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Improving peptide quantification in chitosan nanoparticles

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ABSTRACT

The objective of the present study was to evaluate different methodologies for peptide quantification in the supernatant of chitosan nanoparticles by removing the unliked polymer in the suspension. The ionic gelation method was used to prepare the chitosan nanoparticles encapsulating a 5.3 kDa peptide. Three different methodologies for the processment of the solutions were compared before subjecting the samples to the Bradford protocol or Qubit® kit for protein detection. For the quantification, it was necessary to create a standard peptide curve using different peptide concentrations. The suitable standard curve would be one in which the peptide was diluted in the empty chitosan supernatant (obtained after nanoparticles centrifugation) or in the filtrate of the empty chitosan supernatant. Our results indicated the safest methodology tested for peptide quantification in the supernatant chitosan nanoparticles was filtering the solution before subjecting the sample to the Bradford assay.

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1. Introduction

Today, nanotechnology is found in a wide range of applications [1–4] and allows the manipulation of material creating structures in a small dimension. Nanoparticles around 300 nm are suitable to incorporate drugs or bioactive molecules improving drug delivery formulation, which allows a diverse range of applications in the pharmaceutical field [5]. Polymeric nanoparticles are promising to achieve this goal due to its capacity to promote drug sustained release and the possibility to direct the drug to reach specific sites in the body [6,7]. Chitosan is a good example of a natural polymer used to prepare these nanoparticles since it presents characteristics such as hydrophilicity, biocompatibility, biodegradability and low toxicity [8].

Chitosan is formed by monosaccharide chains linked by glycosidic bonds with polymer amino groups, enabling the formation of chemical complexes with both drugs and biological tissues [9]. The chemical properties of chitosan [α (1 → 4) 2-amino 2-deoxy β-D glucan] are determined by the molecular weight, degree of deacetylation and viscosity [10]. The molecular weight of this polymer can be low (70 kDa), medium (190–310 kDa) or high (500 kDa) [11].

This cationic polymer is soluble in weak acids which presents protonated amino groups in its polymeric chain [12]. In pharmaceutical applications, chitosan nanoparticles play an important role by carrying negatively charged molecules, such as peptides and proteins, and providing a significant protection against their biodegradation by enzymes in physiological fluids [13].

The ionotropic gelation method, containing sodium tripolyphosphate (TPP) as a cross-linker agent, is commonly used to prepare chitosan nanoparticles [14,15]. Usually, during the cross-linking process, the TPP solution, including the negatively charged biomolecule, is slowly added to a chitosan solution resulting in the spontaneous formation of nanoparticles. This simple and mild method of preparation and the advantages of chitosan become these nanoparticles carriers of interest to peptides and other molecules like antibiotics. In this way, chitosan nanoparticles have become the target of studies in the treatment of important pathologies such as cancer and diabetes [16–19].

After the nanoparticle production, it is important to determine the amount of the molecule of interest (drug or a biomolecule) that is linked to or encapsulated in the nanoparticles. The mechanism by which the peptide and protein, like insulin, lipase and others, interacts with chitosan-TPP nanoparticles is, probably, through hydrophobic interactions and H-bonding [20,21].

The physical-chemical characteristics of the compound must be taken into consideration when choosing the quantification method.

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Table 1
Physicochemical properties of chitosan nanoparticles containing the 5,3 kDa peptide.

Formulation	Size (nm)[a]	PdI[a]	Z-potential (mV)[a]
Empty chitosan nanoparticles	183,3 ± 8,32	0.397 ± 0.07	33,7 ± 2,45
Peptide within chitosan nanoparticles	245,9 ± 25,4	0.463 ± 0.01	39,3 ± 4,88

[a] These results comprise the average of three independent preparations (±standard deviation).

Considering several groups aiming to incorporate peptides and proteins within chitosan nanoparticles, the Bradford method for protein quantification could be employed. Since our goal was the incorporation of the peptide in the chitosan nanoparticles, the Bradford method for protein quantification was employed [22]. Medical applications of organic–inorganic hybrid materials within the field of silica-based bioceramics.

In the Bradford protein assay, the Coomassie blue dye BG-250 binds to a basic or aromatic chemical group of proteins [23]. After this interaction, the dye changes to its anionic form and its absorbance is detected in the blue color [24]. However, inspite of the practicality of the method, the dye may react with the chitosan or other polymer that did not bind with the TPP and interfere with the protein quantification [25].

To determine the association efficiency of the peptide to the chitosan nanoparticles, the amount of peptide that was not linked to the nanoparticles present in the supernatant is subtracted from the total amount of peptide added in the preparation. However, some of the peptide, TPP and chitosan that were not linked are also found in the supernatant suspension in different proportions and may also link to the dye, interfering in the Bradford reaction, which leads to false experimental values [26].

Therefore, the present work evaluated different methods for peptide quantification present in the supernatant of chitosan nanoparticles suspension, aiming to remove compounds not associated to the nanoparticles, especially chitosan, which could interfere with the association efficiency results.

2. Methodology

2.1. Chitosan nanoparticles preparation

Chitosan was purchased from Sigma-Aldrich (degree of deacetylation ≥75% and viscosity 20–300 cps where c = 1% in 1% acetic acid) with low molecular weight (as specified by manufacturer). Chitosan nanoparticles were prepared according to the protocol described by Calvo et al. [14] and modified according to Neves et al. [7]. In brief, a chitosan solution was prepared dissolving the polymer in 1% acetic acid (w/v) for 40 min homogenization at 15500 rpm in the Ultra-Turrax equipment.

Peptide-chitosan nanoparticles (PC) were formed by slowly adding dropwise of TPP solution containing peptide (5.3 kDa) in a chitosan solution under gentle magnetic stirring. The TPP was purchased from Sigma-Aldrich and is technical grade (as specified by manufacturer). The final solution was kept under stirring for 1 h. The empty chitosan nanoparticles (EC) were obtained using the same methodology, but instead of adding peptide to the TPP solution, ultrapure water was added. The EC was used as a blank in the association efficiency reaction of the peptide to the chitosan nanoparticles. The mean diameter, given as Z-average, and size distribution, given as polydispersity index (PdI), of the nanoparticle formulations were assessed by dynamic light scattering performed in a Zetasizer Nano ZS ZEN3600 (Malvern Instruments, UK). All the preparations were made in triplicate.

2.2. Scanning Electron Microscopy

The chitosan nanoparticles images were obtained by Scanning Electron Microscopy (SEM). To this purpose, the chitosan or peptide within nanoparticles solution was homogenized for 2 min to avoid agglomeration and then a drop of the sample was put in a copper grid with carbon and dried for 24 h at room temperature. Images were acquired using an electron microscope (Jeol, model JEM-1011) with accelerating voltage of 100 kV.

2.3. Sample processing to evaluate peptide association to chitosan nanoparticles

The association efficiency (AE) of chitosan nanoparticles within the peptide was determined indirectly from the detection of free peptide in the solution. For this purpose, three different methodologies for the processment of the solutions were compared before being submitted to the quantification tests and are described next.

2.4. Methodology 1

1.5 mL of the PC or EC nanoparticles solutions were placed into tubes and centrifuged for 1 h at 16100 *g* and 4 °C to collect the supernatant. For the PC, it was expected that part of the peptide which did not bind to the chitosan would be free in the supernatant. The supernatant from the EC was used as a blank for the efficiency association calculations.

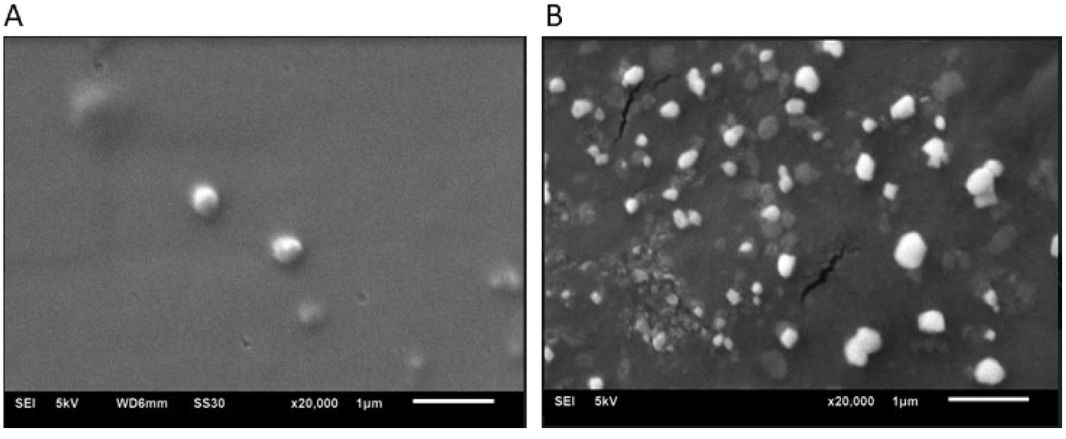

Fig. 1. Scanning electron microscopy of chitosan nanoparticles. (A) Chitosan nanoparticles. (B) Peptide incorporated within chitosan nanoparticles.

Table 2
Absorbance values obtained after reading the peptide solutions at 595 nm according with Methodologies 1, 2, and 3.

	Methodology 1		Methodology 2		Methodology 3	
Peptide (μg/mL)	Absorbance	SD[b]	Absorbance	SD[b]	Absorbance	SD[b]
50	0,221	0,049	0,248	0,004	0,871	0,046
25	0,058	0,048	0,180	0,038	0,613	0,101
12,50	0,006	0,039	0,070	0,011	0,400	0,030
6,25	−0,024	[a]	−0,016	[a]	0,272	0,022
3125	−0,040	[a]	−0,044	[a]	0,138	0,005
1,56	−0,023	[a]	−0,075	[a]	0,097	0,048
0,78	−0,048	[a]	−0,050	[a]	–	–
0,039	–	–	−0,058	[a]	–	–

[a] Negative values.
[b] SD: standard deviation.

2.5. Methodology 2

The early steps of this methodology were the same as presented for Methodology 1. After collecting the supernatant of either the PC or EC chitosan nanoparticles, the pH was adjusted to a range between 7 and 9 using 0.1 M NaOH as proposed by Su and collaborators [26] until it was possible to see the chitosan aggregate in the medium. Next, the resulted solutions were centrifuged at 9300 *g*, 4 °C for 10 min. The supernatants were collected and used for the association efficiency assays.

2.6. Methodology 3

For this Methodology, the PC or EC nanoparticles solutions were placed into Amicon Ultra-15 filters 30 kDa (Millipore®) and centrifuged at 3000 *g*, 4 °C by 40 min. The PC or EC nanoparticles and free chitosan were held in the column while the remaining components (water, free peptide and TPP) passed through the filter named here as a filtrate of the PC solution (FPC) or filtrate of the EC solution (FEC). In the FPC, the remaining peptide that did not bind to the chitosan nanoparticles is expected to be seen. Both the FEC and FPC were stored at 20 °C for use in the association efficiency assays.

2.7. Peptide quantification

The resulted supernatants obtained, according to Methodologies 1 and 2, and the filtrates obtained in Methodology 3, were submitted to the same analysis for quantification of free peptide in the chitosan nanoparticles solution: Bradford protocol and Qubit® kit for the detection of proteins. The Coomassie Protein Assay Reagent used in the Bradford assay was purchased from Sigma-Aldrich (Product Number 27813).

2.7.1. Bradford protocol

The standard peptide curve was created using peptide concentrations of 1.56, 3.125, 6.25, 12.5, 25, and 50 μg/mL. For preparing these solutions, the peptide was diluted in the supernatants or in the filtrate of the EC, according to the methodology analyzed. For the Bradford assay, 40 μL of Bradford's reagent (Sigma-Aldrich) was added to 160 μL of the supernatant or filtrate from each solution and placed into a 96-well microplate. The absorbance was read at 595 nm wavelength. The experiments were performed in triplicate.

2.7.2. Qubit® protocol

The Qubit® 2.0 Fluorometer and the Qubit Protein Assay Kit were used according to the manufacturer's recommendation. As in the Bradford protocol, the peptide was quantified in the supernatants or in the filtrate of the PC. The Qubit Protein Assay Kit contains three standard solutions with concentrations of 0, 10, and 20 μg/ml used to create the calibration curve. From each sample in the test, 20 μL was used. The analyses were performed in triplicate.

2.8. Association efficiency calculation of peptide to chitosan nanoparticles

The amount of non-linked peptide to chitosan detected in each methodology was subtracted from the total peptide amount used to prepare the PC solutions.

3. Results

3.1. Characterization of chitosan nanoparticles

The physicochemical properties of chitosan nanoparticles encapsulating the 5.3 kDa peptide are shown in Table 1. The polydispersity indexes were in the range of 0.3 to 0.4., indicating homogeneity of the preparations. The Scanning Electron Microscopy images of chitosan or peptide within chitosan nanoparticles presented a near-spherical morphology in the nanometer scale for the particles (Fig. 1). In addition, it was possible to visualize no apparent aggregation of the nanoparticles.

3.2. Peptide quantification according to Methodology 1

According to Methodology 1, it was necessary to create a standard curve to detect the peptide in the PC supernatant. As can be noted in Table 2, the absorbance readings for the peptide concentrations below 6.25 μg/mL presented negative values, thus, making it impossible to create a curve using the Bradford method. Although it was not possible to construct the standard curve, the peptide amount was investigated by reading the samples at 595 nm and using the Qubit® kit for the detection of proteins. The results showed mean values for absorbance of 0.342 and it was not possible to estimate the peptide concentration in the sample.

For peptide quantification using the Qubit® kit, the mean peptide concentration in the PC supernatant was 270 μg/ml, while the EC supernatant (blank), which was supposed to have a lower value than the PC, presented a concentration of 286 μg/ml.

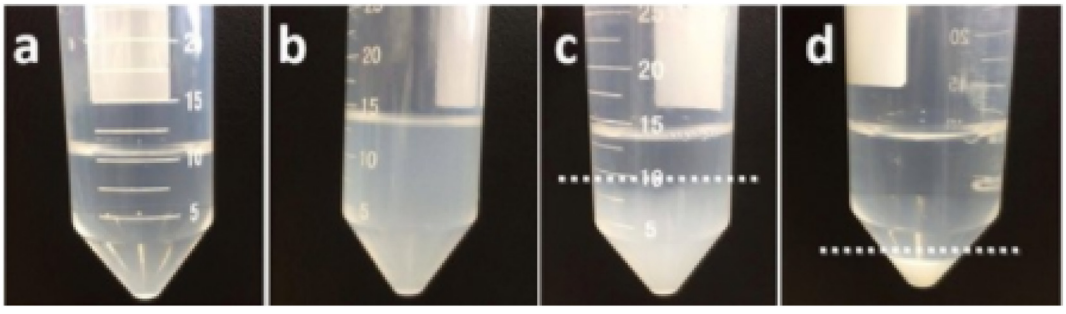

Fig. 2. Chitosan precipitation. (A) Crystalline chitosan solution at pH 6.4. (B) Turbidity chitosan solution immediately after changing for pH 7.5. (C) Chitosan solution at pH 7.5 after 20 min indicating two distinct liquid phases. (D) Chitosan solution at pH 7.5 after centrifuged indicating chitosan sediment. The trace lines determine the phases separations.

3.3. Peptide quantification according to Methodology 2

After changing the pH value to 7.5, the chitosan that did not associate to nanoparticles was precipitated in the supernatant by spontaneous formation of polymeric aggregate as can be seen in Fig. 2.

The peptide concentrations used to create the standard curve were the same as reported in Methodology 1. However, in this case, the peptide was diluted in the EC supernatant obtained after chitosan precipitation with NaOH. The PC or EC supernatants were submitted to the Bradford protocol and values for absorbance at 595 nm are shown in Table 2.

The absorbance values for peptide concentrations obtained by Methodology 2 were also not acceptable for composing the standard curve. As can be seen in Table 2, the absorbance at concentrations below 6.25 μg/mL presented negative values. Despite this, the samples were submitted to the Bradford method in an attempt to quantify the remaining peptide in the PC supernatant after chitosan precipitation. The mean absorbance value obtained in this test was 0.242, also close to the one pointed out in Table 2 for the concentration of 50 μg/mL peptide, and higher than the total peptide amount added for preparing nanoparticles (26 μg/mL).

The Qubit® kit for protein detection was also used to investigate the peptide concentration in the nanoparticles, in which EC presented a supposed protein concentration of 20 μg/mL while the mean peptide concentration in the supernatant of PC was 40.8 μg/mL. Although in this assay, the peptide concentration in the PC supernatant was lower than the result above, it still exceeds the total peptide amount added in the beginning of nanoparticles preparation. In addition, high absorbance values were detected even in the EC, where peptide had not been added.

3.4. Peptide quantification according to Methodology 3

For Methodology 3, the chitosan nanoparticles containing peptide was initially centrifuged using Amicon Ultra-15 filters 30 kDa (Millipore®) and the filtrates (FEC and FPC) were submitted to the Bradford method and Qubit® kit in order to quantify the peptide not linked to the chitosan.

In the Bradford method, the standard curve for peptide quantification was constructed using the FEC with the same concentrations applied in Methodologies 1 and 2. The absorbance values for the samples at 595 nm are presented in Table 2.

As shown in Table 2, all values were positive, creating the standard curve (Fig. 3), in contrast with those observed in the others methodologies tested.

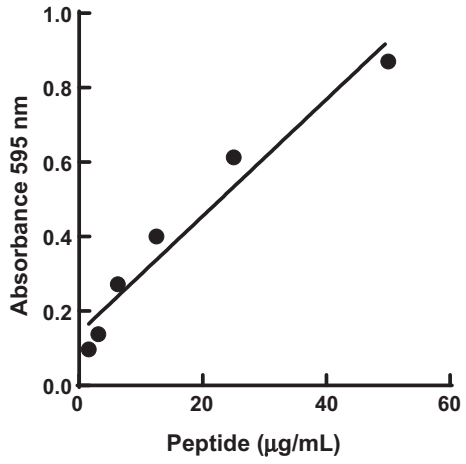

Fig. 3. Standard curve for peptide. y = 0,0157× + 0,1413. R^2 = 0,95.

Table 3
Association efficiency (AE) for peptide determined by Bradford method or using Qubit® kit.

Sample	Bradford		Qubit®		
	Value[a]	μg/mL	AE%	μg/mL	AE%
Q1	0,197	3,55	87.7	28,2	83,4
Q2	0,148	0,42	98,30	25,6	86,15
Q3	0,200	3,70	85,70	28,2	76,15

[a] Average values of three absorbance detections.

After constructing the standard curve, the FPC was submitted to the Bradford methodology. For each preparation, the absorbance detections were also performed in triplicate and the results were inserted in the equation referring to the calibration curve obtained previously. From these results, it was possible to find out the association efficiency of peptide within the chitosan nanoparticles (Table 3). The results signaled that the peptide AE was >85% in all triplicates by using the Centricom® column to remove free chitosan.

The FPC from the three different nanoparticles preparations resultant of this methodology were also submitted to the Qubit® kit and the FEC was used as a reaction blank. The peptide concentration was determined in the FPC as well as the AE of all the preparations by using the mean absorbance values of each triplicate and subtracting it from the reading obtained in the FEC (Table 3).

4. Discussion

Nanotechnology can contribute as a promising way to formulate increasingly efficient drug delivery systems [27]. However, the correct characterization of the formulation is necessary to evaluate, among other physicochemical parameters, whether the amount of the drug or the bioactive principle was encapsulated in a satisfactory amount for the therapeutic effect. In the case of proteins and peptides, the Bradford method is commonly employed [28,29].

The methodology used for the preparation of the chitosan nanoparticles was the ionic gelation, which consist in the spontaneously formation of nanoparticles by crosslinking of chitosan and TPP [15]. The chitosan nanoparticles incorporating the peptide showed an increase in size in relation to the empty nanoparticles, suggesting, by the method used, the incorporation of the peptide [14]. The resulted nanoparticles presented a nanometer size confirmed by dynamic light scattering and SEM images analyses.

Despite the wide application of the Bradford method for the quantification of biomolecules in polymeric formulations, any polymer fragments can interact with the dye and promote light dispersity, interfering with absorbance detection. Carlsson and collaborators [25] explained that chitosan interacts with Coomassie BG-250 and presents absorbance values at all wavelengths, resulting in inaccurate value for protein quantification. In the present work, different methodologies were evaluated to avoid false results for peptide quantification using the Bradford method in the chitosan nanoparticles within the peptide.

For the analysis of free peptide using the Bradford method, it was necessary to create a standard curve. The suitable standard curve would be one in which the peptide was diluted in the EC supernatant (for Methodologies 1 and 2) or FEC (for Methodology 3). However, as can be seen in Table 2, it was not even possible to construct such a curve using Methodologies 1 and 2, since peptide in a concentration equal to or below 6.25 μg/mL presented negative values. Despite knowing that without the standard curve it is not possible to determine a peptide concentration, the PC supernatants were submitted to the Bradford assay. It is possible to assume, by comparing the absorbance values showed in Table 2, that none of these two methodologies would be acceptable since the resultant absorbance value would be higher than the early peptide concentration added in the preparation.

The principle of Methodology 3, which consisted in filtering the suspension of chitosan nanoparticles in an Amicon® filter, was based on the experiments of Hosseinzadeh and collaborators [30]. The hypothesis for using the filter to remove free chitosan, was that peptide is a 5.3 kDa polypeptide and therefore would pass through the filter membrane while both the nanoparticles and free chitosan (molecular mass > 130 kDa) would be retained in the filter membrane. Using this methodology, positive values for absorbance were detected, even at concentrations below 6.25 μg/mL, and, in this way, it was possible to construct the standard curve (Fig. 3). The filtrate resulted from the nanoparticles produced according to Methodology 3 was submitted to the Bradford method and, using the standard curve, it was possible to estimate the concentration of peptide that was associated within the nanoparticles.

The association efficiencies of peptide to chitosan nanoparticles were 87.7%, 98.3%, and 85.7% for three different nanoparticles preparations: Q1, Q2 and Q3, respectively (Table 3). Although, the data obtained after analyzing samples by the Qubit® kit (Table 3) did not exactly present the same values, it showed similar concentrations and preserved the increasing order of the peptide concentration found in the filtrate obtained by the Bradford method (Q2 > Q1 > Q3).

Despite other similar works with chitosan nanoparticles make use of Bradford method to protein quantification [29], it is safe to say that the results obtained may be not the most accurate due the link between chitosan and the Coomassie blue dye [30].

Because of this, some mathematical theories have already been proposed to correct some interferences caused by polymers when using the Bradford method to estimate the protein concentration in solutions containing traces of polymers [25]. However, there is still no mathematical formula for this correction for chitosan. Therefore, it is consistent to say the safest way to obtain accurate results using the Bradford method in solutions containing chitosan is by cutting out as much as possible the presence of this polymer in the test solution.

One way found to remove efficiently chitosan from solution of interest was using Amicon® Ultra-15 filters. Hu et al., 2013 [31] also used this strategy in their work, however for peptide quantification it was used High-Performance Liquid Chromatography (HPLC), which consist in a method more expensive than Bradford and also requires qualified and experienced personnel to handle the equipment, unlike the method here presented. Besides removing chitosan residues for association efficiency analysis as shown here, Millipore® filtres has also been applyed to isolate the produced nanoparticles from the entire solution, as demonstrated by Jayaraman et al. [32].

5. Conclusion

In this way, the results indicated that Methodology 3, based in the separation of free polymers from the rest of the formulation using filter membranes showed better results than previous methodologies. Here, the Amicon filter 30 kDa was suitable to be used with peptides and components smaller than the molecular size of the chitosan nanoparticles. This strategy is efficient for prior processing samples for analysis with the Bradford test since it removes the chitosan interference with the dye, which was the main problem found for detection of peptides in the chitosan nanoparticles using this protein quantification protocol.

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