

Preparation of sub-100-nm β -lactoglobulin (BLG) nanoparticles

SANGHOON KO & SUNDARAM GUNASEKARAN

Department of Biological Systems Engineering, University of Wisconsin – Madison, Madison, WI, USA

(Received 16 November 2005; revised 2 June 2006; accepted 5 June 2006)

Abstract

Sub-100-nm nanoparticles were prepared from β -lactoglobulin (BLG) with a narrow size distribution by a desolvation method using glutaraldehyde for cross-linking. With pre-heating of the BLG solution to 60°C and subsequent pH readjustment to 9.0, nanoparticles of 59 ± 5 nm were obtained with improved uniformity. Bovine serum albumin (BSA) nanoparticles, prepared under similar conditions for comparison, were larger and less uniform. The half-width of 80% particle distribution was used to compare the uniformity of particle size distribution. The stability of the nanoparticles was investigated by degradation tests at neutral and acidic pHs with and without proteolytic enzymes, trypsin and pepsin. The degradation time, determined by a graphical approach, was used to compare the relative stabilities of BLG and BSA nanoparticles. The particles of BLG were more stable than those of BSA in acidic and neutral media with and without added enzymes.

Keywords: Nanoparticle, bovine serum albumin (BSA), β -lactoglobulin (BLG), particle size distribution, degradation, human serum albumin (HSA)

Introduction

Nanoparticles are structured with a dense polymeric network in which an active molecule may be dispersed (Nakache et al. 2000). Nanoparticles offer the feasibility to entrap drugs or bioactive compounds within but not chemically bound to them. Since nanoparticles are sub-micron and sub-cellular in size, they have versatile advantages for targeted, site-specific delivery purposes (Vinogradov et al. 2002) as they can penetrate circulating systems and target sites.

Various biocompatible and biodegradable biopolymers have been used in the formation of nanoparticles to maximize delivery efficiency and increase the desirable benefits (Kreuter 1994; Coester et al. 2000; Rhaese et al. 2003). Albumin nanoparticles have been extensively investigated with respect to their preparation methods and release properties

Correspondence: Sundaram Gunasekaran, Department of Biological Systems Engineering, 460 Henry Mall, University of Wisconsin-Madison, Madison, WI 53706, USA. Tel: 608 262 1019. Fax: 608 262 1228. E-mail: guna@wisc.edu

ISSN 0265–2048 print/ISSN 1464–5246 online © 2006 Informa UK Ltd.

DOI: 10.1080/02652040601035143

(Vural et al. 1990; Lin et al. 1993; Langer et al. 2003). Human serum albumin (HSA) and bovine serum albumin (BSA) have been used as natural matrix materials for delivery devices (Brannon-Peppas 1995).

The delivery of protein particles in the body is mainly influenced by particle size and surface characteristics (Moghimi et al. 2001). Oral delivery systems confront problems such as their breakdown or major irritation caused by harsh environments of the digestive system in the body (Allemann et al. 1998). Desirable delivery systems should pass through the stomach and ultimately release loaded materials in target sites.

Bovine β -lactoglobulin (BLG) is the major component and the primary gelling agent of whey proteins. It is a small (18.3 kDa) globular protein with two disulphide bonds and one free thiol group which is inaccessible to solvent at or below neutral pH (Papiz et al. 1986). BLG is known to be stable at low pH and highly resistant to proteolytic degradation in the stomach. Because it can maintain a stable globular conformation, BLG is resistant to peptic and chymotryptic digestion (Reddy et al. 1988).

Emulsion and desolvation methods have been used for nanoparticle formation of proteins such as HSA, BSA and vicilin (Arshady 1990; Lin et al. 1993; Roser and Kissel 1993; Ezpeleta et al. 1996; Santhi et al. 2000, 2002; Arnedo et al. 2002; Langer et al. 2003). When using the emulsion method it is difficult to remove the oil phase and to obtain a narrow size distribution of the particles formed. However, the desolvation process has been successfully used to prepare HSA nanoparticles of ~ 100 nm diameter (Lin et al. 1993). During particle formation, protein solutions undergo conformational changes with various properties depending on the type of protein, concentration, cross-linking methods and environmental conditions, especially pH (Lin et al. 1993; Langer et al. 2003). The conformational changes in a protein, i.e. the unfolding of protein structure, expose its interactive sites such as disulphide bonds and thiol groups (Kinsella and Whitehead 1989). Subsequently, cross-linking leads to the formation of a network which allows particles to entrap bioactive compounds. For manufacturing particles with an appropriate size distribution and surface properties, a balance between attractive and repulsive forces is necessary. During the particle formation, unfolding of a globular protein makes its disulphide bonds, thiol groups and hydrophobic regions exposed to exterior, which increases intra-molecular cross-linking but decreases hydrophobic interaction (Clark et al. 1981; Harwalkar and Kalab 1985; Ezpeleta et al. 1996). Thus, size and surface properties of protein particles depend on the number of disulphide bonds and thiol groups, degree of unfolding, electrostatic repulsion among protein molecules, pH and ionic strength.

It is hypothesized that increasing unfolding and decreasing hydrophobic interaction of protein molecules are important for preparing nanoparticles of desirable size. Thus, small molecular weight, highly-unfolding and less hydrophobic protein is preferred. Since BLG is smaller and less hydrophobic than BSA, it is a good candidate for preparing nanoparticles. In addition, the optimization of manufacturing procedures to increase unfolding and decrease hydrophobic interaction of protein molecules, e.g. pH far from pI, and pre-heating of protein solution to increase protein unfolding are important. Most of the investigations on protein-based nanoparticles for drug carriers have been studied with albumin nanoparticles or microparticles originating from bovine and human serum; few studies have dealt with the preparation of BLG microparticles (Bhattacharjee and Das 2001). Therefore, this study focused on the preparation of BLG nanoparticles.

The objectives were to: (1) prepare and characterize BLG nanoparticles using the desolvation method, (2) determine the effect of pre-heating on the size and distribution of nanoparticles formed, (3) determine degradation stability of BLG nanoparticles in acidic

and neutral conditions in the presence of proteolytic enzymes and (4) prepare BSA nanoparticles as a reference under similar conditions.

Materials and methods

Materials

BLG (bovine, minimum 80%) and BSA (fraction V, minimum 97%) were obtained from Sigma-Aldrich Co. (St. Louis, MO). Analytical grade glutaraldehyde (50% solution), acetone and other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Preparation of BLG and BSA nanoparticles

BLG and BSA nanoparticles were prepared by a desolvation method (Marty et al. 1978; Lin et al. 1993; Weber et al. 2000; Langer et al. 2003). Two percent (w/v) solution of BLG or BSA in 10 mM NaCl at pH 9.0 was stirred with a magnetic bar at 500 rpm and room temperature. Acetone, a desolvating agent, was added at a rate of 1 mL min⁻¹ until the solution became just turbid. The rate of acetone addition was controlled carefully since it also influences the resulting particle size (Langer et al. 2003). The amounts of acetone added for BLG and BSA formation were 22.5 and 13.0 mL, respectively. At the end of the addition of acetone, the final pH of both solutions was 8.1. After desolvation, 0.01 mL of a 4% glutaraldehyde-ethanol solution was added to induce particle cross-linking by stirring continuously at room temperature for 3 h.

The nanoparticles formed were purified by five cycles of centrifugation and dispersion. For each centrifugation step, BLG and BSA solutions were centrifuged at 25 000 g (Optima LE-80K, Beckman Coulter Inc., Fullerton, CA) for 30 min. After centrifugation, BLG and BSA pellets were redispersed to the original volume of acetone solution at pH 9.0 to prevent particle aggregation. Each redispersion step was performed in an ultrasonication bath (Branson, Danbury, CT). Free BLG or BSA and excess cross-linking agent were removed from the particles through the purification steps. The resulting nanoparticles were stored in absolute ethanol at 4°C.

Pre-heating and pH adjustment of BLG solution

In order to decrease the size of the nanoparticles, the BLG solution prepared as above was heated in a water bath at 60°C for 30 min before desolvation. During acetone addition, solution pH lowered but was subsequently readjusted to 9.0. This step was not performed for BSA.

Determination of particle size and zeta-potential

Average size, distribution and zeta-potential of BLG and BSA nanoparticles were determined by photon correlation spectroscopy (PCS) using a commercial particle size analyser (Zetasizer 3000HSA, Malvern Instruments Ltd., Malvern, UK) which employs a 10-mW Helium-Neon laser operating at 633 nm. For size measurement, the nanoparticles prepared were dispersed in 10 mL of distilled water at a 1:400 (w/v) ratio and the pH was adjusted to 9.0 with NaOH. The dispersions were stirred continuously at room temperature for 30 min and then measured at 25°C with a scattering angle of 90°. The average particle size and its distribution were determined using the commercial software (PCS: zeta mode v 1.51, Malvern Instruments Ltd., Malvern, UK). For the

zeta-potential measurement, 10 mL of nanoparticle dispersion at a 1:400 (w/v) ratio was prepared at 10 pre-defined pH values between 2–11. Both average particle size and zeta-potential measurements were repeated three times for every sample.

Atomic force microscopy (AFM)

Size and morphology of BLG nanoparticles were measured using AFM which scans topological shape of a specimen without any artifact. AFM is an alternative method to determine the size of particles but observes details of individual particles while PCS measures average size of large group of particles. An AFM (MultiView 1000, Nanonics imaging Ltd, Jerusalem, Israel) system was used under tapping mode to measure size and topological shape of BLG nanoparticles. The BLG nanoparticles prepared were spread onto a mica surface and dried in air. The topography and error signal of the samples were generated by recording the vertical movements on the sample during scanning. The AFM scan area was $1 \times 1 \mu\text{m}^2$ and $2 \times 2 \mu\text{m}^2$, respectively, for BLG nanoparticles prepared with and without pre-heating. In both cases, the resolution was set at 348×348 pixels. The particles on the images were analysed to determine their size using the IMAQ vision builder software (v. 6.1, National instruments, Austin, TX). The average particle size was determined as four-times the average hydraulic radius. Hydraulic radius is defined as the ratio of particle area to particle perimeter.

In vitro degradation of BLG and BSA nanoparticles

When sub-100 nm protein nanoparticles are prepared, their stability is a concern. Lowering the particle size disproportionately increases the particle surface area in the surrounding media. Thus, diffusional penetration of water and particle erosion increases in a solution. To determine the degradation stability of BLG and BSA nanoparticles, *in vitro* degradation was performed at 37°C in pH 7.4 phosphate-buffered saline (PBS) following the procedure of Gopferich (1996) under acidic and neutral conditions. For the acidic condition, 30 mL of 0.1 M PBS solution adjusted to pH 2.0 was used with and without 0.6 mL of a 0.1% pepsin solution. For the neutral condition, 30 mL of PBS at pH 7.4 was used with and without 1000 BSEE unit/mL of a trypsin. Prior to mixing the particles, the solutions were pre-incubated at 37°C for 1 h. After mixing, the nanoparticle suspensions were incubated at 37°C in a horizontal shaker water bath at 10 shakings per min.

Aliquots of 1 mL of the solution were sampled at periodic intervals for 98 h. Each sample was centrifuged at 10 000 rpm for 5 min. The centrifugation precipitates regular particles but still disperses the degraded debris. The supernatant was removed and the pellet was redispersed in 1 mL of 20% trichloroacetic acid (TCA) solution. The redispersion step was performed in an ultrasonication bath (Branson, Danbury, CT) to disperse the particles completely. The absorbance of the particles in TCA solution was measured at 800 nm (Magee et al. 1995) and the absorbance was used to represent the uncorrupted BLG and BSA nanoparticles in acidic and neutral environments with and without a proteolytic enzyme.

Results and discussion

Particle size and zeta-potential

Figure 1 shows the distribution of BLG and BSA nanoparticles formed without pre-heating and pH adjustment. For the BLG nanoparticles, the peak of the distribution was at

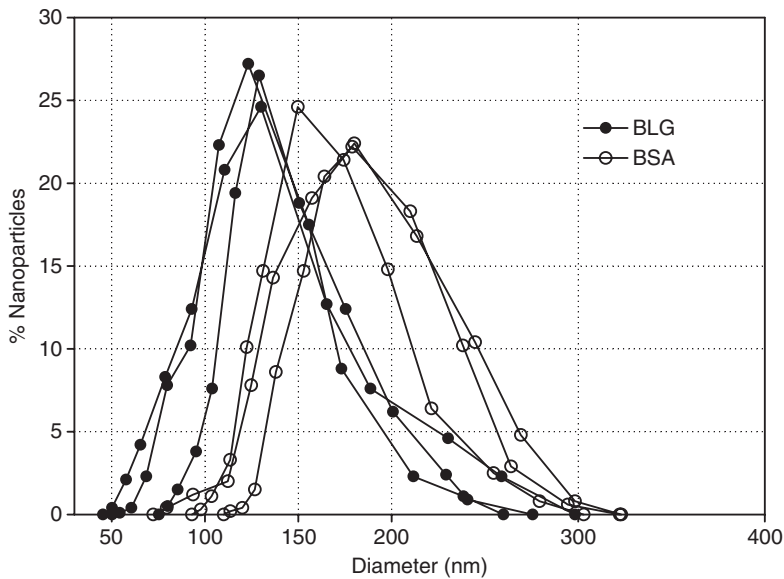


Figure 1. Particle size distribution of BLG and BSA nanoparticles prepared without pre-heating and pH adjustment BLG and BSA.

127 ± 4 nm whereas for BSA nanoparticles it was at 170 ± 17 nm. The number average diameters of BLG and BSA nanoparticles were 131 ± 8 nm and 175 ± 11 nm, respectively. From the particles size distribution data the size range around the peak which contains 80% of the particles was calculated. One half of this 80% particle bandwidth was used as a measure of particle size dispersion. The half-widths of 80% particle bandwidth for BLG and BSA nanoparticles were 36 ± 10 nm and 47 ± 6 nm, respectively. These values are compared in Figure 2.

The AFM (error signal) image of BLG nanoparticles formed is shown in Figure 3. The average size of BLG nanoparticles measured from AFM micrographs was 127 ± 50 nm, which is comparable to the number average particles size (131 ± 8 nm) measured by PCS. Figure 4 shows the zeta-potential of BLG and BSA nanoparticles. Both zeta-potential profiles are similar and decrease with increasing pH. The surface of both particles was charged positively at acidic condition and negatively at neutral and basic conditions with the transition occurring at their respective pIs.

Comparing the BLG and BSA particles, the BSA nanoparticles were larger with broader distribution than the BLG nanoparticles. The size of the BSA nanoparticles has been 250 nm for a phosphodiester oligonucleotide carrier (Arnedo et al. 2002), 120–550 nm from surface modification with primary amine (Roser and Kissel 1993), 400–820 nm for an anti-tumour 5-Fluorouracil carrier (Santhi et al. 2002) and 450–840 nm for Methotrexate delivery (Santhi et al. 2000) depending on the preparation conditions. The relative particles sizes of BLG and BSA can be explained by their surface charge and surface hydrophobicity. Both proteins are characterized by a high content of charged amino acids (Brown 1975; Papiz et al. 1986) with similar pI (Bottomley et al. 1990). At basic pH, the size of the protein aggregates as well as the void spaces within a particle generally decreases (Schmidt 1981). In addition, proteins are generally more unfolded at basic pH which exposes more reactive sites for cross-linking (Kinsella and Whitehead 1989). The unfolding of the BLG and BSA

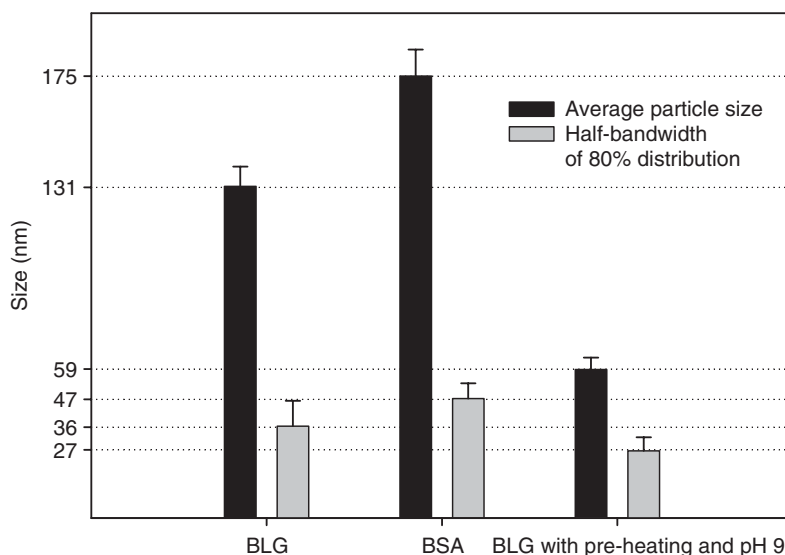


Figure 2. Average particle size and half-bandwidth of 80% distribution of BLG and BSA nanoparticles.

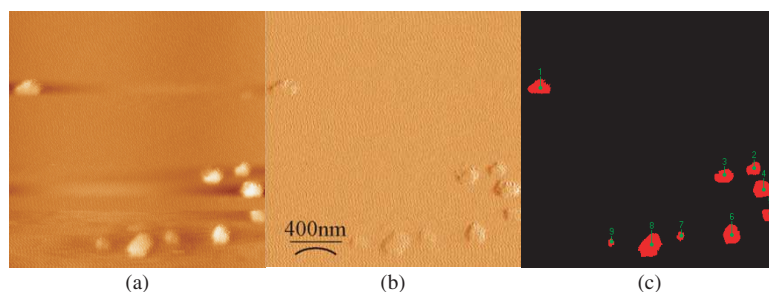


Figure 3. AFM (a) topography, (b) error signal image and (c) particle analysis of BLG nanoparticles without pre-heating and pH adjustment. Average particle size was 127 ± 50 nm.

molecule at basic pH increases thiol-disulphide interchange reaction, which may enhance particle formation but inhibit the formation of large aggregates. These BLG and BSA nanoparticles were manufactured at pH 9.0 whose molecules would have negative charge. This condition offered that both BLG and BSA solutions resulted in small particles charged negatively on their surface since coacervate precipitation was suppressed at pH 9.0.

Another critical factor, the surface hydrophobicity, dictates the propensity to bind non-polar amino acid groups to a hydrophobic part of its surface. Hydrophobic interactions between hydrophobic regions of unfolded polypeptide chains lead to their aggregation, resulting in size increment (Ismond et al. 1988). At basic pH, protein unfolding results in the change of the protein secondary structure. As pH increases, β -sheet formation in a protein increases due to an increase in hydrogen bonding (Krimm and Bandekar 1986). At basic pH, a thiol group or previously hidden hydrophobic groups in BLG becomes exposed and the thiol-disulphide interchange reaction is accelerated. The degree of unfolding is

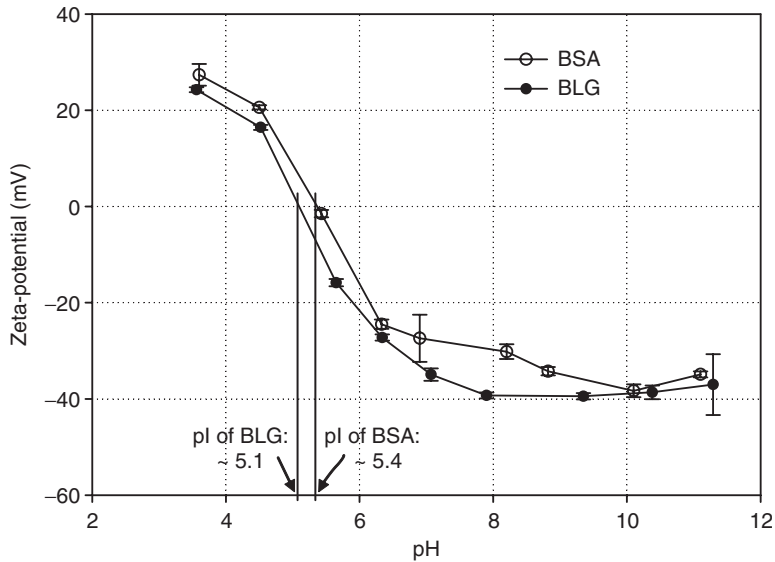


Figure 4. Zeta-potential of BLG and BSA nanoparticles.

related to the amino acid composition (Birdi 1976). The effective hydrophobicity of BLG and BSA is 12.2 and 18.2, respectively, which means BLG has a less hydrophobic portion than BSA (de Wit 1998). Thus, small hydrophobic interactions of BLG suppressed the aggregation of the molecules and then resulted in smaller particles rather than BSA particles. This result supports the original hypothesis with regards to lowering hydrophobic interaction as a means for decreasing the size of protein nanoparticles.

Effect of preheating and pH adjustment

Figure 5 shows the size distribution of BLG nanoparticles formed after pre-heating to 60°C and the pH readjusted to 9.0. The average particle size was 59 ± 5 nm with the majority of the particles of size 50 ± 4 nm. In addition, the half-bandwidth of 80% of particles was narrower (27 ± 5 nm) than what was obtained without pre-heating and pH readjustment. This is a substantial improvement in both lowering the average particle size and improving the particle size uniformity. Analysis of AFM images of BLG nanoparticles formed with pre-heating and pH adjustment (Figure 6) resulted in an average particle size of 51 ± 18 nm which is comparable to the PCS size data.

Pre-heating makes protein molecules unfold so that the hydrophobic interactions between them are suppressed, which reduces self-aggregation. Further, by maintaining pH 9.0 high repulsive forces were generated between BLG molecules and increased their unfolding. Therefore, it is thought that optimizing pre-heating and pH adjustment may be the key in preparing uniform BLG nanoparticles of sub-100-nm size range.

Degradation of nanoparticles

Degradation plots of the BLG and BSA nanoparticles under acidic and neutral conditions are shown in Figures 7 and 8, respectively. All the degradation curves exhibited a typical rapid initial decrease in absorbance followed by a fairly stable tail region. By linearizing

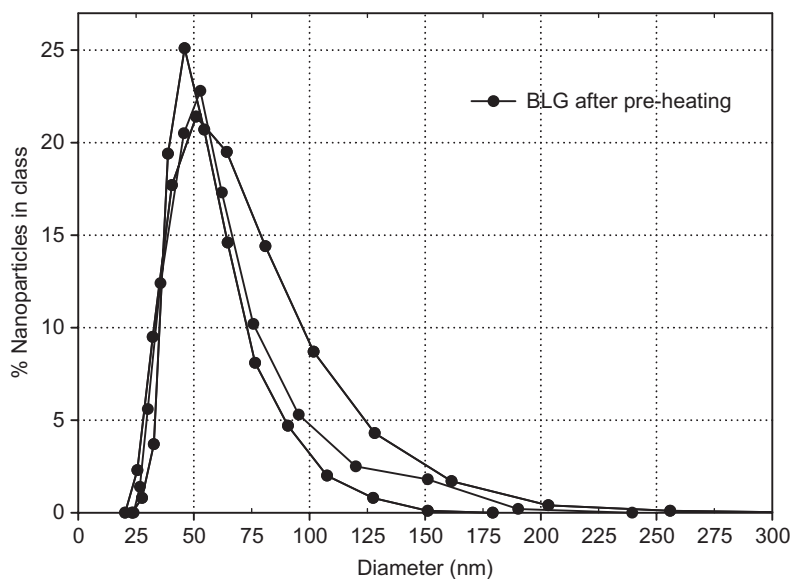


Figure 5. Particle size distribution of BLG nanoparticles prepared with pre-heating at 60°C and pH adjustment at 9.0.

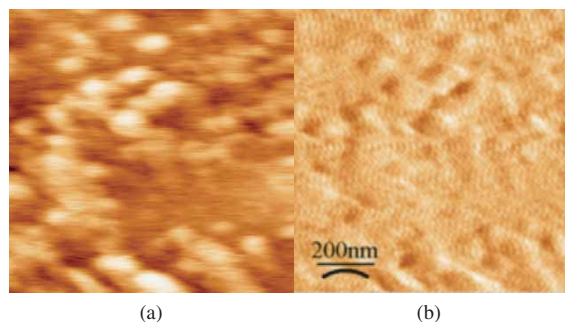


Figure 6. AFM (a) topography and (b) error signal image of BLG nanoparticles with pre-heating to 60°C and pH adjustment at 9.0. Average particle size is 51 ± 18 nm.

the initial and final regions, as illustrated in Figure 9, the degradation time (D_t) was determined at the intersection of the two linear segments. The average D_t values are listed in Table I.

BLG particles were relatively more stable in the acidic environment (pH 2.0) than the BSA nanoparticles (Figure 7). The BSA particles degraded completely after 7.4 h (Table I). At the acidic pH far from pI, BSA is known to unfold fully, but BLG unfolds only partially (Kinsella and Whitehead 1989). As unfolding continues, it is easier for hydration and proteolytic degradation to occur. The degradation rates increased substantially for both BLG and BSA when pepsin was added, though BLG was relatively more stable than BSA. The D_t values of BLG and BSA nanoparticles were 7.3 and 5.6 h, respectively,

At neutral pH of 7.4, both BLG and BSA nanoparticles were highly stable (Figure 8). Only less than 20% of the initial amount degraded over 4 days. Addition of trypsin

Table I. Degradation time (D_t) of the BLG and BSA nanoparticles.

Degradation time	Acidic condition (pH 2.0)				Neutral condition (pH 7.4)			
	No enzyme		Pepsin		No enzyme		Trypsin	
	BLG	BSA	BLG	BSA	BLG	BSA	BLG	BSA
D_t (h)	22.1±	7.4±	7.3±	5.6±	—*	—	15.4±	8.1±
	12.4	2.0	0.3	0.5			1.4	1.8

*No intersection of the two linear segments.

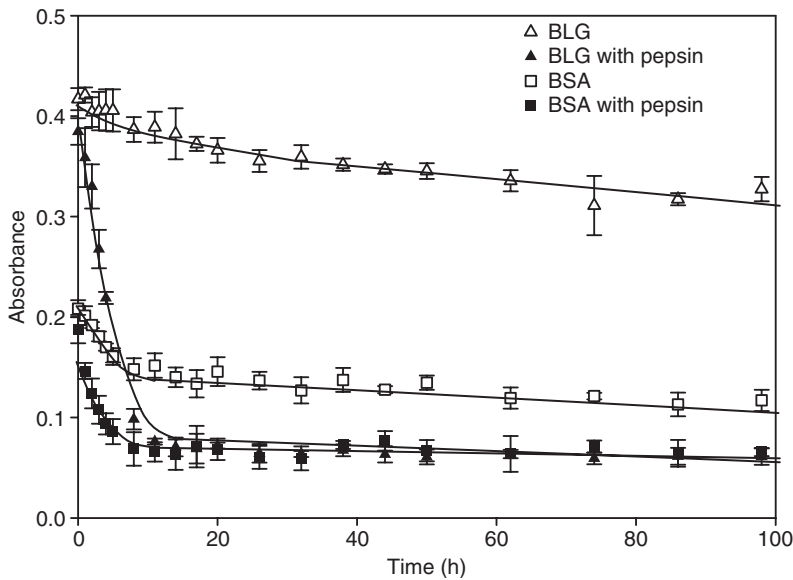


Figure 7. Degradation of BLG and BSA nanoparticles with and without pepsin at pH 2.0.

accelerated their degradation. However, BLG particles were relatively more stable than BSA particles as measured by D_t . For BLG particles D_t was 15 h and for BSA particles it was 8.1 h. At neutral pH, trypsin is expected to attack specific sites on the surface and in the interior of the protein particles. The number of susceptible peptide bonds is likely important in determining the rate and extent of degradation. For trypsin, it has been reported that the main site at which hydrolysis occurs is the carboxyl group of basic amino acids, such as lysine and arginine (Magee et al. 1995). BLG has 15 lysine and three arginine residues whereas BSA has 59 lysine and 23 residues (Brown 1975; Papiz et al. 1986; Carter and Ho 1994). The portions of lysine and arginine residues in BLG are 9.3 and 1.9%, respectively, while those in BSA are 10.2 and 4.0%. In the PBS containing trypsin (pH 7.4), the BLG particles degraded more slowly than the BSA particles. The proteolytic enzyme activity on the surface of the BLG particles were less since the amount of basic amino acids was limited.

Several factors such as particle preparation technique, degradation environments, enzyme activity, surface area, porosity, tortuosity and size can affect the degradation on the matrix of protein nanoparticles. The development of a dense cross-linking matrix for nanoparticles

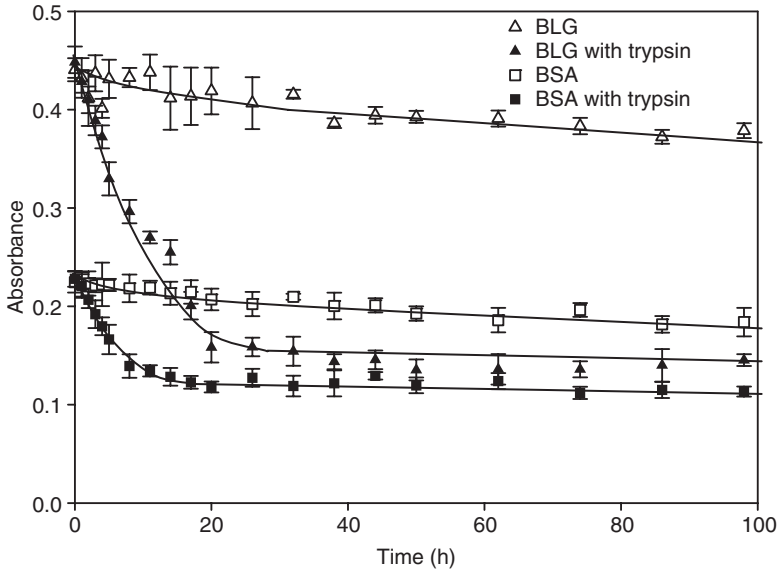


Figure 8. Degradation of BLG and BSA nanoparticles with and without trypsin in pH 7.4 PBS.

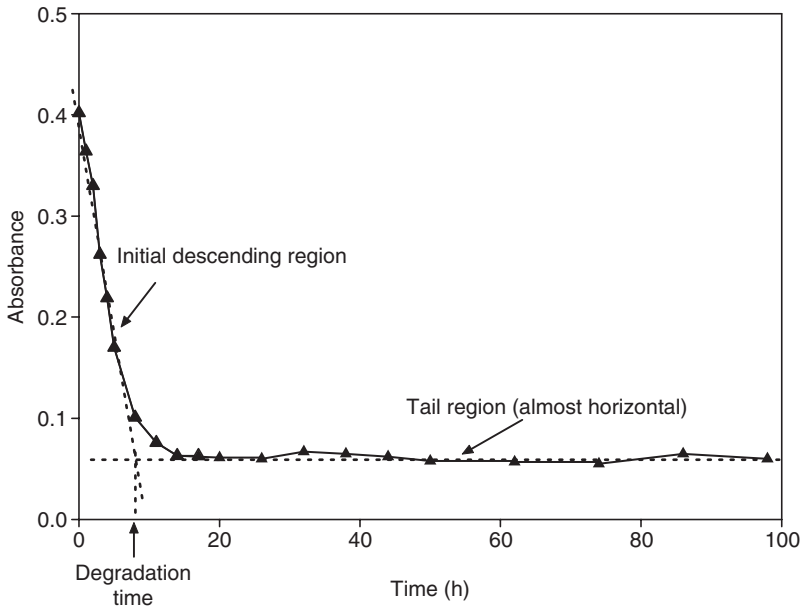


Figure 9. Determination of degradation time (D_t) using linear fits to the initial and tail regions (shown by dotted lines) of the absorbance *vs.* time curves. D_t is the time at which the two straight lines intersect.

offers resistance against the proteolytic degradation since it is difficult for the enzymes to penetrate into the particles. For BLG and BSA nanoparticles manufactured under similar processing conditions, the BLG showed better resistance against enzyme degradation under both neutral and acidic environments. Only 11.2% basic amino acid residues retarded the

hydrolysis of the BLG nanoparticles. Thus, one could attribute the resistance of the BLG nanoparticles against the enzyme attack to the dense structure and small portion of basic amino acid composition.

Conclusions

BLG nanoparticles were prepared in the sub-100-nm size range with a narrow size distribution by a desolvation method. Pre-heating of the BLG solution to 60°C and subsequent pH adjustment to 9.0 helped to lower particle size. The average particle size obtained with and without the pre-heating step were 131 ± 8 nm and 59 ± 5 nm, respectively. The pre-heating step also improved the uniformity of the particles as measured by the half-bandwidth of 80% distribution. Under similar conditions, BLG nanoparticles were smaller and more uniform than those produced using BSA due to lower hydrophobic interactions of BLG than that of BSA. The BLG nanoparticles were also more stable than BSA nanoparticles in acidic and neutral environments with and without an added proteolytic enzyme. This could be attributed to the denser matrix structure and to a lesser amount of the basic amino acid composition of BLG compared to BSA.

References

- Allemann E, Jean-Christophe L, Gurny R. 1998. Polymeric nano- and microparticles for the oral delivery of peptides and peptidomimetics. *Advanced Drug Delivery Reviews* 34:171–189.
- Arnedo A, Espuelas S, Irache JM. 2002. Albumin nanoparticles as carriers for a phosphodiester oligonucleotide. *International Journal of Pharmaceutics* 244:59–72.
- Arshady R. 1990. Albumin microspheres and microcapsules: Methodology of manufacturing techniques. *Journal of Controlled Release* 14:111–131.
- Bhattacharjee C, Das KP. 2001. Characterization of microcapsules of beta-lactoglobulin formed by chemical cross linking and heat setting. *Journal of Dispersion Science and Technology* 22:71–78.
- Birdi KS. 1976. Interaction of insulin with lipid monolayers. *Journal of Colloid and Interface Science* 57:228–232.
- Bottomley RC, Evans MTA, Parkinson CJ. 1990. Whey proteins. In: Harris P. *Food gels*. London: Elsevier Applied Science. pp 435–466.
- Brannon-Peppas L. 1995. Recent advances on the use of biodegradable microparticles and nanoparticles in controlled drug delivery. *International Journal of Pharmaceutics* 116:1–9.
- Brown JR. 1975. Structure of bovine serum albumin. *Federation Proceedings* 34:591.
- Carter DC, Ho JX. 1994. Structure of serum albumin. *Advances in Protein Chemistry* 45:153–203.
- Clark AH, Judge FJ, Richards JB, Stubbs JM, Suggett A. 1981. Electron microscopy of network structures in thermally-induced globular protein gels. *International Journal of Peptide and Protein Research* 17:380–392.
- Coester C, Kreuter J, Briesen Hv, Langer K. 2000. Preparation of avidin-labelled gelatin nanoparticles as carriers for biotinylated peptide nucleic acid (PNA). *International Journal of Pharmaceutics* 196:147–149.
- De Wit JN. 1998. Nutritional and functional characteristics of whey proteins in food products. *Journal of Dairy Science* 81:597–608.
- Ezpeleta I, Irache JM, Stainmesse S, Gueguen J, Orecchioni AM. 1996. Preparation of small-sized particles from vicilin (vegetal protein from *Pisum sativum* L) by coacervation. *European Journal of Pharmaceutics and Biopharmaceutics* 42:36–41.
- Gopferich A. 1996. Mechanisms of polymer degradation and erosion. *Biomaterials* 17:103–114.
- Harwalkar VR, Kalab M. 1985. Thermal-denaturation and aggregation of beta-lactoglobulin at pH 2.5 – effect of ionic-strength and protein-concentration. *Milchwissenschaft* 40:31–34.
- Ismond MAH, Murray ED, Arntfield SD. 1988. The role of non-covalent forces in micelle formation by vicilin from *Vicia Faba*. The effect of urea, guanidine hydrochloride and sucrose on protein interactions. *Food Chemistry* 29:189–198.
- Kinsella JE, Whitehead DM. 1989. Proteins in whey: Chemical, physical, and functional properties. *Advances in Food and Nutrition Research* 33:343–438.

- Kreuter J. 1994. Nanoparticles. In: Kreuter J. Colloidal drug delivery systems. New York: Marcel Dekker. pp 219–342.
- Krimm S, Bandekar J. 1986. Vibrational spectroscopy and conformation of peptides, polypeptides, and proteins. *Advances in Protein Chemistry* 38:181–364.
- Langer K, Balthasar S, Vogel V, Dinauer N, Briesen Hv, Schubert D. 2003. Optimization of the preparation process for human serum albumin (HSA) nanoparticles. *International Journal of Pharmaceutics* 257:169–180.
- Lin W, Coombes AGA, Davies MC, Davis SS, Ilium L. 1993. Preparation of Sub-100 nm human serum albumin nanospheres using a pH-coacervation method. *Journal of Drug Targeting* 1:237–243.
- Magee GA, Halbert GW, Wilmott N. 1995. Effect of process variables on the in-vitro degradation of protein microspheres. *Journal of Controlled Release* 37:11–19.
- Marty JJ, Oppenheimer RC, Speiser P. 1978. Nanoparticles-a new colloidal drug delivery system. *Pharmaceutica Acta Helveticae* 53:17–23.
- Moghimi SM, Hunter AC, Murray JC. 2001. Long-circulating and target-specific nanoparticles: Theory to practice. *Pharmacological Reviews* 58:283–318.
- Nakache E, Poulain N, Candau F, Orecchioni AM, Irache JM. 2000. Biopolymer and polymer nanoparticles and their biomedical applications. In: Nalwa HS, editor. *Handbook of nanostructured materials and nanotechnology: Organics, polymers, and biological materials*. San Diego: Academic Press. pp 577–635.
- Papiz MZ, Sawyer L, Eliopoulos EE, North ACT, Findlay JBC, Sivaprasadarao R, Jones TA, Newcomer ME, Kraulis PJ. 1986. The structure of beta-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* 324:383–385.
- Reddy IM, Kella NKD, Kinsella JE. 1988. Structural and conformational basis of the resistance of D-lactoglobulin to peptic and chymotryptic digestion. *Journal of Agricultural and Food Chemistry* 36:737–741.
- Rhaese S, Briesen Hv, Rubsamen-Waigmann H, Kreuter J, Langer K. 2003. Human serum albumin-polyethylenimine nanoparticles for gene delivery. *Journal of Controlled Release* 92:199–208.
- Roser M, Kissel T. 1993. Surface-modified biodegradable albumin nanospheres and microspheres. 1. Preparation and characterization. *European Journal of Pharmaceutics and Biopharmaceutics* 39:8–12.
- Santhi K, Dhanaraj SA, Joseph V, Ponnusankar S, Suresh B. 2002. A study on the preparation and anti-tumor efficacy of bovine serum albumin nanospheres containing 5-fluorouracil. *Drug Development and Industrial Pharmacy* 28:1171–1179.
- Santhi K, Dhanaraj SA, Koshy M, Ponnusankar S, Suresh B. 2000. Study of biodistribution of methotrexate-loaded bovine serum albumin nanospheres in mice. *Drug Development and Industrial Pharmacy* 26:1293–1296.
- Schmidt RH. 1981. Gelation and coagulation. In: Cherry JP, editor. *Protein functionality in foods*. Washington, DC: American Chemical Society. p 131.
- Vinogradov SV, Bronich TK, Kabanov AV. 2002. Nanosized cationic hydrogels for drug delivery: Preparation, properties and interactions with cells. *Advanced Drug Delivery Reviews* 54:223–233.
- Vural I, Kas HS, Hincal AA, Cave G. 1990. Cyclophosphamide loaded albumin microspheres: 2. Release characteristics. *Journal of Microencapsulation* 7:511–516.
- Weber C, Coester C, Kreuter J, Langer K. 2000. Desolvation process and surface characteristics of protein nanoparticles. *International Journal of Pharmaceutics* 194:91–102.