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Article

Challenges in estimating the encapsulation efficiency of proteins in polymersomes – Which is the best method?

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Polymersomes are nanometric vesicles that can encapsulate large and hydrophilic biomolecules, such as proteins, in the aqueous core. Data in literature show large variation in encapsulation efficiency (%EE) values depending on the method used for calculation. We investigated different approaches (direct and indirect) to quantify the %EE of different proteins (catalase, bovine serum albumin-BSA, L-asparaginase and lysozyme) in Pluronic L-121 polymersomes. Direct methods allow quantification of the actual payload of the polymersomes and indirect methods are based on the quantification of the remaining non-encapsulated protein. The protein-loaded polymersomes produced presented approximately 152 nm of diameter (PDI ~ 0.4). Higher %EE values were obtained with the indirect method (up to 25%), attributed to partial entanglement of free protein in the polymersomes poly(Ethylene Glycol) corona. For the direct methods, vesicles were disrupted with chloroform or proteins precipitated with solvents. Reasonable agreement was found between the two protocols, with values up to 8%, 6%, 17.6% and 0.9% for catalase, BSA, L-asparaginase and lysozyme, respectively. We believe direct determination is the best alternative to quantify the %EE and the combination of both protocols would make results more reliable. Finally, no clear correlation was observed between protein size and encapsulation efficiency.

Keywords: Polymersomes. Encapsulation efficiency. Biomolecules encapsulation. Poloxamer. Vesicle disruption.

INTRODUCTION

In recent years, several nanostructures have been investigated as potential drug delivery systems (Mitchell *et al.*, 2020). Indeed, the use of this technology gains special relevance when it comes to the formulation and administration of biopharmaceuticals such as protein drugs. It helps to overcome the challenges of high molecular weight and hydrophilic nature of macromolecules that usually results in degradation and poor penetration/absorption. In addition, nanoencapsulation can also contribute to lower the immunogenicity usually associated to protein drugs (Mitchell *et al.*, 2020), improve protein stability and *in vivo* distribution, promote solubility, prolong blood circulation time, allow controlled and targeted release and protect from biodegradation by plasma enzymes, thus increasing treatment effectiveness and decreasing side effects. Among the main nanocarriers investigated for this purpose we highlight self-aggregated systems such as liposomes, cubosomes, hexosomes and polymersomes (Gao, Wang, 2023).

Liposomes are phospholipid vesicles of one or more concentric lipid bilayers surrounding an aqueous compartment. They are one of the first nanostructures used for medical purposes owing to safety and biocompatibility (Gao, Wang, 2023). Cubosomes and hexosomes are nanostructured liquid crystalline systems that can encapsulate both hydrophobic and hydrophilic substances and have a high tolerance to environmental stresses and potential for controlled release (Tan, Hosseini, Jafari,

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2022). Nonetheless, all these nanostructures present lipids in their composition, therefore are susceptible to oxidation and microbial degradation.

Polymersomes, on the other hand, are nanometric vesicles formed by the self-aggregation of amphiphilic copolymers in contact with an aqueous medium. Owing to the polymeric composition, they present high physicochemical stability and mechanical strength when compared to other self-aggregated nanostructures (Kansız, Elçin, 2023). In addition, polymersomes offer a great opportunity to encapsulate large and hydrophilic biomolecules in the aqueous core (Gao, Wang, 2023; Kansız, Elçin, 2023). Several research groups are focused on the study of these nanocarriers, mainly on novel materials, systems composition, preparation techniques and strategies for controlled release (Mitchell *et al.*, 2020).

Usually, the encapsulation of biomolecules in uncharged (nonionic) polymersomes is a physical process in which an aqueous volume of the biomolecule solution is encompassed by the polymeric membrane during self-aggregation. Therefore, similar encapsulation rates are expected for biomolecules when no other interaction is present, such as electrostatic or affinity ones (Farzin *et al.*, 2023). In this sense, encapsulation efficiency (%EE) would be influenced by biomolecule concentration in solution and probably biomolecule size. However, data in literature shows large variation in protein encapsulation efficiency values depending on the method used for its calculation (Apolinário *et al.*, 2018, 2019; Blackman *et al.*, 2018; Oliveira *et al.*, 2020). This parameter is extremely important, among others, for dose estimation. Also, the differences observed in literature impair comparisons among the systems proposed and a proper understanding of their therapeutic potential. Both direct and indirect methods can be used to determine %EE. Direct methods allow quantification of the polymersomes actual payload (protein mass) and techniques such as spectroscopy (Fourier-transform infrared spectroscopy-FT-IR, Raman, Ultraviolet–visible) or immunoassays are used (Goyal *et al.*, 2021). The polymersomes must be disrupted to release the payload. Indirect methods, on the other hand, are based on the quantification of the remaining non-encapsulated protein. The %EE is then calculated by subtracting the non-encapsulated protein mass from the initial protein mass used to prepare the system (Apolinário *et al.*, 2018; de Souza Guimarães *et al.*, 2022).

In this work, we investigated different direct and indirect approaches to quantify the %EE of proteins in Pluronic L-121 polymersomes and found that direct determination of %EE provides a more reliable value than indirect determination, which usually overestimates this parameter. This is an important information giving the number of papers reporting %EE values calculated based on indirect method. Globular proteins of different sizes and charges were studied to demonstrate that, for this class of biomolecules, no significant correlation between size and %EE is observed.

MATERIAL AND METHODS

Material

L-asparaginase (ASNase, 140 kDa, pI = 5.5) was purchased from ProSpec Tany® (Ness-Ziona, Israel); egg white lysozyme (LYS, 14.5 kDa, pI = 10.7), bovine catalase (CAT, 240 kDa, pI = 5.4) and bovine serum albumin (BSA, 55.5 kDa, pI = 4.7) were from Sigma-Aldrich (Saint Louis, MO, United States). The Pluronic® L-121 (PEG_5-PPO_{68}-PEG_5) was kindly donated by Basf SE. All other reagents were purchased from Sigma Aldrich (Sigma Aldrich Co., Saint Louis, MO, United States): Sepharose 4B, chloroform and Bicinchoninic Acid Kit Assay. All materials were used as received, without any further purification. Purified water was used in all experiments (Milli-Q plus 185 water purification apparatus, Millipore/Merck, Darmstadt, Germany).

Polymersomes preparation and purification

Polymersomes systems nanoencapsulating different proteins (ASNase, CAT, BSA and LYS) were prepared to evaluate the influence of size (molar mass) and charge on encapsulation efficiency. Pluronic L-121 (5 mg/mL) and the protein (2 mg/mL) were added to 1X phosphate-saline buffer pH 7.4 (PBS) and magnetically stirred at 400 rpm, 4°C for 1 h for complete dissolution of the copolymer. To produce the polymersomes, the system was kept overnight under magnetic stirring at 800 rpm, room temperature.

Finally, the systems were centrifuged (1,000 x *g*, 5 minutes) to remove any residual bulky polymer. Blank polymersomes were also prepared, *i.e.* without any loaded protein. All experiments were performed in triplicate.

Protein-loaded polymersomes were purified by size exclusion chromatography (SEC) with Sepharose 4B used as the stationary phase in a 16 mL glass column. Each sample was placed on top of the column and the elution was performed with 1X PBS buffer pH 7.4. The eluted fractions were collected in a 96-well microplate (Corning UV Plate 96 Well, Flat Bottom) and absorbance read at $\lambda = 600$ nm in a Spectra Max M2 spectrophotometer (Molecular Devices, CA, United States). Fractions presenting absorbance values higher than 0.1 were collected and combined to result in the final polymersomes system.

Polymersomes size and zeta potential determination

The nanostructures produced were evaluated by dynamic light scattering (DLS) to determine size and polydispersity index (PDI) using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK) operating with a 633 nm He-Ne laser and the detector set at an angle of 173°. Samples were previously diluted 10X in PBS pH 7.4 filtered (Whatman Uniflo 30mm Syringe Filter 0.2um PES w/GF Prefilter) and transferred to polystyrene cuvettes (Malvern, DTS0012). Measurements were taken in triplicate at count rates from 100 to 200 kcps (counts per second x 10^3) and results presented as the average hydrodynamic diameter based on the diffusion coefficient, using the Stokes-Einstein equation (Apolinário *et al.*, 2019). Additionally, the zeta potential (z) of the empty polymersomes was determined by capillary electrophoresis using the same Zetasizer Nano ZS instrument. Samples were diluted five times in PBS and three cycles of measurements were conducted for each analysis.

Determination of total protein concentration

Two methods were employed to quantify the total protein concentration and calculate the encapsulation efficiency, namely the BCA and absorbance reading at $\lambda = 280$ nm.

Bicinchoninic Acid Method (Micro BCA)

The QuantiPro BCA (Sigma-Aldrich kit, St Louis, MO, USA) was used following the manufacturer's protocol with a calibration curve for each assay based on bovine serum albumin standard diluted in 1X phosphate-saline buffer pH 7.4, at a concentration range from 0.0 to 40.0 µg/mL. The QuantiPro working reagent was prepared by mixing 25 parts of the QA reagent (sodium carbonate, sodium tartrate and sodium bicarbonate in 0.2 M sodium hydroxide, pH 11.25) with 25 parts of the QB reagent (a solution of bicinchoninic acid at 4% (w/v), pH 8.5) and once the mixture was homogenized, the QC reagent (4% solution (w/v) of copper (II) sulfate pentahydrate) was added. Subsequently, 150 µL of each sample was mixed with 150 µL of QuantiPro working reagent and incubated at 37°C for 2 hours. All tests were performed in triplicate. Absorbance reading was performed at $\lambda = 562$ nm in a Spectra Max M2 spectrophotometer (Molecular devices).

Absorbance reading at λ = 280 nm

Samples of 100 µL were transferred to a quartz cuvette and the absorbance read at $\lambda = 280$ nm in a spectrophotometer Spectra Max M2 (Molecular devices, CA, United States). The protein concentration in mg/mL was calculated based on the molar extinction coefficient for each protein according to Equation 1. The molecular mass data and molar extinction coefficients were estimated using ProtParam tool from Expasy and the following values were obtained: bovine serum albumin MM = 66432.96 Da and $\varepsilon_{molar} = 42925\ M^{-1}\ cm^{-1}$, catalase MM = 59784.10 Da and $\varepsilon_{molar} = 64540\ M^{-1}\ cm^{-1}$, L-asparaginase MM = 34593.94 Da and $\varepsilon_{molar} = 23505\ M^{-1}\ cm^{-1}$, lysozyme MM = 14313.14 Da and $\varepsilon_{molar} = 37970\ M^{-1}\ cm^{-1}$.

$$\text{Protein concentration (g/L)} = \frac{Abs_{280} \times MW}{\varepsilon_{molar} \times l} \quad \textbf{(Eq. 1)}$$

in which Abs_{280} is the absorbance measured at $\lambda = 280$ nm, MW is the molar mass of the monomer (Da), ε_{molar} is the molar extinction coefficient ($M^{-1}\ cm^{-1}$) and l is the length of the path (cm).

Determination of the Encapsulation Efficiency (%EE)

Different approaches were used to determine the encapsulation efficiency, as depicted in Figure 1 and detailed below.

Indirect Method

After purification of the polymersomes systems by SEC, the fractions corresponding to the free protein were identified by reading the absorbance at λ = 280 nm. Following, the fractions were combined and the total protein mass determined based on the absorbance at λ = 280 nm (Section 2.4.2). The mass of encapsulated protein was obtained subtracting the mass of non-encapsulated protein ($P_{non\text{-}encapsulated}$) from the total mass of protein added to the system (P_{total}), and %EE was calculated according to Equation 2. Assays were performed in triplicate.

$$\%EE = \frac{(P_{total} - P_{non-encapsulated})}{P_{total}} \times 100 \qquad \textbf{(Eq. 2)}$$

Direct Method by polymersomes disruption with chloroform

Samples of 500 µL of purified polymersomes were mixed with equal volumes of chloroform to disrupt the vesicles. The mixture was subjected to centrifugation at 4°C, 2,655 x *g* for 90 minutes and subsequently the total protein concentration of the aqueous phase was determined by both Micro-BCA and absorbance reading at 280 nm. The values obtained were used to estimate the total protein mass encapsulated ($P_{encapsulated}$) and the %EE was calculated based on Equation 3 (Oliveira *et al.*, 2020). Assays were performed in triplicate.

$$\%EE = \frac{P_{encapsulated}}{P_{total}} \times 100 \qquad \textbf{(Eq. 3)}$$

Direct Method by encapsulated protein quantification after precipitation in organic solvents

After the purification process, the total volume of the polymersomes system was dried using a Concentrator PLUS (Eppendorf), at 30°C, V-AQ mode (vacuum-aqueous). Next, the dried samples were resuspended in a mixture of chloroform:methanol (1:1) and left at 4°C for 2 hours for complete dissolution of the polymer and precipitation of the protein. The samples were centrifuged at 15,000 x g for 20 minutes and the supernatant containing the dissolved polymer was discarded. Another wash of the precipitated proteins with the same mixture of organic solvents was carried out followed by centrifugation under the same conditions (15,000 x *g* for 20 minutes). The precipitated proteins were then dissolved in 1X phosphate saline buffer pH 7.4 (Amini *et al.*, 2017; Alanezi, Neau, D'mello, 2020) and the total protein concentration determined by absorbance reading at λ = 280 nm in a Spectra Max M2 spectrophotometer (Molecular devices). The %EE was calculated based on Equation 3 and assays were performed in triplicate.

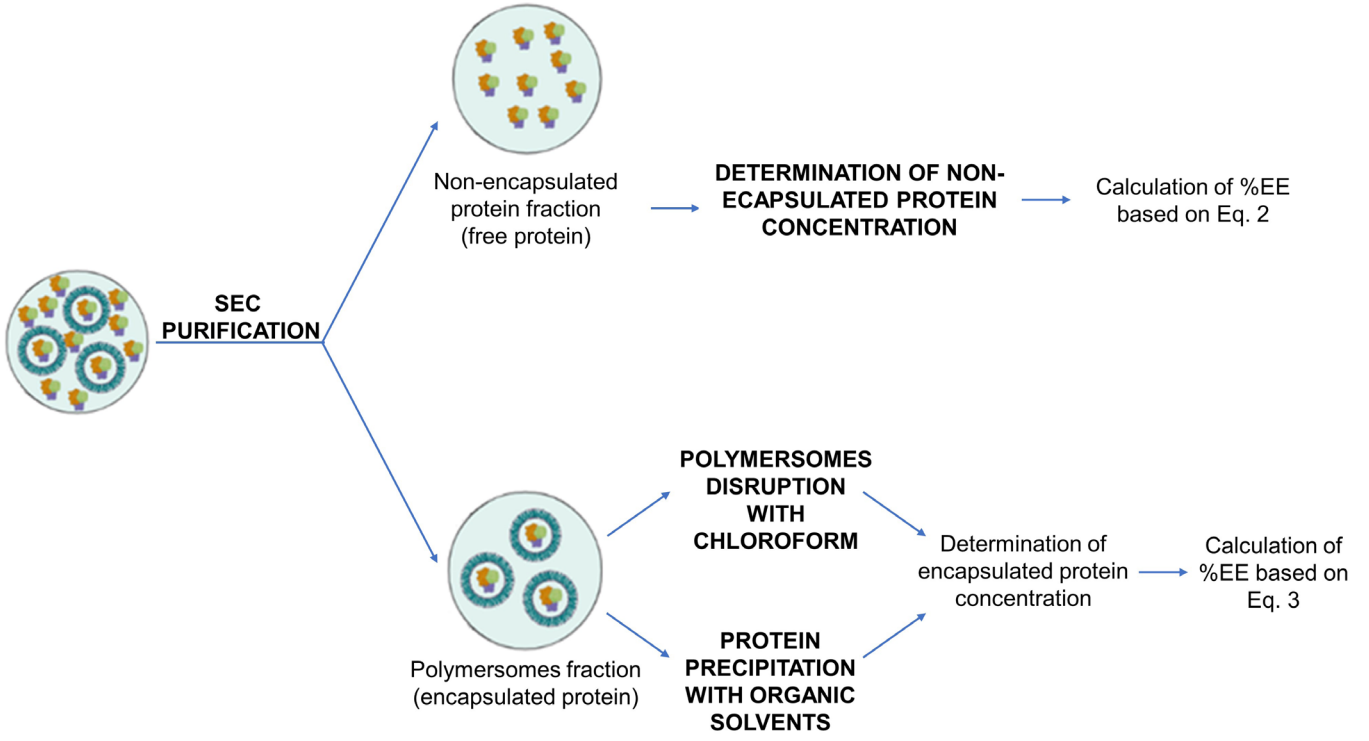

FIGURE 1 – Representative scheme of the different approaches (indirect and direct methods) used to determine the encapsulation efficiency (%EE) after purification by SEC (size exclusion chromatography).

RESULTS AND DISCUSSION

Polymersomes preparation and characterization

The DLS curves (Supplementary material) show that all the systems presented expected size, corresponding to Pluronic L-121 polymeric vesicles (Apolinário *et al.*, 2019; Oliveira *et al.*, 2020). Table I presents the summary of the hydrodynamic diameter (H_D) and polydispersity index (PDI) values obtained. As can be seen, overall the polymeric vesicles presented sizes around 130 to 190 nm and PDI values between 0.2 and 0.5. It is important to stress that our goal here is to investigate encapsulation efficiency and therefore systems were not extruded to adjust size and lower PDI, what explains the high PDI values observed in the presence of some of the proteins. Besides, it is relevant to mention that the PDI is not related to %EE, but only to stability and consequently to future applications of the system (Dimov *et al.*, 2017). In addition, we must consider that the cumulant method used to analyze DLS data and determine the average particle size and particle size distribution is based on the assumption that only one particle population is present (monomodal) and a gaussian distribution is expected for the particle size. Samples presenting even very low number of larger molecules (what might happen for polymersomes) might result in larger PDI values that does not actually reflect the variation on the mean particle size distribution (Stetefeld, McKenna, Patel, 2016).

TABLE I - Characterization of Pluronic L-121 polymersomes nanoencapsulating different proteins, as well as the empty vesicles (blank PL), (values presented as mean ± standard deviation)

Protein	Hydrodynamic diameter - Dh (nm)	Polydispersity index (PDI)
Catalase	181 ± 9	0.332 ± 0.027
BSA	138 ± 0.3	0.498 ± 0.021
L-asparaginase	141 ± 7	0.243 ± 0.020
Lysozyme	152 ± 15	0.457 ± 0.027
Blank PL	150 ± 16	0.474 ± 0.017

Protein encapsulation did not result in significant changes in the polymersomes size, which suggests a lack of interaction between the proteins and Pluronic L-121 during the vesicles formation. In other words, protein nanoencapsulation is understood as a passive process in which a certain volume of the protein solution is incorporated inside the forming vesicles during the polymer auto-aggregation process (Farzin *et al.*, 2023). Under this premise, we measured the z-potential of the empty vesicles and a value of - 4.95 ± 0.22 mV was obtained, corresponding to approximately neutral vesicles, what we attributed to the PEG corona of the Pluronic L-121 polymersomes. This result also suggests there would be no significant electrostatic interactions between the polymer and the proteins, regardless of whether they are cationic or anionic. However, a discrete increase in the hydrodynamic diameter was observed for the catalase formulation (180 nm), which may be related to a possible interaction and entrapment of this large protein (240 kDa) in the PEO corona of the vesicles. This finding has already been discussed in previous works using Pluronic L-121 vesicles (Apolinário *et al.*, 2019; Oliveira *et al.*, 2020).

Determination of encapsulation efficiency

Table II presents the %EE results for all the strategies evaluated. As mentioned in the materials and methods section, after disrupting the systems with chloroform, the protein concentration was determined both by Micro-BCA and absorbance reading at λ = 280 nm. However, since protein concentration was very low owing to the dilution of the system, apparently the Micro-BCA was not sensitive enough to detect protein, especially considering the interference of the blank. Unexpected absorbance values were detected after performing Micro BCA on blank polymersomes samples (without protein) submitted to disruption with chloroform, suggesting that the polymer concentration in the aqueous phase is considerably high enough to interfere with protein quantification (data not shown). This phenomenon was also observed by Hussain, Forbes, Perrie, (2019) when quantifying encapsulated proteins in liposomes; in this case lipids in the aqueous phase interfered with the assay. Therefore, results for %EE after polymersomes disruption with chloroform (Table II) are based on protein quantification by absorbance reading at λ = 280 nm. It is important to mention that total disruption of the polymersomes in the presence of chloroform was confirmed by DLS measurements. The scattering profile showed the absence of vesicles in the aqueous fraction, confirming complete rupture and, consequently, release of the protein payload. Regarding a possible denaturing effect of chloroform on the proteins, it is usually associated with time of contact, concentration of solvent and miscibility with water in mixtures. Here, the systems were immediately centrifugated after vortexing the chloroform/water biphasic system formed. In addition, immiscible solvents such as chloroform tend to cause less denaturation than soluble ones in mixture with water (Asakura, Adachi, Schwartz, 1978). Therefore, we are confident no significant protein denaturation took place.

TABLE II - Encapsulation efficiency for the different proteins encapsulated in Pluronic L-121 polymersomes based on different indirect and direct methods

Protein	%EE (Mean ± SD)		
	Indirect Method	Direct Methods	
		Polymersomes disruption with chloroform	Protein precipitation with organic solvents
Catalase	15 ± 4	6 ± 2	8 ± 2
BSA	8 ± 5	9 ± 3	6 ± 3
L-asparaginase	22.7 ± 5	17.6 ± 0.4	13.8 ± 0.0
Lysozyme	25 ± 9	0.5 ± 0.2	0.9 ± 0.2

As can be seen from Table II, differences were observed between direct and indirect determination of %EE. In general, higher values were obtained with the indirect method, suggesting an overestimation of the %EE. In our previous work Apolinário *et al.* (2019), Pluronic L-121 polymersomes were used to encapsulate L-asparaginase and %EE ~ 69% was obtained based on the indirect method by centrifugation. In contrast, %EE = 4.9% was calculated by direct method using vesicle disruption with 2% Triton X-100 and protein quantification by BCA. Other reported values for L-asparaginase encapsulation in poly(ethylene glycol)–poly(lactic acid) (PEG-PLA) and poly(ethylene glycol)-poly(ε-caprolactone) (PEG-PCL) polymersomes were approximately 20% and 23%, respectively, also using the indirect method (Apolinário *et al.*, 2018; Pachioni- Vasconcelos *et al.*, 2020). demonstrating an overestimation when compared to the results obtained by direct method. Similarly, in another work Pluronic L-121 polymersomes were used for catalase encapsulation and the indirect method by ultracentrifugation provided %EE values 13 to 15 fold higher compared to the direct method, reaching up to 70% for the indirect method (Oliveira *et al.*, 2020). Finally, %EE ~ 25% were reported for BSA encapsulated in PEG-PCL polymersomes based on the indirect method (Pachioni-Vasconcelos *et al.*, 2020). Another similar value (24%) was obtained for BSA encapsulated in PMPC-PDPA polymersomes by indirect quantification of the non-encapsulated protein after purification by size exclusion chromatography (Wang *et al.*, 2012).

Regarding the direct determination of %EE, reasonable agreement was found between the two methods, *i.e.* vesicle disruption with chloroform and protein precipitation with solvents. Therefore, we believe the two methods would be appropriate and the combination of both would provide more accuracy to the results. It is also important to note that no clear correlation was observed between protein size and encapsulation efficiency. In fact, the smallest protein (lysozyme) presented the lowest %EE value, while for the other proteins differences were smaller. If we consider encapsulation in non-charged vesicles as a physic process with no significant chemical interactions between polymer and proteins, as we described earlier, encapsulation should be proportional to protein concentration in solution and similar %EE values would be expected for all the proteins investigated. Even if interactions were present, higher values would be expected since lysozyme presents a positive net charge at pH 7.4 (pI = 10.7) and the vesicles are neutral to slightly negative. This hypothesis does not explain the low encapsulation efficiency observed. One explanation could be the widely reported lysozyme propensity to self-aggregation, both in unsaturated and supersaturated solutions (Price, Tsuchiya, Arata, 1999). Therefore, it is important to consider factors that modify protein solubility and aggregation like pH, temperature, ionic strength and protein concentration. An alternative

to lower protein aggregation would be the addition of osmolytes. It has been earlier reported in a study of L-asparaginase formulation that osmolytes like sucrose and sorbitol reduce protein aggregation (Wlodarczyk *et al.*, 2019). In this sense, osmolytes allow working with higher protein concentrations/molar ratios and increase encapsulation efficiency, probably resulting in better therapeutic profiles. Protein:liposome molar ratios of 2000 for example resulted in higher internalization rate of β-galactosidase in HeLa cells *in vitro* than molar ratios of 1000 and 1500 owing to the higher protein mass loaded in the vesicles (Chatin *et al.*, 2015).

Here we compared indirect and direct methods to calculate encapsulation efficiency of proteins in polymersomes and significant differences were observed between them. Direct methods were found to be more accurate and reliable than the indirect method, which has high variability and usually overestimates %EE. We highlight the importance of considering possible overestimation of this parameter when using data from the literature on protein nanoencapsulation in polymersomes and liposomes, since indirect determination is the most used method. Also, we advise the use of direct methods and, if possible, more than one. The results presented in this work will serve as a guide for further investigations of biomolecules encapsulation in nanocarriers as well as the interpretation of encapsulation values previously reported in the literature.

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REFERENCES

Alanezi AA, Neau SH, D'mello AP. Development and Application of a Modified Method to Determine the Encapsulation Efficiency of Proteins in Polymer Matrices. AAPS PharmSciTech. 2020 Oct;21(7):1–8.

Amini Y, Amel Jamehdar S, Sadri K, Zare S, Musavi D, Tafaghodi M. Different methods to determine the encapsulation efficiency of protein in PLGA nanoparticles. Biomed Mater Eng. 2017;28(6):613–20.

Apolinário A, Magoń M, Pessoa Jr. A, Rangel-Yagui C. Challenges for the Self-Assembly of Poly(Ethylene Glycol)–Poly(Lactic Acid) (PEG-PLA) into Polymersomes: Beyond the Theoretical Paradigms. Nanomaterials [Internet]. 2018 May 26 [cited 2020 Jul 4];8(6):373. Available from: http://www.mdpi.com/2079-4991/8/6/373

Apolinário AC, Ferraro RB, de Oliveira CA, Pessoa A, de Oliveira Rangel-Yagui C. Quality-by-Design Approach for Biological API Encapsulation into Polymersomes Using "Off-the-Shelf" Materials: a Study on L-Asparaginase. AAPS PharmSciTech. 2019;20(6):1–12.

Asakura T, Adachi K, Schwartz E. Stabilizing effect of various organic solvents on protein. Journal of Biological Chemistry. 1978 Sep 25;253(18):6423–5.

Blackman LD, Varlas S, Arno MC, Houston ZH, Fletcher NL, Thurecht KJ, et al. Confinement of Therapeutic Enzymes in Selectively Permeable Polymer Vesicles by Polymerization-Induced Self-Assembly (PISA) Reduces Antibody Binding and Proteolytic Susceptibility. ACS Cent Sci [Internet]. 2018 Jun 27 [cited 2023 Mar 22];4(6):718–23. Available from: https://pubs.acs.org/doi/full/10.1021/acscentsci.8b00168

Chatin B, Mével M, Devallière J, Dallet L, Haudebourg T, Peuziat P, et al. Liposome-based Formulation for Intracellular Delivery of Functional Proteins. Mol Ther Nucleic Acids. 2015 Jun 23;4:e244.

Dimov N, Kastner E, Hussain M, Perrie Y, Szita N. Formation and purification of tailored liposomes for drug delivery using a module-based micro continuous-flow system. Scientific Reports 2017;7:1 [Internet]. 2017 Sep 21 [cited 2023 Mar 30];7(1):1–13. Available from: https://www.nature.com/articles/s41598-017-11533-1

Farzin A, Beheshtizadeh N, Vaez A, Siminzar P, Varzandeh M, Azami M. Encapsulation: Controlled drug delivery. Principles of Biomaterials Encapsulation: Volume 2. 2023 Jan 1;2:149–200.

Gao X, Wang M. Nanoparticles for intracellular protein delivery. Encyclopedia of Nanomaterials. 2023 Jan 1;590–603.

Goyal AS, Salvador C, Mathe C, Candeias A, Caldeira AT. Optimization of protein extraction and ELISA immunodetection from protein-based paint models with mesoporous silica nanoparticles and MCM41. The European Physical Journal Plus 2021 136:6 [Internet]. 2021 Jun 25

[cited 2023 Mar 15];136(6):1–16. Available from: https://link.springer.com/article/10.1140/epjp/s13360-021-01628-0

Hussain MT, Forbes N, Perrie Y. Comparative Analysis of Protein Quantification Methods for the Rapid Determination of Protein Loading in Liposomal Formulations. Pharmaceutics. 2019 Jan 1;11(1).

Kansız S, Elçin YM. Advanced liposome and polymersome-based drug delivery systems: Considerations for physicochemical properties, targeting strategies and stimuli-sensitive approaches. Adv Colloid Interface Sci. 2023 Jul 1;317:102930.

Mitchell MJ, Billingsley MM, Haley RM, Wechsler ME, Peppas NA, Langer R. Engineering precision nanoparticles for drug delivery. Nature Reviews Drug Discovery 2020 20:2. 2020 Dec 4;20(2):101–24.

Oliveira CA, Forster C, Léo P, Rangel-Yagui C. Development of triblock polymersomes for catalase delivery based on quality by design environment. J Dispers Sci Technol [Internet]. 2020;0(0):1–10. Available from: https://doi.org/10.1080/01932691.2020.1823232

Pachioni-Vasconcelos J de A, Apolinário AC, Lopes AM, Pessoa A, Barbosa LRS, Rangel-Yagui C de O. Compartmentalization of therapeutic proteins into semi-crystalline PEG-PCL polymersomes. https://doi.org/101080/1539445X20201812643 [Internet]. 2020 [cited 2022 Apr 2];19(2):222–30. Available from: https://www.tandfonline.com/doi/abs/10.1080/1539445X.2020.1812643

Price WS, Tsuchiya F, Arata Y. Lysozyme Aggregation and Solution Properties Studied Using PGSE NMR Diffusion Measurements. J Am Chem Soc. 1999 Dec 15;121(49):11503–12.

de Souza Guimarães M, Cachumba JJM, Bueno CZ, Torres-Obreque KM, Lara GVR, Monteiro G, et al. Peg-Grafted Liposomes for L-Asparaginase Encapsulation. Pharmaceutics [Internet]. 2022 Sep 1 [cited 2023 Mar 22];14(9):1819. Available from: https://www.mdpi.com/1999-4923/14/9/1819/htm

Stetefeld J, McKenna SA, Patel TR. Dynamic light scattering: a practical guide and applications in biomedical sciences. Biophys Rev [Internet]. 2016 Dec 1 [cited 2023 May 31];8(4):409–27. Available from: https://pubmed.ncbi.nlm.nih.gov/28510011/

Tan C, Hosseini SF, Jafari SM. Cubosomes and Hexosomes as Novel Nanocarriers for Bioactive Compounds. J Agric Food Chem [Internet]. 2022 Feb 9 [cited 2023 Aug 22];70(5):1423–37. Available from: https://pubs.acs.org/doi/abs/10.1021/acs.jafc.1c06747

Wang L, Chierico L, Little D, Patikarnmonthon N, Yang Z, Azzouz M, et al. Encapsulation of biomacromolecules within polymersomes by electroporation. Angew Chem Int Ed Engl [Internet]. 2012 Oct 29 [cited 2023 Mar 26];51(44):11122–5. Available from: https://pubmed.ncbi.nlm.nih.gov/23023772/

Wlodarczyk SR, Costa-Silva TA, Pessoa-Jr A, Madeira P, Monteiro G. Effect of osmolytes on the activity of anti-cancer enzyme L-Asparaginase II from Erwinia chrysanthemi. Process Biochemistry. 2019 Jun 1;81:123–31.

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