Cationic beta-lactoglobulin nanoparticles as a bioavailability enhancer: effect of surface properties and size on the transport and delivery *in vitro*

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ABSTRACT

BLG (beta-lactoglobulin) and CBLG (cationic BLG developed by our lab) were evaluated as potential nutraceutical/drug carriers. The cationic corona conferred CBLG with superior integrity and drug retention under gastrointestinal conditions, at most 40-fold higher mucoadhesion, up to 30-fold greater transepithelial permeation and, at most, 285% higher cellular uptake, compared to BLG. Furthermore, the more hydrophilic CBLG species exhibited better mucoadhesion, while the more hydrophobic one exhibited higher cellular uptake. Intriguingly, protein molecules were more cytotoxic and exhibited up to 175% higher tight junction-opening capacity than did protein nanoparticles, whereas the nanoparticles displayed up to 770% higher mucoadhesion, greater transepithelial permeation, and elevated cellular uptake. Finally, all these surface properties and performances were significantly altered as CBLG bound to serum proteins. Possible mechanisms underlying these findings are considered in detail. This research sheds some light on the development of protein-based nanoencapsulants and their performance upon oral administration.

Keywords: Beta-lactoglobulin (BLG); Cationic polymers; Surface properties; Nanoparticles; Encapsulation; Delivery systems; Mucoadhesion

33 **List of abbreviations**

- 34 BLG : (bovine) beta-lactoglobulin
- 35 CBLG: cationic beta-lactoglobulins. These include C-EDA and C-P600 in this study.
- 36 EDA: ethylenediamine
- 37 PEI: (branched) polyethyleneimine
- 38 EDC: N-(3-dimethylaminopropyl)-N-ethylcarbodiimide
- 39 C-EDA: ethylene-derived cationic beta-lactoglobulin
- 40 C-P600: polyethyleneimine (MW 600)-derived cationic beta-lactoglobulin
- 41 PBS: phosphate buffer saline
- 42 HBSS: Hank's balanced salt solution
- 43 DMEM: Dulbecco's modified Eagle's medium
- 44 FBS: fetal bovine serum
- 45 BSA: bovine serum albumin
- 46 FITC: fluorescein isothiocyanate
- 47 PSM: porcine stomach mucin
- 48 TEER: transepithelial electrical resistance
- 49 MTT assay: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide assay
- 50 DLS: dynamic light scattering
- 51 QCM: quartz crystal microbalance
- 52 GI tract: gastrointestinal tract
- 53 SGF: simulated gastric fluid
- 54 SIF: simulated intestinal fluid

1. Introduction

Protein-based nanoparticles are gaining increasing interest as bioavailability enhancers of poorly absorbed nutraceuticals and drugs. Proteins are naturally abundant, biodegradable, and biocompatible (Hawkins, Soon-Shiong, & Desai, 2008; Panyam & Labhasetwar, 2003). Moreover, they are amphiphilic polymers which could interact adequately with both the solvent and different types of drugs. Compared to polysaccharides that possess rigid polymeric chains (Ogawa, Yui, & Okuyama, 2004), proteins tend to adopt a more compact and flexible conformation that favours the formation of nanoparticles and entrapment of nutraceuticals/drugs. Beta-lactoglobulin (BLG) is a major component of whey protein. It is a highly water-soluble globulin that possesses inherent patches for binding lipophilic nutrients (Dufour, Genot, & Haertlé, 1994). It also exhibits a unique resistance against pepsin and moderate digestibility by trypsin, both of which imply the possibility of preparing it as a delivering vehicle with controlled release properties. Recently, we have developed a novel protein derivative, cationic beta-lactoglobulin (CBLG), which inherited the advantages of native BLG, and exhibited remarkable mucoadhesion due to its positive surface charge (Teng, et al., 2014). Like native BLG, CBLG self-assembled into nanoparticles in the presence of organic solvent, suggesting its potential for nutraceutical/drug carriers. Therefore, it is of our great interest to evaluate the nutraceutical/drug delivering capacities of BLG- and CBLG-based systems.

The transport and delivery of the encapsulated nutraceutical/drugs in the human body is a complex process that involves a cascade of biological processes, ranging from digestion to their

internalization by targeted cells or tissues. Many of these processes require delicately designed nanocarriers with desirable surface properties, which facilitate the interaction with specific biological surfaces or compounds. In this study, we choose BLG- and CBLG-based nanocarriers as model delivering systems. The superior performances of CBLG over BLG in a few key biological processes will be first demonstrated through a set of *in vitro* tests. Thereafter, the different behaviours of these nanocarriers will be explained by their structures and surface properties. Specifically, the contrast between CBLG and BLG represented the role of surface charge. Moreover, since the two CBLG species synthesized in our study exhibited similar surface charges but distinct surface hydrophobicities, a comparison between them may illustrate the importance of hydrogen bonds and hydrophobic interactions. Additionally, investigations of the different performances between protein molecules and nanoparticles may provide some useful information on the effect of size. Lastly, the influence of buffers and serum proteins in the physiological environment will be assessed.

2. Materials and methods

2.1. Materials

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): bovine BLG (90% purity), ethylenediamine dihydrochloride (EDA, 98% purity), branched polyethyleneimine (MW 600, abbreviated as P600), fluorescein isothiocyanate (FITC) N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC, 97% purity), curcumin (98% purity),

pepsin (3,200-4,500 units/mg), and trypsin (10,000 BAEE units/mg). Porcine stomach mucin (PSM, type III, that contains 0.5-1.5% sialic acid) was obtained from Himedia Co., India. All reagents involved in cell study (e.g. cell culture medium, PBS) were purchased from Life Technologies (Grand Island, NY, USA). All other reagents (e.g. Tween 20, simulated gastric and intestinal fluid) were of analytical grade.

2.2. Synthesis and characterization of CBLG molecules and nanoparticles

CBLG was synthesized via an EDC-aided coupling reaction, and nanoparticles were prepared with BLG and CBLG by organic solvent desolvation, as previously described (Teng, Li, Luo, Zhang, & Wang, 2013). In brief, the coupling process was initiated by incubating BLG and cationizer (EDA or PEI) at pH 4.75 in the presence of EDC, and it was terminated after 4 h by adding sodium acetate buffer. The product was dialyzed to remove unreacted cationizer molecules and freeze-dried afterwards. To produce the nanoparticles, the protein was dissolved in deionized water at pH 7.5 at 0.75 to 1 mg/ml, mixed with acetone to initiate particle formation, crosslinked by glutaraldehyde, and evaporated to remove acetone. The obtained dispersion containing the nanoparticles was filtered through a 0.22 μ m Acrodisc syringe filter membrane (Pall Co., Newquay, UK). The filtrate was then subjected to dialysis centrifugation (5,000 g, 30 min, molecular weight cutoff = 100 kDa) to remove free protein molecules and unreacted crosslinkers. Afterwards, the retentate was diluted properly to achieve a nanoparticle concentration of 5 mg/ml, which was determined by weight measurement after lyophilization. The CBLG prepared, using EDA and P600 as cationizers, were defined as C-EDA and C-P600,

114 respectively. The nanoparticles were defined as the acronyms of the proteins, followed by the
115 term “NPs”. *Detailed procedures for synthesizing CBLG molecules and nanoparticles are*
116 *included in the Supplementary Information.*

117 The dispersions of BLG and CBLG (both C-EDA and C-P600, same hereinafter) molecules and
118 nanoparticles were diluted in three buffers: Hank’s balanced salt solution (HBSS, pH 7.0),
119 Dulbecco's modified Eagle's medium (DMEM, pH 7.4), and DMEM supplemented with fetal
120 bovine serum (FBS). The dispersions in DMEM with FBS were subjected to dialysis
121 centrifugation, as described above, to remove unbound serum protein, while the other dispersions
122 were measured without such treatment. All the dispersions were measured for their zeta
123 potentials by laser Doppler velocimetry, using a Nano ZS90 Zetasizer (Malvern Inc., Malvern,
124 UK). The surface hydrophobicity (So) of the samples was measured, using
125 8-anilino-1-naphthalenesulfonic acid (ANS) as a hydrophobic probe (Laligant, Dumay, Casas
126 Valencia, Cuq, & Cheftel, 1991). The particle sizes and count rates (an index that reflected the
127 total amount of scattered photons) were measured separately by dynamic laser scattering (DLS).
128 No dilution was carried out before the measurement. The data were analyzed using the cumulant
129 algorithm, and the z-average diameter was reported. *Complete procedures for these*
130 *measurements are included in Supplementary Information.*

131 2.3. Determination of releasing profile and particle disintegration

132 Curcumin, as a model drug, was incorporated into BLG or CBLG nanoparticles, which were
133 analyzed for their releasing profiles following a previously reported method (Teng, Li, & Wang,

2014). The release was initiated by mixing 2 ml of the sample with 8 ml of SGF (commercial SGF containing 5 mg/ml of Tween 20 and pepsin at a substrate/enzyme ratio of 100:1, w/w), followed by incubation at 37 °C in a reciprocal shaking bath (120 rpm). At predetermined time intervals, 0.5 ml of the mixture was withdrawn, diluted with 2.0 ml of PBS (pH 7.2, 100 mM, containing 5 mg/ml of Tween 20) and analyzed for its particle size and count rate by DLS. Another aliquot of 0.5 ml was withdrawn and diluted, using the same procedure, followed by dialysis centrifugation (5,000 g, 30 min), using a Macrosep® centrifuge tube (MW cutoff = 100 kDa, Pall Corp., Ann Harbor, MI). The curcumin-containing Tween-20 micelles were recovered from the filtrate, diluted appropriately with PBS, and then measured for the content of released curcumin spectrophotometrically at the wavelength of 426 nm. When a surfactant, such as Tween 20, is added, the released curcumin maintained its stability, as was demonstrated in previous reports (Lin, Lin, Chen, Yu, & Lee, 2009). A fresh mixture of SGF and Tween 20 was supplemented after each measurement to maintain sink condition.

Meanwhile, a further batch of the samples was mixed with SGF at the same ratio and digested for 60 min at 37 °C. The digesta were diluted with PBS and centrifuged as mentioned above, and the retentate containing intact nanoparticles was supplemented with PBS to 2 ml. The dispersion was incubated at 37 °C for 15 min and then mixed with 8 ml of preheated SIF (commercial SIF treated with 5 mg/ml of Tween 20 and trypsin at a BLG/trypsin ratio of 100:1, w/w). The drug release, particle size, and count rate in SIF were monitored for up to 4h, following the similar procedure as for SGF.

2.4. Determination of mucoadhesive properties

The mucoadhesion of BLG/CBLG molecules and nanoparticles was assessed by quartz crystal microbalance (QCM) analysis (Wiecinski, et al., 2009), using a gold-coated AT-cut quartz crystal with a fundamental frequency of 4.95 MHz (QSX-301, Q-Sense Co., Linthicum, MD, USA). In brief, the crystal was washed, dried, and then mounted on a Q-Sense E1 microbalance (Q-Sense Co., Linthicum, MD, USA). Different dispersions were passed through the sample chamber in the following sequence: PBS, mucin dispersion (400 µg/ml in PBS, filtered through a 220 nm membrane), PBS, sample dispersion (diluted with PBS to 100 µg/ml), and PBS. The frequency change due to the deposition of biomacromolecules on the crystal was recorded by QTools 3 software (Q-Sense Co., Linthicum, MD, USA), and the fifth overtone of the frequency was converted to deposited mass (in ng/cm² crystal surface), using the Sauerbrey model. Mucoadhesion was expressed as the mass ratio of different samples to mucin deposited on the crystal surface.

2.5. Cell culture

Human colon adenocarcinoma cell line (Caco-2) was provided by the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10% (v/v, same in this section) FBS and 1% penicillin-streptomycin in a humidified incubator with 5% CO₂ at 37 °C. The growth medium was changed every other day, and the cells were subcultured after reaching 80-90% confluence.

2.6. Transport of nanoparticles via Caco-2 cell monolayer

Caco-2 monolayer was cultured and treated with different samples to evaluate the permeability across the small intestine lining according to Hubatsch, Ragnarsson, & Artursson (2007) with slight modification. Meanwhile, another set of the Caco-2 monolayers was incubated with the same batch of samples and monitored for its transepithelial electrical resistance (TEER) for 2 h. The samples were then aspirated and replaced with fresh HBSS, and the recovery of TEER was measured for a further 22 h. *Procedures for these measurements are introduced in detail in the Supplementary Information section.* For both measurements, the monolayers treated with sample-free HBSS were assayed as control (for TEER) or background (for cell uptake).

2.7. Cellular uptake

Prior to the cellular uptake study, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was carried out to establish the non-toxic range of concentrations (Das, Kasoju, & Bora, 2010). Thereafter, BLG/CBLG molecules and empty nanoparticles were fluorescently labelled, using an FITC labelling kit (Thermo Fisher Scientific Inc., Rockford, IL, USA), following manufacturer's protocol. The reasons why curcumin-loaded nanoparticles were not adopted in this part were: (1) the considerable cellular uptake of free curcumin due to its hydrophobicity, (2) the overlapping fluorescent spectra of curcumin and FITC, and (3) the possible conformational change of proteins induced by the incorporation of curcumin. The cells were seeded in a black 96-well plate at 3×10^4 cells/well, incubated at 37 °C to achieve 80%

confluence, treated with different concentrations (50, 100, and 200 µg/ml) of samples for 4 h, washed three times with PBS, disintegrated with a lyse buffer (0.5% Triton X-100 in 0.2 M NaOH solution), and finally measured for fluorescence intensity, using a multilabel microplate reader (Victor X3, PerkinElmer 2030, PerkinElmer, Waltham, MA, USA). The cellular uptake was estimated as the fluorescence intensity of the lysate divided by that of the feed. *Detailed protocol for cell uptake measurement is presented in Supplementary Information.*

2.8. Statistics

All measurements were performed in triplicates. The results were expressed as means $\pm$ standard error. To identify statistically significant differences, the data were subjected to analysis of variance, followed by Tukey's test with an experimentwise confidence level of $\alpha=0.10$, using SAS 9 software (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Characterization of BLG and CBLG nanoparticles

Table 1 illustrates the structures of BLG/CBLG molecules and nanoparticles, and it compares several characteristics that are important for nutraceutical/drug delivery. Interestingly, the surface hydrophobicity (S_o) of C-EDA was 13-fold higher than that of BLG, whereas the S_o of C-P600 was 60% lower than that of BLG, although both C-EDA and C-P600 carried a similar amount of positive surface charge. When the samples were dispersed in biological buffers, such as HBSS (pH 7.0) and DMEM (pH 7.4), the zeta potential decreased (in terms of the absolute value, same

hereinafter) by up to 48%, together with a significant decrease in the surface hydrophobicity. When FBS was introduced into DMEM, the zeta potentials for CBLG molecules and nanoparticles changed their signs to negative, although their magnitudes were not significantly changed. In addition, all samples showed higher surface hydrophobicity and increased average size in the presence of FBS.

3.2. Particle disintegration and nutraceutical/drug release in the GI tract

The releasing profiles of BLG/CBLG nanoparticles are presented in Figure 2A. Using curcumin as a model drug and a protocol reported in our previous study (Teng, Li, & Wang, 2014), we observed a loading capacity and encapsulation efficiency of 9.7% and 98%, respectively, for all samples (preliminary data not shown); these values were considerably higher than those achieved with many other biopolymers, such as zein (Patel, Hu, Tiwari, & Velikov, 2010) and alginate-chitosan complex (Das, Kasoju, & Bora, 2010). When incubated in SGF, more than 80% of the encapsulated curcumin was released from BLG nanoparticles in the first 30 min, and the percentage continued to increase to 95% after the nanoparticles were placed in SIF. By contrast, less than 25% of the curcumin was released from CBLG nanoparticles.

We further investigated the stability of BLG/CBLG nanoparticles. When dispersed in SGF or SIF without Tween 20, the average diameters of the nanoparticles were comparable to those observed in HBSS (50~70 nm, preliminary data not shown) at the beginning of digestion. However, the initial particle size increased significantly to over 100 nm when Tween 20 was introduced. During the incubation in SGF, BLG nanoparticles showed an increase in their average size (by

6-fold) accompanied by a 39% decrease in the count rate (Figure 2C). When transferred into the SIF, both the size and count rate declined continuously. At the end of treatments, only 17% of the count rate was retained. These results suggested considerable disintegration of BLG nanoparticles. By contrast, CBLG nanoparticles maintained remarkable stability against peptic and tryptic digestions, as suggested by the nearly constant particle size and count rate.

3.3. Mucoadhesion

As shown in Figure 3A, the protein/mucin ratios of C-EDA and C-P600 nanoparticles reached 4.28 and 10.0, respectively. These values were over 15 and 40 times higher than that of BLG nanoparticles (0.254), and they were considerably higher than that achieved by other biopolymers such as chitosan (Chayed & Winnik, 2007). According to our previous report, CBLG molecules also displayed higher mucoadhesion than did BLG (Teng, Li, Luo, Zhang, & Wang, 2013). However, it was noteworthy that the C-EDA and C-P600 nanoparticles showed 770% and 475% higher mucoadhesion than did their constituent molecules, although the former exhibited less positive surface charges than did the latter (Table 1).

3.4. Interaction with the small intestine epithelia

The capability of BLG/CBLG samples to open the tight junctions between epithelial cells was analyzed by measuring the change in transepithelial electric resistance (TEER). As shown in Figures 3B and 3C, BLG samples (both molecules and nanoparticles) did not reduce the TEER values significantly during the 2-hour period of treatment, which was similar to the sample-free

buffer (results not shown due to data overlapping). On the other hand, C-EDA and C-P600 molecules (Figure 3B) were capable of reducing the TEER by up to 27% and 88%, respectively. On the other hand, C-EDA and C-P600 nanoparticles (Figure 3C) exhibited lower capability of tight junction disruption than the protein molecules (Figure 3B), causing a decrease of at most 17% and 32%, respectively, in the TEER. Regardless of the type and concentration of samples, a satisfactory recovery in TEER was observed after the termination of treatment, with the TEER values reaching more than 95% of the original values after 22 h. Such a result was much better than that achieved using pure PEI molecules (Ranaldi, Marigliano, Vespignani, Perozzi, & Sambuy, 2002). Similar recovery was also observed with cationic polymers such as chitosan (Vilasaliu, et al., 2010). However, chitosan could open the tight junction only at acidic pH, whereas CBLG was able to exhibit such an effect at neutral pHs.

The permeability of both the protein molecules and nanoparticles decreased in the same order (C-P600>C-EDA>BLG) as for their tight junction-disrupting capacities (Figure 3D). However, unlike for the tight junction opening, nanoparticles exhibited, at most, two orders of magnitude higher permeability than their constituent molecules, and these permeabilities were comparable with chitosan and PANAM dendrimers (El-Sayed, Ginski, Rhodes, & Ghandehari, 2002). Also noteworthy was that the basolateral-to-apical permeabilities were approximately 86% higher than those observed in the apical-to-basolateral analysis. However, the ranking of these values among different samples was not altered.

3.5. Cell viability

Figure 4 compares the viability of Caco-2 cells exposed to different samples. The buffer containing no proteins did not exhibit any noticeable cytotoxicity (data not shown). When dispersed in FBS-free DMEM (Figure 4A), C-P600 molecules exhibited significant cytotoxicity, reducing the viability of Caco-2 cells to 71% and 52% at concentrations of 200 and 500 µg/ml, respectively. C-P600 nanoparticles, on the contrary, did not show any observable toxicity. In addition, the cytotoxicity of C-P600 molecules was significantly lowered by the addition of FBS (Figure 4B). All other samples, regardless of the concentration and buffer, did not exhibit any observable cytotoxicity (Figure 4B).

3.6. Cellular uptake

Among different protein molecules, C-P600 demonstrated the highest cellular uptake (up to 5.74% in DMEM without FBS, same hereinafter), followed by C-EDA (up to 1.86%) and BLG (up to 0.13%), as shown in Figure 5A. On the other hand, the cellular uptake of nanoparticles followed the order of C-EDA (up to 26.5%)>C-P600 (up to 15.8%)>BLG (up to 6.82%). Comparable results have been reported in many previous studies on cationic nanoparticles using various assays, such as fluorescent analysis, confocal microscopy and flow cytometry (Huang, Ma, Khor, & Lim, 2002; Lu, et al., 2005; Luo, Teng, Li, & Wang, 2015; Patil, Sandberg, Heckert, Self, & Seal, 2007; Yue, et al., 2011).

As shown in Figure 5B, the influence of the absorbed serum protein varied greatly among different samples. For native BLG and C-P600, the change in cellular uptake caused by serum protein adsorption was minimal. Conversely, the FBS in DMEM reduced the cellular uptake of

290 C-EDA nanoparticles by at most 40%.

291 **4. Discussion**

292 *4.1. Chemical composition and buffering system determine the properties of protein molecules* 293 *and nanoparticles*

294 The surface properties of protein-based delivery systems are highly dependent on their chemical
295 structure. Although both P600 and EDA possess net positive charges, they exhibit significant
296 hydrophobicity due to their chemical structure. The hydrophobic backbone of EDA (Mattarella
297 & Richardson, 1983) and hydrophilic branches of P600 (Bronstein, et al., 1998) were
298 responsible for the different surface hydrophobicities of C-EDA and C-P600. The shift of zeta
299 potential towards negative values after particle formation was probably due to the consumption
300 of amine groups by glutaraldehyde, the crosslinker. The concomitant increase in surface
301 hydrophobicity could be explained by the partial protein unfolding induced by organic solvents
302 (Zhang, Liang, Tian, Chen, & Subirade, 2012). It is also worth mentioning that the positively
303 charged amine groups are more densely distributed on the polymeric branches of P600,
304 compared to the cationic monomer, EDA. This creates a multivalent environment which may
305 benefit the mucoadhesion and cellular uptake of C-P600 (Hong, et al., 2007), as will be shown in
306 later sections.

307 The physiological environment to which the delivering systems are exposed may also alter their
308 surface properties. Conventional buffers, such as HBSS and DMEM, contain abundant ions that

diminish the surface charges of the samples. This resulted in the decrease in zeta potential (Figure 1A), weaker electrostatic repulsion, and extensive aggregation of BLG/CBLG molecules and nanoparticles as indicated by greater average sizes (Figure 1C). Moreover, the reduction in electrostatic repulsion allowed the proteins and nanoparticles to adopt a more compact structure with less hydrophobic chains exposed (Zhang, Liang, Tian, Chen, & Subirade, 2012), leading to a lower surface hydrophobicity (Figure 1B). In FBS-containing DMEM, all the samples exhibited negative zeta potentials at around -20 mV and increased surface hydrophobicities. These changes were possibly due to the acquisition of a serum protein corona, considering that BSA (the major protein in FBS) is known for its abundant negative charge and high surface hydrophobicity (Cardamone & Puri, 1992). The absorbed serum protein resulted in a significant increase in the particle size (Figure 1C), which is in accordance with previous literature (Fleischer & Payne, 2012). Amongst all samples, C-EDA nanoparticles exhibited the greatest increase in average size, possibly due to its high surface hydrophobicity that resulted in more significant aggregation via hydrophobic interaction. It was also noteworthy that the nanoparticles prepared with native BLG also underwent the same changes as described above. This phenomenon suggested that BLG was also capable of absorbing serum proteins, although both of them are negatively charged. This was concurred with previous reports (Fleischer & Payne, 2012). In the following sections, the roles of different surface properties in the performance of protein molecules and nanoparticles will be discussed in details.

4.2. Surface properties determine the performances of protein molecules and nanoparticles as

329 *delivery systems*

330 *4.2.1. Effect of steric hindrance*

331 The conjugation of cationic moieties to BLG did not only confer it with abundant positive
332 charges but also altered the accessibility of site-specific molecules (e.g., enzymes). Native BLG
333 nanoparticles were prone to peptic and tryptic digestion, as shown in Section 3.2., leading to
334 rapid release of encapsulated curcumin. This was probably due to the ability of these enzymes to
335 break down either the peptide bonds in BLG molecules or the bonds formed between
336 glutaraldehyde and the lysine residues that maintained the particle structure (Teng, Li, & Wang,
337 2014; Weber, Coester, Kreuter, & Langer, 2000). When BLG was grafted with EDA or P600, its
338 peptide bonds might have become less accessible to enzymes due to increased steric hindrance.
339 In addition, the covalent bonds formed between glutaraldehyde and the grafted EDA or P600
340 moieties might be more difficult to digest, because the geometric properties of these two
341 molecules were distinct from those of lysine.

342 *4.2.2. Effect of surface charge*

343 Electrostatic attraction and repulsion play a critical role in the interaction between the
344 nanocarrier and biological surfaces. Cationic polymers generally exhibit better adhesion to
345 biological surfaces, such as intestinal epithelia, which are densely covered by negatively charged
346 glycoproteins. This was supported by our results, where C-EDA and C-P600 exhibited
347 significantly better mucoadhesion than unmodified BLG. The mucoadhesion of the CBLGs was

comparable to that of chitosan or poly (amido amine) (PANAM) dendrimers (Smart, 2005). Meanwhile, CBLGs also displayed the capability of opening the tight junction between the Caco-2 monolayers, probably by weakening the negative charge on the cell membrane and contacting proteins, such as cytoskeletal F-actin. (Ranaldi, Marigliano, Vespignani, Perozzi, & Sambuy, 2002). A conformational change of these proteins is then triggered, leading to the dislocation of tight junction proteins, including ZO1 and occludin. On the other hand, cationic nutraceutical/drug carriers were able to breach the negatively charged cell membranes (Merhi, et al., 2012), which facilitates translocation into the cytoplasm and interaction with specific cellular components (Thach & Finkelstein, 2013). These processes induce significant cytotoxicity, as observed for the most cationic sample (C-P600 molecule) in our study. However, the cytotoxicity was mitigated after the neutralization by serum proteins.

The cellular uptake of BLG molecules or nanoparticles was also significantly improved after cationization, as discussed in Section 3.6. The reason why we chose a cancer cell line (Caco-2) in this study was that particles with a diameter of 100-600 nm tend to pass through the loose blood vessels surrounding the tumor tissues, a phenomenon known as enhanced permeation and retention (EPR) or passive targeted delivery (Maeda, Wu, Sawa, Matsumura, & Hori, 2000). The advantage of polycations in cellular uptake has been well documented (Bannunah, Villasaliu, Lord, & Stolnik, 2014; Fleischer & Payne, 2012), and it could be explained by the different mechanisms of cellular internalization. In the absence of serum proteins, cationic polymers or nanoparticles could be trafficked into cells by both direct translocation (membrane breaching) and calthrin-mediated endocytosis (Lin & Alexander-Katz, 2013), whereas anionic samples enter

the cells mainly via caveolae-mediated endocytosis (Bannunah, Vllasaliu, Lord, & Stolnik, 2014). When serum is introduced, both anionic and cationic molecules and nanoparticles are able to acquire a serum protein corona, as discussed in Section 4.1. However, the behaviour of the nanoparticle/serum complex is highly dependent on the charge of the incorporated nanoparticles. Typically, serum proteins enhance the binding of cationic nanoparticles to the scavenger receptors on the cell membrane, thus promoting the cellular uptake. Anionic particles, on the other hand, show reduced cell uptake in the presence of serum proteins, because both of them bind to the native protein receptors (Fleischer & Payne, 2012). Lastly, the permeation across the small intestine epithelium depends on the adhesion to the mucin layer (mucoadhesion) and the cross-membrane transport. The latter includes paracellular transport through the opened tight junction and transcellular transport via cellular uptake. Since CBLG performed better, in all these processes, than did BLG, the enhanced permeability for CBLG observed in our study was reasonable.

4.2.3. Effect of surface hydrophobicity

The comparison between two CBLGs (C-EDA and C-P600), which showed similar surface charges but distinct surface hydrophobicities, provided a good illustration of the role of surface hydrophobicity for nanocarriers. The more hydrophilic C-P600 showed more than two-fold higher mucoadhesion than did C-EDA, which was probably due to its capability of extensive hydrogen bonding (Bronstein, et al., 1998). Some neutral (e.g., cellulose derivatives) or anionic (e.g., sodium alginate) macromolecules also demonstrated considerable mucoadhesion, probably

through extensive hydrogen bonding (Smart, Kellaway, & Worthington, 1984). On the other hand, the high surface hydrophobicity of C-EDA might have led to better permeation through the phospholipid bilayer in the cell membrane (Deshayes, Morris, Divita, & Heitz, 2005), contributing to higher cellular uptake than C-P600, despite its slightly lower surface charge.

4.2.4. Comparison between protein molecules and nanoparticles

As discussed in previous sections, delivering systems comprising protein molecules behaved quite differently from those composed of protein aggregates (nanoparticles). The processes investigated in our study could be roughly divided into three categories.

First, the processes that mainly involve contact with a charged surface (e.g., mucoadhesion) may take place more effectively in the presence of larger nanoparticles. One possible reason for this lay in the multivalency of nanoparticles. When individual protein molecules aggregated into compact nanoparticles, the total surface area was greatly reduced. As a result, the charged or hydrophilic groups might have been densely distributed on the newly formed surface of the nanoparticles in order to maximize their contact with water. This created a multivalent surface that could facilitate the interaction with the mucin layer. This phenomenon has also been used to explain the enhanced performance of other multivalent polymers, such as dendrimers (El-Sayed, Ginski, Rhodes, & Ghandehari, 2002).

Second, the procedures requiring rapid diffusion and interaction with certain biomacromolecules (e.g., cytoskeletal F-actin and cytoplasmic proteins) may proceed more rapidly for protein

molecules. Compared to the nanoparticles, protein molecules exhibited smaller size, which indicated larger specific surface area and higher diffusion rate, both of which might have facilitated their adhesion to and interaction with the target compounds. This is supported by a number of studies (Bannunah, Vllasaliu, Lord, & Stolnik, 2014; Pan, et al., 2007), in which smallest cationic (e.g., those smaller than 10 nm) particles are usually more toxic than are larger ones.

Third, for the processes that involve both surface contact and diffusion, an optimal size that balances the performances in all these steps may exist. Examples for these processes are the permeation across the Caco-2 monolayer and cellular uptake. The former process represents a combination of mucoadhesion, cellular uptake, and tight junction opening. In our study, larger nanoparticles exhibited better permeation than did smaller molecules, possibly owing to their better performances in the first two processes. Cellular uptake, on the other hand, is a rather complex process. As discussed in Section 4.2.2, it involves at least two mechanisms, direct translocation and endocytosis. The first process is size-independent, but it becomes deactivated as soon as it diminishes the transmembrane potential (Leroueil, et al., 2008). The latter one requires a proper size to trigger the wrapping of cell membrane (Chithrani, Ghazani, & Chan, 2006). Put together, the cellular uptake is size-dependent, and protein nanoparticles exhibited better cellular uptake in our study, possibly because their sizes were more suitable for endocytosis.

5. Conclusions

In this study, we investigated the performance of CBLG molecules and nanoparticles as novel nutraceutical/drug carriers. Owing to the grafted cationic moieties, CBLG exhibited good particle integrity against digestive enzymes, sustained release in the GI tract, remarkable mucoadhesion, strong and reversible tight junction opening capacity, desirable permeability across Caco-2 monolayer, and elevated cellular uptake. In a comparison between the two synthesized CBLGs, the more hydrophilic C-P600 demonstrated higher mucoadhesion, while the relatively hydrophobic C-EDA displayed better cellular uptake. Furthermore, clear size-dependence was observed in many biological processes, in which protein molecules and nanoparticles behaved distinctly. Finally, the surface properties and performances of all samples were significantly affected by the buffering system. Binding of serum proteins in FBS mitigated the cytotoxicity of CBLG, at the expense of lowered cellular uptake. Our study shed some light on the design of protein-based drug/nutraceutical carriers with well-defined surface properties and controllable release/delivery properties. Follow-up studies on the performance of CBLG nanoparticles in various cell lines and their *in vivo* efficacy have been initiated.

Acknowledgement

This work was supported by the University of Maryland and the USDA National Institute of Food and Agriculture under the Project Number MD-NFSC-5491. The authors are grateful to Dr. Lucy Liangli Yu, Dr. Haiqiu Huang, and Mr. Boyan Gao for their generous assistance in fluorescent analysis.

Supplementary information

The following experimental procedures on BLG and CBLG are described in the Supplementary Information section: preparation of BLG/CBLG nanoparticles, characterization of BLG/CBLG molecules or nanoparticles (molecular weight, net charge, surface hydrophobicity, particle size, and count rate), cellular uptake analysis, and Caco-2 monolayer transport study. This material is available free of charge via the Internet at <http://www.sciencedirect.com>.

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Tables

Table 1 Surface structures and properties of BLG/CBLG molecules and nanoparticles (NPs) *.

Sample	Structure	Zeta potential (mV)	So ** (dimensionless)	Size (nm)
BLG		-35.8 ± 1.2 e ***	108.5 ± 7.4 d ***	4.7 ± 0.6 d
C-EDA		39.4 ± 1.4 b ***	1419.4 ± 50.7 b ***	6.2 ± 1.2 d
C-P600		46.8 ± 2.5 a	41.3 ± 5.3 f	9.8 ± 1.4 c
BLG NPs		-42.6 ± 0.9 d	198.4 ± 14.5 c	58.4 ± 4.2 b
C-EDA NPs		33.7 ± 1.3 c	1782.3 ± 66.8 a	73.2 ± 6.6 a
C-P600 NPs		39.9 ± 1.6 b	78.3 ± 10.6 e	50.4 ± 2.5 b

* Data with different superscripts indicate significant ($P < 0.10$, experimentwise) difference. The schematics are provided for comparing the surface structures of different samples. They do not represent the actual scale of the molecules and nanoparticles.

** So: surface hydrophobicity index.

*** These data were reported in a previous study (Teng, Li, Luo, Zhang, & Wang, 2013) and are presented here for comparison purpose.

Figure(s)

Figure 1. Zeta potential (A), surface hydrophobicity (B), and particle size (C) of BLG/CBLG molecules and nanoparticles

Figure 2. Effect of digestion on the particle integrity and drug release. Figure 2A shows the in vitro release of curcumin from the nanoparticles. Figure 2B and 2C show the progression in particle size and count rate respectively as a function of incubation time. The magnified insets are intended for visualizing the variation in particle sizes and count rates of CBLGs as a function of time.

Figure 3. Transport of BLG/CBLG across small intestine epithelia. Figure 3A represents the mucoadhesion properties of BLG/CBLG nanoparticles. Figures 3B and

3C depict the change in transepithelial electrical resistances (TEER) of Caco-2 monolayers by BLG/CBLG molecules and nanoparticles, respectively. A zoomed in version of Figure 3B can be found as Supplementary Figure S1. Figure 3D represents the permeabilities of BLG/CBLG molecules or nanoparticles across the Caco-2 monolayer

Figure 4. Cytotoxicity of Caco-2 cells of BLG/CBLG molecules or nanoparticles dispersed in DMEM without (A) or with (B) FBS

Figure 5. Uptake of BLG/CBLG molecules or nanoparticles by Caco-2 cells in DMEM without (A) or with FBS (B)