desiccator (n=3). The biological activity of trypsin was investigated after the disruption of the polymeric shell and performing the enzymatic assay of trypsin to measure its ability to cleave ester link in *N*-benzoyl-l-arginine ethyl ester (BAEE), as described by [25, 26]. Also. The physical integrity of the encapsulated trypsin was studied by measuring how intact protein was retained without any change in the molecular weight by utilising SEC by applying a previously developed and validated method in our lab.

Table 2. Moisture contents (%) of polymeric trypsin-PEGylated-NCs post-storage for six months (in a desiccator at five ˚C and 21 ˚C at76% RH), (n=3).

NC ID	% Moisture content	
	Stored in desiccator at 5 °C ± SD	**Stored in 76% RH at 21 °C ± SD**
NC1	0.89 %± 0.46	10.23 %± 1.55
NC2	2.13%± 1.02	6.33 %± 1.12
NC3	5.22%± 2.71	7.25%± 1.56
NC4	2.87 %± 1.45	10.23%± 2.86

4. Discussion

The development of polymeric nanocapsules for protein faces several challenges ranging from the reduction in the percentage of encapsulated proteins to changing the conformational stabilities and consequently losing their activities. In this study, nanocapsules were developed based on the previously reported conditions in the literature; the factors affecting particle size, EE%, release and protein stability were also considered. After careful characterisation of the four formulations and investigating the role of each formulation factors, it was revealed that trehalose plays crucial roles in determining several properties of the nanocapsules, including particle size, biological activity, and shelf life. Besides, it was concluded that dominantly polycaprolactone copolymer is able to confine a high amount of protein; however, adding PEG enhances the release.

No significant difference was reported between the four formulations in term of encapsulation efficiency; this confirms that none of the changed variables may impact the encapsulation efficiency. The high encapsulation efficiency observed in this work (~80%) is attributed to utilising a copolymer with a high content (60%) of the semicrystalline and hydrophobic monomer (ε-Caprolactone). Formulating polymeric nanocapsules with high molecular weight, hydrophobic and crystalline polyester polymer results in high encapsulation efficiency due to the low leakage of confined protein during the formulation process [28]. One of the main features of polycaprolactone is its permeability due to the abundance of pores on its surface; they are however very tiny which limits the release of large molecules, e.g. protein [29, 30]. Hence, the copolymerisation of caprolactone with other amorphous and hydrophilic monomers, e.g. lactide enhances drug release significantly [31]. Also, PEGylation is a process of conjugating the therapeutic agent or drug carriers with polyethylene glycol (PEG). PEG is a universal polymer with different molecular weights and various physicochemical properties; PEGs possess many features which make them key molecules in many dosage forms. The PEGylation of nanoparticles enhances their bioavailability, biodistribution, pharmacokinetic properties, and biocompatibility [32].

In oral polymeric drug delivery systems, PEGylation of polymeric nanoparticles enhances their drug release profile. It makes them more suitable for drug release within the time of journey of oral drugs. PEG comprises different hydrophilic functional groups which tend to soften the polymeric nanocarrier by collecting water around the particles. In this study, PEG played a crucial role in increasing the permeability of PCL by entering the surrounding aqueous media, e.g. Gastric and intestinal fluids; thus, enlarging the existing PCL pores for the improvement of more protein release [33]. The above-discussed assumption is also supported by the findings of *Ammar et al., 2018*; the release of lamotrigine from two poly (D, L-Lactide-co-Caprolactone) copolymers with 80% or 25% caprolactone content was slow. The PLC

polymeric nanocapsules needed around ten days to release 90 % of encapsulated lamotrigine; no PEGylation process took place [34]. The case is, however, different in the current study; utilising PEG as a plasticiser for the developed nanocapsules accelerated drug release where (~80%) of protein was released within 24 hours.

Furthermore, the same copolymer (poly (D, L-Lactide-co-Caprolactone)) was investigated previously at different monomer ratios, and it showed that the encapsulation efficiency reached the maximum value when the caprolactone formed more than half of the copolymer weight. The role of caprolactone refers to its hydrophobic nature as it solidifies quickly during the preparation process, which reduces the protein leakage and consequently increases the encapsulation efficiency [16]. Although the used hydrophilic polymer limited the leakage of the drug outside the NC core during the process of preparation, release profile was high in the intestinal conditions. The shown release profile was attributed to the PEGylation of the copolymer. The release profile of trypsin may have been enhanced due to the interaction between NCs with PEG, which plasticised the polymer by increasing the water content around the particles.

Trypsin release from PNCs depends on the medium of dissolution and the entire time of the experiment. Protein efflux from nanocapsules in SGF (within 4 hours of monitoring) was biphasic. The first phase was initial burst release when 10%-15% of trypsin was release within 15 – 30 minutes which may have happened due to the liberation of the adsorbed protein onto the surface of nanocapsules. Then, an equilibrium phase had taken place for the rest of the experiment time when a very slow release was reported, around more 5%. At the other side, trypsin release in SIF was also in a biphasic pattern; the adsorbed protein on the polymeric surface (10% -15%) was released with 15 – 30 minutes. Afterwards, the remaining encapsulated trypsin was liberated in a sustained release manner until the end of the release experiment. The total percentage of the released protein was around 80% within 24 hours with some variations between the formulations; however, no significant difference was found between any of these four formulations, $P > 0.1$. Drug release mechanisms are variant and depend on the materials, type of formulation, method of preparation, nature of the incorporated drugs, and/or the media of release. For polymeric nanoparticles, there are many mechanisms of release suggested to explain the pattern of drug liberation, e.g. polymer degradation or erosion, diffusion, or dissolution-controlled release. In our previous study, the mechanism of protein release from PEGylated and non-PEGylated poly (D, L lactide-co-caprolactone) nanocapsules was investigated in details, and it was concluded that main means of drug release was diffusion, as illustrated in TEM images performed before and after drug release [16]. Also, PEGylating nanocapsules enlarged the pore sizes at the surface of polymeric nanocapsules and enhanced the release of trypsin as macromolecule. The differences were observed between the release in SGF and

SIF may be attributed to different factors; firstly, the entire time of experiment in SIF is longer than its counterpart in SGF which allowed more protein to be released. Secondly, SIF media conditions may have affected the nature of the interaction between PEG and the used copolymer. The interaction may have enlarged the pores within the shell of nanocapsules, increased the SIF penetration towards the core, and consequently, enhanced trypsin release with time.

Although the role of trehalose in protecting proteins was proven in many studies and well explained, its role in stabilising proteins in nanocapsules was not well investigated. Also, most of the studies utilising extremolytes, namely, trehalose examined their effectiveness in comparison with ones without protectants. However, the quantitative investigation of trehalose in nanocapsules is not common. Here, the role of trehalose was obvious in maintaining the biological activity of trypsin either at time zero or after the storage. Increasing the amount of the used trehalose from 1 mM to 10 mM enhanced the biological activity of trypsin significantly and even when the NCs were at harsh conditions; 21 ˚C at 76%RH.

Trehalose works as an extremolyte which protects the biological molecules from degradation by a phenomenon called preferential hydration [35, 36]. 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 [37]. Trehalose can stabilise proteins by playing a “water substitutes” role during the drying state of the lyophilisation process. Freeze-drying of proteins removes the hydrogen bonds from protein hydration shell; hence, increasing protein unfolding rate. However, the existence of a sufficient amount of trehalose can maintain hydrogen bonding with protein by linking thee hydroxyl group of sugar to the surface of proteins to reduce unfolding and denaturation of protein [38]. Moreover, trehalose affects the surrounding environment in the protein solution by increasing hydration around the protein molecules and minimising the mobility of the molecules, which stabilises the protein indirectly [39]. On the other hand, trehalose stabilises proteins in solid or lyophilised state 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 to, eventually, restrict protein unfolding and denaturation [40, 41], [42].

Encapsulating trypsin in the solid-state (s/o/w) also protected it from the degradation by lowering the rate of hydrolysis by avoiding the cleavage of amide bonds in the protein structure due to the zero-energy contained in the system [37, 43, 44]. Considering the physical stability of encapsulated trypsin performed by SEC reveals that even when a small amount of trehalose was used, trypsin was able to retain its conformational stability and was protected from the aggregation processes. The obtained stability results conclude that the reduction of biological activity was due to chemical changing in the active site of the protein without any changing in the particle size of the protein itself. 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. Storing the formulations at 21 ˚C and 76% RH increased the exposure of the encapsulated protein to the surrounding moisture as confirmed by the moisture content results, and consequently reduced the protein stability. 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 [45] [46].

5. Conclusion

Many studies were conducted to deliver oral trypsin in conventional dosage forms. In this study, we encapsulated trypsin in polymeric nanocapsules to attain sustained release pattern. The results revealed the impacts of the different formulation attributes, which affected the quality characteristics of the polymeric nanocapsule formulations. The PEGylated poly (D, L-Lactide-co-Caprolactone) nanocapsules were prepared by the double emulsion solvent evaporation method. High encapsulation efficiency and high release profile in SIF were reported with a tiny percentage of released trypsin in SGF; this agrees with the aim of this work of developing targeted PNCs to deliver trypsin to the small intestine. The formulated PNCs protected trypsin from the harsh conditions and enhanced the biological activity of trypsin by encapsulating more trehalose with the protein. Accelerated stability study concluded that trypsin had retained its activity and physical stability over the entire storage time when trehalose amount was high. Also, it was shown how the humidity of the storage place plays a crucial role in the stabilising or destabilising the encapsulated trypsin. This study increased the understanding of the role of different

material attributes, process parameters and storage conditions in affecting the expected therapeutic outcome of the delivered protein.

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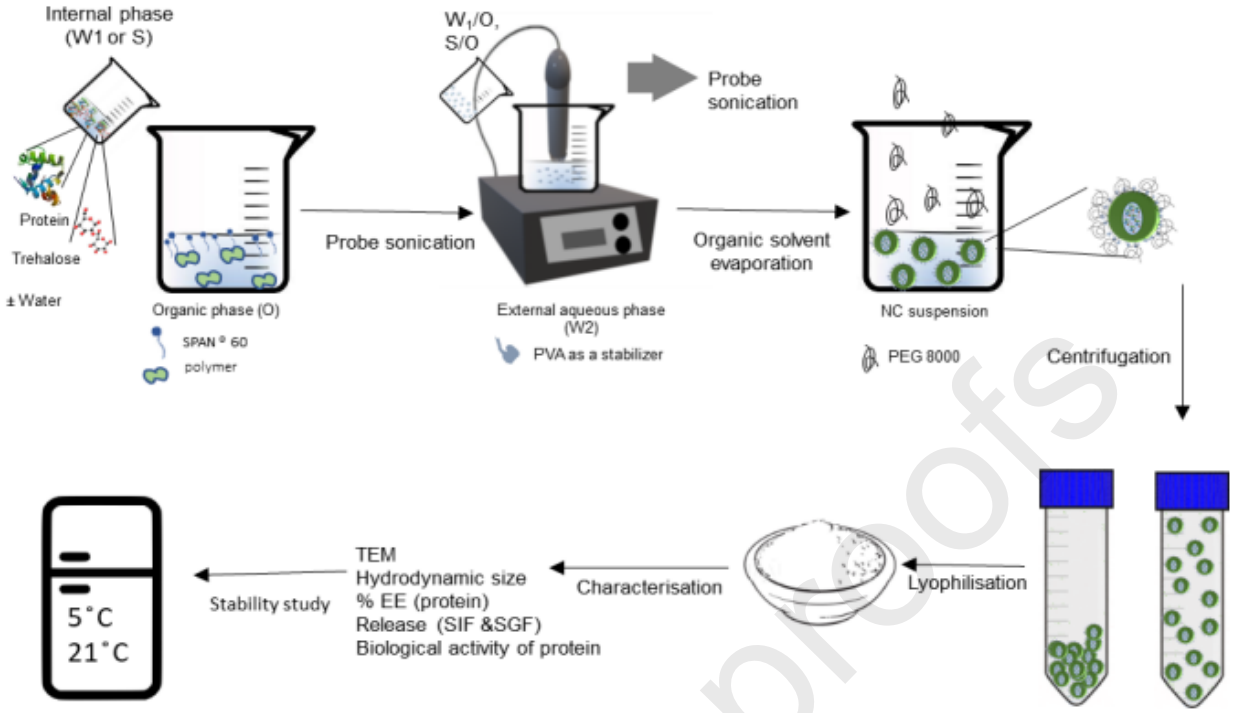

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Omar S. Abu Abed: Conceptualization, Methodology, Writing- Original draft preparation, Investigation, Writing- Reviewing and Editing, Visualization, Formal analysis , Validation, and Resources

Lee Williams: Supervision, Validation, Methodology,

Cheng Chaw: Supervision, Methodology,

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