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Optimisation of the production of fish gelatine nanoparticles as a carrier for sunflower-derived biopeptide

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Abstract

Gelatine obtained from fish skin has become a potential source of preparing nanoparticles and encapsulation of bioactive compounds. Within these fish skin, gelatine nanoparticles show potent benefits for application in pharmaceutical and cosmetic industry. The encapsulated bioactive ingredients within nanoparticles have improved bioavailability, delivery properties, and solubility of the nutraceuticals within the human body and blood stream. Many of such bioactive peptides (biopeptides) are potent antioxidants; and as oxidative stress is the main cause of the onset of various chronic diseases, encapsulation of antioxidant biopeptides within fish gelatine nanoparticles could be a potential remedy to prevent or delay the onset of such diseases and for better health prospects. The purpose of the present work was to prepare a simple, safe, and reproducible novel food delivery nanoparticle system encapsulating a desirable antioxidant biopeptide. An optimisation study was conducted to produce a desirable size of gelatine nanoparticles which showed a higher encapsulation efficiency of an antioxidant biopeptide. Sunflower biopeptide was chosen as the antioxidant biopeptide, as the activity of this protein hydrolysate is quite high at DPPH of 89% and FRAP assay of 968 µm/L. Tilapia fish was used as gelatine source at an average yield of the process at 10% wt/wt. Effects of parameters such as pH, biopeptide concentration, and cross-linking agent 'glutaraldehyde' on the size, stability, and encapsulation efficiency on the nanoparticles were studied. The average diameter of the biopeptide loaded gelatine nanoparticle was between 228.3 and 1,305 nm. Encapsulation efficiency was 76% at an optimal pH of 2, glutaraldehyde concentration of 2 mL, and biopeptide concentration of 0.1 mg/mL exhibited DPPH at 92% and FRAP assay of 978 µm/L. To understand the absorption of sunflower biopeptide in stomach, blood stream, and biopeptide release of the gelatine nanoparticles, biopeptide loaded gelatine nanoparticles were subjected to simulated gastrointestinal conditions mimicking human stomach and intestine; and showed peptide release of 0.1464 and 0.277 mg/mL upon pepsin and pancreatin digestion, respectively.

Introduction

Food and pharmaceutical industry worldwide are experiencing an exponential demand for gelatine due to its wide attributes as a natural, non-toxic, biodegradable, readily available, and cheap polymer. Gelatine polymer has also gained wide popularity for production of nanoparticles with numerous available active sites for attaching targeting molecules and drug or nutraceutical delivery systems, aiming to improve the therapeutic effects, targeted delivery, and reducing the side effects. With the ever growing demand of gelatine due to biocompatibility and availability, production, and utilisation of fish-based gelatine, it not only satisfies the need of the industry but also serves as a means to utilise the discarded by-products of the fish industry (Akbar *et al.,* 2017). One of the major applications of this fish-based gelatine is in nanotechnology for encapsulating bioactive compounds and pharmaceutical agents and their targeted release in the body.

Hydrolysed proteins from many animal and plant sources have been found to possess antioxidant activity; and many sources of bioactive peptides have been exploited including the defatted oil meal cakes (Nimalaratne, 2015). Bioactive peptide is usually inactive when exists as a part of the parent protein but can be released during food processing and enzymatic hydrolysis. The size and composition of a peptide determines its antioxidant and free radical scavenging

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properties. Therefore, formulating such biopeptides into a nanoparticulate system can improve its bioavailability into the cellular components (Ribeiro *et al.,* 2011). Biopeptide fractions from various protein sources can be incorporated, suspended, and dispersed or encapsulated into different forms such as emulsions, liposomes, nutraceuticals, and other edible biopolymers to achieve their optimum functionality, bioavailability, stability, and targeted effectiveness (Amar-Yuli *et al.,* 2010; Livney, 2010; Patel and Velikov, 2011; Elzoghby *et al.,* 2013).

The use of fish gelatine nanoparticles to encapsulate antioxidant biopeptide would give the food and pharmaceutical industry an ingredient with greater functional flexibility and permit the industry to easily maintain kosher/halal status, thus giving the consumers new opportunities for functionally developed foods, and providing more religiously acceptable foods. Nano-encapsulated antioxidant biopeptides are highly permeable through the human intestines where fast degradation and better uptake of peptides into the blood stream takes place, therefore incorporation into food systems can provide many health benefits (Ribeiro *et al.,* 2011).

Therefore, the objective of the present work was geared towards establishing fish waste as a potential halal gelatine alternative based on its rheological properties for application as nanocarriers in the food industry. Biopeptides were obtained from defatted sunflower cakes left behind as by-products of the oil industry. With the demand for protein on the rise, it proved to be a viable yet cheap alternative to meet our current demands. An antioxidant biopeptide previously derived from defatted sunflower cake by enzymatic hydrolysis was used in the present work and exhibited high antioxidant activity. The sunflower-derived antioxidant biopeptide was encapsulated in the gelatine nanoparticles produced in the present work and the biopeptide-loaded gelatine nanoparticles were studied for various factors. The antioxidant biopeptide obtained from the defatted oil meal cakes encapsulated in gelatine polymer was checked for their cellular uptake in the simulated gastrointestinal cavity.

Materials and methods

Reagents

Chemicals required for the assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) and FRAP assay reagents, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The solvents used were of analytical grade and purchased from Merck (Darmstadt, Germany). All other reagents were purchased from Aldrich (Steinheim, Germany).

Materials

Sunflower seeds were purchased from a local market. One hundred kilogram of tilapia fish (average individual fish weight of 5 kg, 6 months old fishes, average length of less than one foot) were purchased from the wet market in Malaysia. They were washed twice with water; heads and viscera were separated, rinsed with cold distilled water, and then stored in sealed plastic bags in a freezer until further use.

Enzymes

Alcalase® (endopeptidase from *Bacillus licheniformis*, 2.4 L) and Flavourzyme® (exopeptidase and endoprotease complex from *Aspergillus oryzae*, 500 L) were purchased from Sigma-Aldrich.

Antioxidant biopeptide fraction obtained from defatted sunflower meal

An antioxidant biopeptide was obtained from previous research. The defatted sunflower "meal" was subjected to sequential enzymatic hydrolysis by an exo and endo peptidase i.e. alcalase and flavourzyme, respectively, to release the protein hydrolysates fraction of < 60 kDa in weight, which was later assessed for its antioxidant activity by DPPH set at 89% and FRAP assay at 968 μm/L.

Gelatine extraction from fish skin tilapia

Gelatine extraction was carried out according to Alfaro *et al.* (2009) with slight modifications. The extracted gelatine was concentrated at 70°C for 5 h and stored in the refrigerator at 5 - 10°C for 30 min, and dried at 60°C for 24 - 36 h until a solid gelatine sheet was obtained. Gelatine sheets were milled and packaged in vacuum plastic, and stored in a desiccator for subsequent process.

Preparation of gelatine nanoparticles

The biopeptide-loaded gelatine nanoparticles were prepared using a two-step desolvation technique as described by Coester *et al.* (2000). The gelatine solution at the second desolvation step was adjusted between pH 2 - 12 by the addition of 0.1 N HCl or 0.1 N NaOH. The pH was monitored continuously during the second desolvation step. Antioxidant biopeptide loading was done at this step following Ofokansi *et al.* (2010) with slight modifications. The suitable concentration of the biopeptide ranging from 0.1 - 1 mg/mL were added to the gelatine solution and incubated at room temperature for 3 h. The solution was desolvated again by drop-wise

addition of ethanol until a permanent faint turbidity was obtained under constant stirring at 1,200 rpm for 30 min. At the end of the process, the formed gelatine nanoparticles were cross-linked with glutaraldehyde (25% aqueous solution, wt/v; 0.25 - 2 mL) at room temperature and stirred for 12 h at 1,200 rpm to cross-link the particles (Ofokasni *et al.,* 2010; Mohtar *et al.,* 2014). The excess of glutaraldehyde was neutralised by adding 5 mL of 12% sodium metabisulfite and were sonicated for 2 min. The particles were purified by centrifugation at 16,000 rpm for 20 min. The effect of parameters such as pH, amount of glutaraldehyde and biopeptide concentration on the nanoparticle size, and entrapment efficiency of the gelatine nanoparticles were studied.

Characterisation of the gelatine nanoparticles loaded with antioxidant biopeptide

Particle size was determined by photon correlation spectroscopy (PCS) by using Zetasizer 3000 (Zetasizer Nano ZS, Malvern Instruments, UK). The samples were measured in suspension after particle preparation, without further dilution. Each sample was measured three times, after which the average value was used for further calculations.

Encapsulation efficiency

The encapsulation efficiency was calculated following Vandervoort and Ludwig (2004) methods, as the amount of biopeptides present in the nanoparticles was compared with the amount of biopeptide initially used in their production. Binding of the biopeptide to gelatine nanoparticles was measured by centrifuging part of the particle suspension at 14,000 rpm for 2 h. A sample of the supernatant was analysed by a spectrophotometer (Angstrom, Model UV/VIS Scanning Spectrophotometer) to determine the amount of the non-entrapped biopeptide. The encapsulation efficiency (% EE) was calculated using Equation 1:

$$\% \text{ EE} = \frac{(\text{Mass of biopeptides in GNPS} \times 100)}{(\text{Mass of biopeptides used in formulation})} \quad \text{(Eq. 1)}$$

Antioxidant activity of the gelatine nanoparticles encapsulating the sunflower-derived antioxidant biopeptide

The scavenging activities of gelatine nanoparticles loaded with biopeptide against the DPPH radical were determined using a previously described method (Aluko and Monu, 2003), with slight modifications as per Jamal *et al.* (2016). The FRAP of the biopeptide loaded nanoparticles was measured according to a previously reported method (Zhang et al., 2008), which was modified as per Girgih *et al.* (2015).

Cellular effect and body digestion in gastrointestinal tract

To simulate the influence of the physiological conditions during digestion, a model system was used as devised by Ribeiro *et al.* (2011). The pH was monitored and controlled to specific values for the stomach and small intestine. The temperature was kept at 37°C as these conditions simulate the conditions inside the stomach and small intestine where digestion on proteins takes place.

For simulation of the gastric fluid digestion, antioxidant biopeptide loaded in gelatine nanoparticles were incubated for 1 h at 37°C, in a 0.32% (w/v) pepsin solution containing 30 mM NaCl, and 0.7% (v/v) of 0.2 N HCl, pH 1.2 in a 1/20 (w/w) enzyme/protein hydrolysate relation. For simulation of the intestinal fluid digestion, antioxidant biopeptide loaded in gelatine nanoparticles were incubated for 3 h at 37°C in a 1% (w/v) pancreatin solution containing 0.05 M KH_2PO_4 and 19% (v/v) of 0.2 N NaOH, pH 7.5 in a 1/20 (w/w) enzyme/protein hydrolysate relation (Megías *et al.,* 2009). The protein content for both samples was measured following a modified Lowry method; and the digestion with pepsin and pancreatin was studied to determine the amount of biopeptide released by the gelatine nanoparticles inside stomach and small intestine.

Results and discussion

Extraction of gelatine from tilapia skin and scales

Tilapia (*Oreochromis* spp.) is a freshwater species and the third most widely cultured fish after carp (*Cyprinus carpio*) and salmon (*Salmo salar*) (El-Sayed, 2006). It accounts for a major portion of fish supply in Malaysia, and the production of tilapia has shown an increase in the past few decades (Jamilah and Harvinder, 2002). Its resistance towards many diseases, durability, and fast growth, as opposed to many other fish varieties, have led to the remarkable upscaling of this fish in the market. Tilapia has high protein content and is comparable with meat sources from other poultry and fish stocks.

Karim and Bhat (2009) set the yield of gelatine extraction on an average lower than that of mammalian gelatine, which is approximately 6 and 19% (expressed as grams of dry gelatine per 100 g of

clean skin) and the average yield of tilapia skin gelatine was 5.10 g/100 g. Jamilah and Harvinder (2002) reported values for black skin gelatine at 5.39% and red skin gelatine at 7.81%, which however are considerably lower than the yields reported by Grossman and Bergman (1992) for tilapia species gelatine (15%), and Holzer (1996) who described a method for skin gelatine extraction that could reach yields higher than 20%. According to Cho *et al.* (2006), the average protein content (wet basis) found in the tilapia skin was 21.30%, which indicates a potential source of fish gelatine production. In our study, the lower extraction yield of tilapia skin gelatine at 10% was established and this low value could be attributed to the loss of collagen through leaching during the series of washing steps. Moreover, incomplete collagen hydrolysis could be a possibility for lower yields as temperature affects the extraction of gelatine from the skin. Low temperatures result in lower yields and incomplete extraction; while higher temperatures tend to degrade the produced gelatine, thus affecting its quality (Alfaro *et al.,* 2009). Since the temperature for gelatine extraction was similar as per previous studies, the low yield of gelatine could be due to collagen loss during the pre-treatment and washing steps.

Optimisation of the process conditions for the preparation of gelatine nanoparticles encapsulating antioxidant biopeptide

The gelatine nanoparticles in the present work were prepared by the two-step desolvation procedure and showed high stability in both water and cell medium. The nanoparticles obtained did not sediment or flocculate, and the stability of the particle size remained the same during the three months investigation time. An optimum desolvation process was established for maximum biopeptide uploading and dependent on three factors namely pH (2 - 12) (Saxena *et al.,* 2005; Nahar *et al.,* 2009), glutaraldehyde concentration which helps in cross-linking the nanoparticles, stability and biopeptide concentration (0.1 - 1.0 mg/mL) (Vandervoort and Ludwig, 2004; Ofokansi *et al.,* 2010).

Characterisation of the gelatine nanoparticles loaded with antioxidant biopeptide

The gelatine nanoparticle sizes obtained varies from 228 to 1305 nm. When the biopeptide gelatine particles were prepared, ethanol was added until a faint turbidity was observed. Glutaraldehyde was added after cross-linking started as depicted in Figure 1; followed by the addition of 5 mL of water to bring the system back to the beginning of coacervate formation. The formed aggregates, which were cross-linked, were smaller than the ones obtained when preparing pilocarpine HCl-loaded particles (Vandervoort and Ludwig, 2004), which varied from 310 to 500 nm. The agitation by the propeller stirrer, however, remained the same for all preparations ensuring a reduction in aggregate formation and preparing smaller sized nanoparticles at a lower pH. 200 mg gelatine extracted from the tilapia skins and scales were dissolved in distilled water (10 mL) under constant mild heating and stirring (40°C; 500 rpm). Contrary to the gelatine solution before the second desolvation step, the pH was adjusted to values ranging between 2 to 12 before nanoparticles were generated by the addition of ethanol. Gelatine, therefore, does not necessarily have to contain an increased amount of high molecular weight components to produce stable and homogeneous colloidal spheres as stated by Farrugia and Groves (1999). The temperature of preparation of the nanoparticles (40°C) was selected to ensure that the molecular weight distribution of gelatine remained relatively constant during incubation. Similarly, the selected pH range which was clearly accepting the isoelectric point of gelatine (IEP of 4.7 - 5.2); as such the gelatine molecules would be sufficiently uncharged to remain sensitive to desolvation but sufficiently charged to prevent their aggregation during nanoparticle formation.

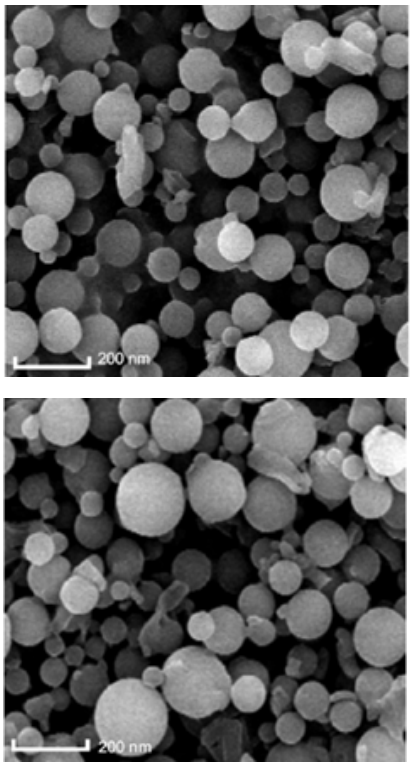

Figure 1. Scanning electron microscopy of the unloaded gelatine nanoparticles and sunflower biopeptide-loaded gelatine nanoparticles.

The mean particle size of the unloaded and FITC-D-loaded gelatine nanoparticles, which were determined by photon correlation spectroscopy, was found to be 253 and 281 nm, respectively, with a narrow-size distribution (Ofokansi *et al.,* 2010). The optimum amount of glutaraldehyde needed for effective cross-linking of the nanoparticles was found to be 37.5 mg per 200 mg of gelatine. According to Ofokansi *et al.* (2010), preliminary experiments used 1 mL of glyoxal to cross-link the gelatine nanoparticles, and as reported by earlier studies, caused abrupt particle aggregation and sedimentation of the nanoparticles prepared. Therefore, glutaraldehyde was a better choice to cross-link the nanoparticles *in situ*. The main mechanism for glutaraldehyde cross-linking is due to its non-zero length cross which helps promote polyfunctional or bifunctional cross-links into the network structure of some biopolymers. It builds bridges for free amino groups of lysine or hydroxyl lysine of protein-based biopolymers such as gelatine. In the present work, zero accumulation and sedimentation of the nanoparticles occurred upon glutaraldehyde addition. The resulting particles remained stable after the processing and washing steps for more than 10 months storage under refrigerated conditions at 2 - 8°C.

The smallest nanoparticle size of 228.3 nm with an encapsulation efficiency of 76% was obtained. Particle size is one of the important properties that influence *in vivo* performance. The smaller particle sizes will ensure lowered level of reticuloendothelial system (RES) uptake, improve utilisation ratio of drug, and diminish drug side effects. Table 1 and Figure 2 depict particle size and polydispersity index for various nanoparticle preparations at various parameters of pH, and amount of biopeptide and glutaraldehyde concentration. Rajan and Raj (2012) prepared nanoparticles of chitosan, polylactic acid, and rifampicin (CS–PLA–RIF); obtained particle size diameter of 187 nm and showed a zeta potential of 21 ± 2.2 mV. It was also observed that by increasing the amount of RIF coating for the nanoparticle preparation from 10 to 50%, particle size saw an increase of up to 214 nm and the zeta potential up to 29 ± 1.6 mV respectively. The reasoning behind was set at the interaction between the RIF and the CS–PLA surface. All particle sizes of biopeptide loaded gelatine nanoparticles were measured in the nanometer range between 228 and 814 nm. The best average size for particles prepared with the gelatine was 228 nm, showing a polydispersity index of 0.320. The particle size increased slightly along with the glutaraldehyde additions, representing 627 and 739 nm with the biopeptide concentration of 0.1 g/g, respectively. As the pH value changed drastically, a significant difference was implied in respect of particle size; however, the polydispersity value came to a relatively constant value.

Table 1. Optimisation study of gelatine nanoparticles encapsulating antioxidant biopeptide.

Run	pH	Biopeptide (mg/mL)	Glutaraldehyde (mL)	Nanoparticle Size (nm)	Particle Density	Encapsulation Efficiency
1	7	0.55	1.125	631.4	0.571	67%
2	2	0.1	0.25	627	0.612	48%
3	12	1	0.25	446.1	0.475	78%
4	2	1	2	385.3	0.601	48%
5	7	0.55	1.125	629.9	0.568	68%
6	12	0.1	2	739.5	0.634	58%
7	2	1	0.25	814.9	0.406	69%
8	7	0.55	1.125	630.0	0.570	70%
9	7	0.55	1.125	628.6	0.567	68%
10	12	0.1	0.25	544.1	0.603	59%
11	12	1	2	482.3	0.557	78%
12	2	0.1	2	228.3	0.320	76%
13	12	0.55	1.125	317.7	0.481	72%
14	2	0.55	1.125	692.8	0.570	68%
15	7	0.1	1.125	638.5	0.568	65%
16	7	0.55	2	1305	0.932	48%
17	7	0.55	1.125	625.9	0.571	66%
18	7	1	1.125	571.0	0.460	68%
19	7	0.55	0.25	560.6	0.643	72%
20	7	0.55	1.125	628.1	0.571	68%

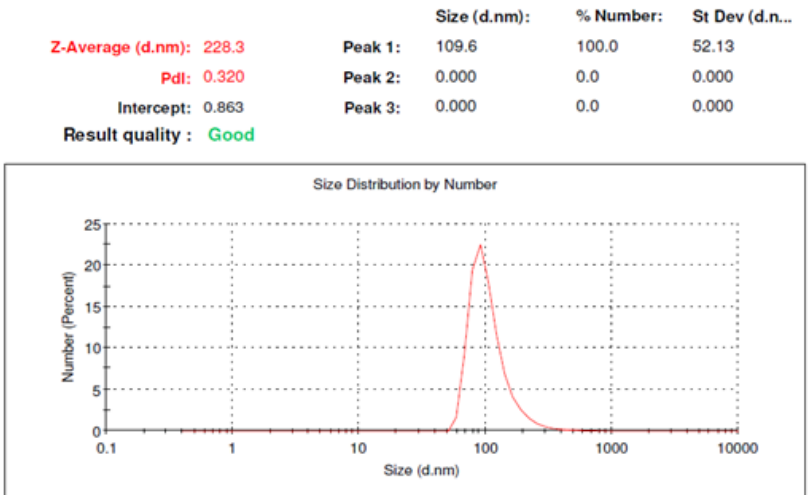

Figure 2. Optimal nanoparticle size distribution and the particle density index of the sunflower biopeptide-loaded gelatine nanoparticles.

The effect of the pH on the particle size was clearer. At a pH 2, the average particle size obtained was 228 nm, as compared to 630 nm at pH 7 and 441 nm at pH 12. This effect is illustrated in Figure 3, in which the response surface has a marked slope in the direction of the pH axis. This significant difference in particle size between the three pH levels tested indicated that the protonation or deprotonation of the amino or carboxylic acid residues present in the gelatine molecules influenced the way the gelatine molecules folded together as particle formation occurred. According to Vandervoort and Ludwig (2004), a possible explanation for the difference in particle size to produce gelatine nanoparticles loaded with hydrocortisone drug might be the difference in cross-linking between the pH levels. If the cross-linking reaction occurred at pH 2, more cross-links would be formed resulting in a denser network and a reduction in particle size. Controlling the particle size offers a possibility to regulate peptide release. Smaller particles have a larger total external surface allowing for a more intense interaction with the medium in which they are dispersed, thus resulting in a faster peptide release, as was demonstrated by Wakiyama *et al.* (1981) for local anaesthetics incorporated in poly (lactic acid) microspheres. The distribution *in vivo* is also dependent on the particle size. A possible uptake in the first cell layers of the cornea, for example, would largely depend on the particle size, as was demonstrated by Calvo *et al.* (1996), who showed that PECL nanoparticles penetrate the first corneal cell layers, while microspheres do not.

Weber *et al.* (2000) performed a study in which they showed that the concentration of the cross-linking agent influenced the amount of free amino groups at the surface of gelatine nanoparticles prepared by a desolvation technique. However, when a constant amount of glutaraldehyde was used and gelatine types were compared, they also demonstrated a difference between gelatine type A and B.

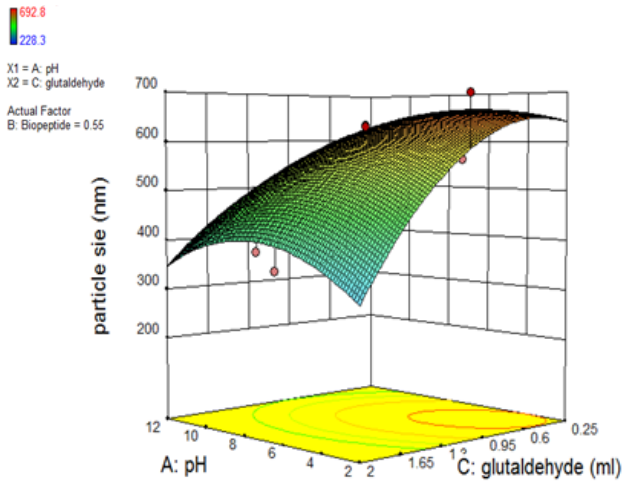

Figure 3. Surface interaction plot of pH and glutaraldehyde on the particle size of sunflower biopeptide-loaded gelatine nanoparticles.

Drug encapsulation efficiencies varying from 48 to 76% were observed. This is somewhat lower than the values obtained for pilocarpine HCl-loaded nanoparticles by Vandervoort and Ludwig (2004), but still in the range of drug loadings found in the literature. One could state that the hydrophilicity plays a role, as pilocarpine HCl is more hydrophilic than hydrocortisone. The hydrocortisone-cyclodextrin complex, however, can also be considered as hydrophilic. According to Souza *et al.* (2014), the encapsulation efficiency of quercetin into lecithin/chitosan nanoparticles presented values higher than 95% for all used concentrations (10, 50, and 70 µg/mL). These efficiencies are higher than those obtained by Ghosh *et al.* (2011) and Pool *et al.*

(2012) for PLGA nanoparticles (encapsulation efficiency below 80%). The high encapsulation efficiency obtained with lecithin/chitosan nanoparticles may be related to the fact that both lecithin and quercetin are hydrophobic molecules, which allows for strong affinity between them.

Other factors involved could be the difference in molecular weight or the difference in initial biopeptide amount added to the preparation during the nanoparticle preparation. For the biopeptide loaded particles, an interaction between the charged drug molecule and the different gelatine polymers at different pH levels was expected to lead to the differences in encapsulation efficiency. High encapsulation efficiency was achieved for gelatine nanoparticles showing a size of 228 nm at 76% entrapment, as both are amino acids in nature and show good affinity for each other. Consequently, larger amount of biopeptide was preferably inserted into the protein nuclei of the nanoparticles, and only a small amount of sunflower biopeptide was lost in the aqueous phase during the preparation process. However, formulations containing 2 mg/mL of biopeptide showed less entrapment efficiency. Therefore, nanoparticles with 0.1 mg/mL, pH 2, and a glutaraldehyde concentration of 2 mL were chosen to be used in further analysis.

The surface interaction plot of pH and glutaraldehyde as shown in Figure 3 further reinforces the conclusion that volume of glutaraldehyde influenced the overall particle size, as lower glutaraldehyde volumes produced larger size nanoparticles. The effect of pH is much less pronounced on the overall nanoparticle size. The surface plot also shows that the usage of low glutaraldehyde volumes (0.25 - 1 mL) with low pH values (2 - 6) produced nanoparticles of generally larger size. Figure 3 also shows the effect of the various factors on the encapsulation efficiency. Although the interaction of biopeptide and glutaraldehyde volume does not seem to have an impact on the optimisation of the encapsulation efficiency, it is shown that there is an optimum pH and biopeptide concentration for maximising the potential encapsulation efficiency. This is seen at pH of 2 - 6 and biopeptide concentration of 0.4 - 0.7 mg/mL.

The size and polydispersity index are essential analyses for the characterisation of nanoparticles, since they influence important parameters such as loading, release, and stability of the compound inside the nanoparticles. It is known that the smaller the particle, the greater the exposed surface area, which leads to a faster release of encapsulated drugs or biopeptide. According to Coester *et al.* (2000), smaller particles also have an increased risk of aggregation during storage; it is important in the development of nanoparticles with a low PDI to achieve maximum stability by a better control (and lesser dispersion) of particles' size. It is worth mentioning that the reproducibility of parameters such as stability and release is directly connected to a low PDI (≤ 0.4), since a high PDI means that there is no uniformity in the size distribution of the sample. Figure 4 shows that increasing biopeptide concentrations in gelatine nanoparticle formations leads to higher values of average size and polydispersity index. These results are in agreement with Ghosh *et al.* (2011) and Pool *et al.* (2012), who demonstrated that when encapsulating quercetin in poly (lactic-co-glycolic acid) (PLGA), nanoparticles obtained were in the particle sizes of 13.28 ± 7.8 nm and 399.67 ± 10.86 nm, respectively; and when the nanoparticles were prepared with values higher than 100 µg quercetin/mL, nanoparticle aggregation was observed few minutes after production. This behaviour can be explained by the presence of high concentrations of quercetin that will interfere with the process of self-organisation of nanoparticles by blocking the repulsive forces between the nanoparticles due to the decrease of spacing between adjacent nanoparticles, thus leading to aggregation. A similar behaviour was observed by Barbieri *et al.* (2013) for lecithin/chitosan nanoparticles with high loading of tamoxifen citrate. Based on these results and previous studies, low biopeptide loading leads to better entrapment, particle size, and polydispersity index of the nanoparticles.

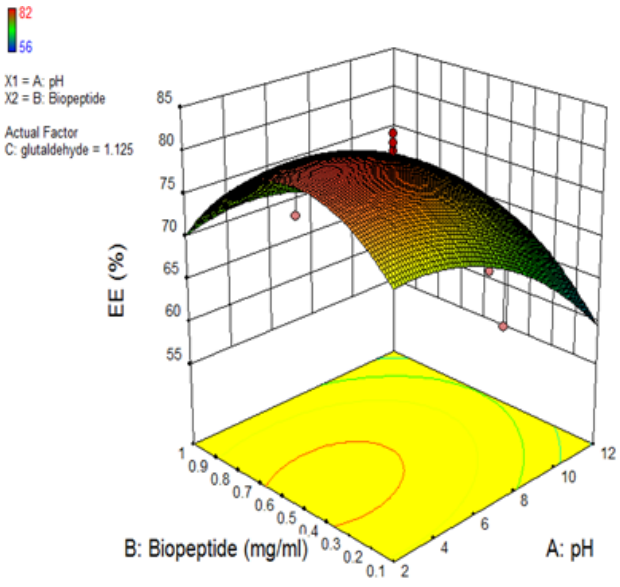

Figure 4. Surface interaction plot of biopeptide and pH on encapsulation efficiency of sunflower biopeptide-loaded gelatine nanoparticles.

The results obtained in the present work corroborate those obtained in the literature. The influence of the number of preparation parameters like gelatine type and pH of the dispersion on nano-particle properties (particle size, zeta potential value, and drug loading or drug release profile) had been studied by Vandervoort and Ludwig (2004). They concluded that particle size and zeta potential do not seem to be influenced by the two gelatine types (A and B), since they have different isoelectric points. The effect of pH on the particle size is more significant. Weber *et al.* (2000) performed a study in which they showed that the concentration of the cross-linking agent had an influence on the amount of free amino groups at the surface of gelatine nanoparticles prepared by desolvation technique. However, when a constant amount of glutaraldehyde was used and gelatine types were compared, they also demonstrated a difference between gelatine type A and B. Nahar *et al.* (2009) suggests a pH-dependent behaviour of gelatine nanoparticles due to its polyelectrolyte nature (contains both amino and carboxylate-terminated chains at its isoelectric point).

Leo *et al.* (1997) studied the involvement of drug having an amino group on the cross-linking degree of the nanoparticle prepared based on the coacervation-phase separation technique, and found possible involvement of amino group of drug in the cross-linking process with glutaraldehyde, and that there was a competition between the amino groups of gelatine and the drug. Similarly, Cascone *et al.* (2002) observed that increasing glutaraldehyde concentration decreased the size of nanoparticle as the cross-linking degree was increased thus gelatine nanoparticle had more dense structure. Nahar *et al.* (2009) reported that when the glutaraldehyde was varied from 50 to 400 µL, there was an approximately 10-fold reduction in the particle size. This was also seen in the results where an increase in the volume also decreased the nanoparticle size. This could be attributed to cross-linking of free amine groups at the nanoparticle surface of glutaraldehyde, which caused hardening of particles, thereby, leading to reduction in their size.

Antioxidant activity of the biopeptide loaded gelatine nanoparticles

The DPPH scavenging capacity assay was used to evaluate the ability of free biopeptide and gelatine nanoparticle to donate protons. DPPH assay shows the capacity of biopeptide to donate hydrogen, in order to stabilise free radicals, and it was maintained after encapsulation in the present work. Biopeptide-loaded gelatine nanoparticles were capable to reduce higher numbers of DPPH molecules when compared with free-biopeptide or unloaded gelatine nanoparticles, which can be related with improved dissolution properties of the sunflower biopeptide after encapsulation. This phenomenon was also observed by Souza *et al.* (2014) when encapsulating quercetin in nanoparticles based on polyvinyl alcohol and Eudragit. The DPPH assay was observed at 92% which was higher than the DPPH scavenging capacity of free biopeptide (89%).

Our results here show an increment in the reducing power of free sunflower biopeptide as compared to the unloaded gelatine nanoparticle and the biopeptide-loaded gelatine nanoparticles. Biopeptide-loaded gelatine nanoparticles were more effective in reducing Fe^{3+} to Fe^{2+} with a value set at 978 µm/L, in comparison with free sunflower biopeptide showing FRAP assay value of 968 µm/L. This property of nanoparticles is attributed exclusively to the ability to donate protons of the phenolic groups of the biopeptide, since the unloaded nanoparticles were not able to reduce Fe^{3+}. The increased reducing power of biopeptide after encapsulation again can be probably explained by an improvement in the dissolution properties of biopeptide in aqueous medium. The oxidation process of food products during production and storage causes a sequence of particularly unfavourable changes in the sensory properties of the product (appearance of rancidity, and changes in colour and texture) responsible for quality downgrading and economic losses. Souza *et al.* (2014) also monitored quercetin-loaded nanoparticles to establish their ferric-reducing property and confirmed that encapsulation enables improvement in the reduction of the ferric ions due to better dissolution of quercetin in the nanoparticle matrix.

Cellular effect and body digestion in gastrointestinal tract

The prepared antioxidant-loaded gelatine nanoparticles were then subjected to a two-step study, where conditions similar to stomach and gastrointestinal tract were applied. A two-step approach was utilised so that we could mimic the conditions in the human body as close as possible, as well as be able to effectively isolate where the test results correlate well and where they are anomalous in nature.

The results of the Lowry Protein test are displayed in Figure 5. An increase in absorbance after each stage suggests the breakdown of the gelatine nanoparticles thus releasing the encapsulated biopeptides. Prior to digestion, the amount of biopeptides released by the gelatine nanoparticles

was 0.0068 mg/mL. This was seen to increase to 0.1464 mg/mL after pepsin digestion, followed by 0.277 mg/mL after pancreatin digestion. These results indicate that the breakdown of the gelatine nanoparticles is satisfactory and corresponds to stable gelatine nanoparticles being produced.

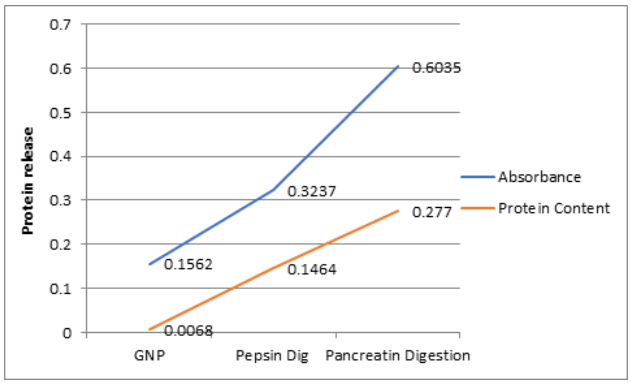

Figure 5. Variance of absorbance *versus* protein content at stages of digestion of sunflower biopeptide-loaded gelatine nanoparticles.

For nanoparticles and the consequent biopeptides to survive in the gastrointestinal tract, it depends on the polymer used, the biopeptide characteristic, and its resistance to the low pH (pH values from 1 - 3 to 3 - 0) in gastric juice, and to the bile salts found in the small intestine. One strategy which has been proposed is to enhance the survival of nanoparticles in dairy products and mayonnaise (Khalil and Mansour, 1998); and in simulated gastrointestinal conditions (Lee and Heo, 2000) is the immobilisation of such biopeptides in large alginate spheres (1 - 2 mm in diameter). Other food-grade encapsulation materials such as gelatine, xanthan, and alginate starch mixtures have also been proposed for encapsulation of biopeptides in spheres with diameters between 1 and 3 mm (Sultana *et al.,* 2000). Preliminary trials here revealed that this sphere size is too large to allow for direct incorporation in food products such as milk, yoghurt, and sour cream without adversely affecting the feel in the mouth. Reduction of the sphere size to less than 100 mm and to choose nanomaterials would be advantageous for texture considerations and allow direct addition of encapsulated biopeptides to a multitude of foods.

Our results here correlate with the results suggested by Lee and Heo (2000), which also saw an increase in the protein content as shown by the Lowry method, which was a result of peptide digestion in the stomach and the small intestines once the nanoparticles were subjected to pepsin and pancreatin digestions. The major portion of digestion of proteins and its breakdown into amino acids takes place in the small intestine by action of pancreatin, which was confirmed in our study as a sharp increase in protein content was observed at 0.277 mg/mL. According to Chen and Li (2012), who set a two-stage *in vitro* gastrointestinal digestion model to assess the survivability of antioxidant peptide fractions with different molecular weights, showed that peptides above 3,000 Da were more easily digested by gastric digestion than those below 3,000 Da. Therefore, it can be said that we were able to simulate the gastrointestinal conditions for the uptake of gelatine nanoparticles in the human body.

Conclusion

An optimisation study was carried out for gelatine nanoparticle production which presented the relationship between three independent variables, i.e. pH, glutaraldehyde concentration and biopeptide concentration, and the response of gelatine nanoparticle size and encapsulation efficiency. The best size of nanoparticles was achieved at 228.3 nm with an encapsulation efficiency of 78%, and showed antioxidant activity of 92% higher than that of the free biopeptide. The gastrointestinal digestion of the antioxidant encapsulated nanoparticles was thoroughly studied and an increase in the protein absorption concluded that the gelatine nanoparticles encapsulating the antioxidant biopeptide could be digested by pepsin and pancreatin enzymes. While studies continue to improve nanoparticle release technology, further studies are required to understand the sorption as well as biological release profile within the human matrix of a wider range of bioactive molecules from such gelatine nanocarriers.

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