

Feature Article

BIOPOLYMER BASED NANO-DELIVERY SYSTEMS FOR ENHANCING BIOAVAILABILITY OF NUTRACEUTICALS*

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Abstract Recently, there has been an increasing interest in the development of efficient food-grade oral nano-delivery systems for encapsulation, protection and target delivery of nutraceuticals to enhance their bioavailability, further to prevent disease and promote human health and well-being. Food proteins represent promising candidates for efficient nutraceutical nanocarriers due to their exceptional characteristics, namely biodegradability, nonantigenicity, high nutritional value, abundant renewable sources and extraordinary binding capacity to various nutraceuticals. In addition, their biocompatibility, biodegradability, low toxicity, low cost, and non-starch polysaccharides possess many favourable characteristics such as stability in the harsh gastric environment, resistance to digestive enzymes, and mucoadhesiveness to intestinal mucosal surface. This review describes the design and formation of nanoscaled delivery systems for nutraceuticals using food-grade proteins (including peptides), polysaccharides and their associative complexes. The toxicity and cellular uptake fate of the nanostructures, as well as their effects on the intestinal absorption of the encapsulated nutraceuticals were also discussed.

Keywords: Food biopolymer; Nano-delivery systems; Bioavailability; Nutraceuticals.

INTRODUCTION

Oral delivery *via* nanoparticles is an attractive route to deliver therapeutics and bioactive compounds due to its ease of administration and patient compliance, especially when long term or daily use is required. Polymer-based delivery systems that trap molecules of interest within networks have been developed extensively for the biomedical and pharmaceutical sectors to protect and transport bioactive compounds to target functions. The main mechanisms involved in the enhanced bioactive molecule absorption by polymeric nanoparticles are: (i) protection of the bioactive molecule from the harsh environment of the GI tract, (ii) prolongation of the residence time in the gut by mucoadhesion, (iii) endocytosis of the particles and/or (iv) permeabilizing effect of the polymer^[1]. In spite of successful elaboration of many synthetic polymers as delivery systems, these cannot be used in oral delivery systems that require compounds generally recognized as safe (GRAS). In the purpose of oral consumption and minimizing carrier-induced undesirable cytotoxicity, we believe that no better are food-grade polymers suitable in developing such delivery systems. Biopolymers of food origin are fascinating and important materials, making up some of the most complex examples of soft condensed matter with which we

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interact daily^[2]. They are natural sources of bio-polymeric soft materials, not only biodegradable and biocompatible, but also biofunctional^[3, 4]. Nanostructured vehicles such as association colloids, lipid based nanoencapsulator, nanoemulsions, biopolymeric nanoparticles, nanotubes and nanofibers formed with food-grade ingredients including food biopolymers (proteins, carbohydrates), fats, low molecular weight surfactants and co-polymers (protein-carbohydrate conjugates) have been employed to deliver a range of functional ingredients in pharmacy and foods^[5, 6].

As a portmanteau of the words “nutrition” and “pharmaceutical”, nutraceuticals represent the dietary compounds with health and medical benefits, including the prevention and treatment of disease. Nutraceuticals offer an excellent opportunity to improve public health. There is an increasing body of evidence supporting the biological and pharmacological effects of nutraceuticals including antioxidative, anticancer and chronic disease prevention properties which were demonstrated in numerous *in vitro* and *in vivo* studies^[7–10]. Despite promising results in preclinical settings, the applicability of nutraceuticals to humans has met with limited success largely due to inefficient systemic delivery and poor oral bioavailability of the promising active agents^[11]. The major challenge of dietary nutraceuticals is their poor oral bioavailability, and one key barrier to the absorption of nutraceuticals is intestinal epithelium because it is difficult for many nutraceuticals to diffuse across the cells through the lipid-bilayer cell membranes. In addition, insufficient gastric residence time, low solubility within the gut, as well as instability under conditions encountered in food processing (temperature, oxygen, light) or in the gastro-intestinal (GI) tract (pH, enzymes, presence of other nutrients) also limit the activity and potential health benefits of nutraceutical molecules.

This review considers the design and formation of nanoscaled delivery systems for nutraceuticals using food grade polymers, proteins (peptides), polysaccharides and their associative complexes. Recent progresses in their toxicity, cellular uptake fate, as well as their effects on the intestinal absorption of the encapsulated nutraceuticals are also discussed.

FOOD PROTEIN BASED NANO-DELIVERY SYSTEMS

Food proteins have excellent functional properties including emulsification, gelation, foaming and water binding capacity^[12–14]. There is growing interest in developing protein nanocarriers as GRAS drug or nutraceuticals delivery devices due to their exceptional characteristics, including biodegradability, nonantigenicity, high nutritional value, abundant renewable sources and extraordinary binding capacity of various drugs or nutraceuticals. In addition, protein nanoparticles can be easily prepared and scaled up during manufacture^[13, 14]. Furthermore, protein nanoparticles were accepted as metabolizable naturally occurring components. Hydrolysis of proteins by digestive enzymes generates bioactive peptides that may exert a number of physiological effects *in vivo*^[12].

Milk Proteins and Peptides

Over the past a few years, milk proteins have attracted intensive research and development efforts to harness them to deliver additional health-promoting and other bioactive compounds in food and drug applications, which resulted in a wide variety of novel applications. Among milk proteins, caseins and whey proteins are the major building materials widely applied in fabrication of food grade nanoscaled delivery systems.

Caseins

The caseins are proline-rich, open-structured rheomorphic proteins, which have distinct hydrophobic and hydrophilic domains. Consisted with α 1-, α 2-, β - and κ -caseins (CN), most of the caseins (95%) are naturally self-assembled into casein micelles that are spherical colloidal particles with the diameter in the range of 50–500 nm (average 150 nm). α 1-CN, α 2-CN and β -CN are characteristic with their serine-phosphate residue centers. They are held together in the casein micelle mainly by hydrophobic interactions, and by calcium-phosphate nanoclusters, bridging between their serine-phosphate residues. κ -CN is a glycoprotein, with 2 cysteines forming intermolecular disulfide bridges. It forms a “hairy layer” on the surface of the micelles, stabilizing them sterically and electrostatically against aggregation. It has found that caseinate^[15], and isolated

β -CN^[16] can bind vitamin D, apparently by hydrophobic interactions, and β -CN has been shown to bind a chemotherapeutic drug, mitoxantrone, by combination of hydrophobic and ionic interactions^[17]. The co-assembly process of α s1-CN, α s2-CN, β -CN and κ -CN forming casein micelles can be harnessed for nanoencapsulation of hydrophobic nutraceuticals. Semo *et al.* first reported using casein micelles as nanovehicles for hydrophobic bioactive compounds: after binding vitamin D to soluble caseinate, casein micelle was induced to reformation by reconstituting the original mineral composition of milk^[15]. Casein micelles with average size distribution of < 200 nm was also applied as nanodelivery vehicles for loading with curcumin, a flavorant with anticancer properties, which can enhance the cytotoxicity of curcumin towards HeLa cancer cells^[18]. Incorporation of a hydrophobic drug into casein micelles was also achieved through solvent-mediated high-pressure homogenization method^[19]. In addition, the individual caseins can self assemble in pure solutions. The excellent self-assembly properties of β -casein were utilized for entrapment and solubilization of a hydrophobic chemotherapeutic drug, to facilitate its oral delivery^[17]. The fact that caseins have evolved to be easily digestible in the stomach provides a target-activated release mechanism. Livney *et al.* have recently studied β -casein's temperature-dependent step-wise micellization^[20], and its co-assembly with bioactive ligands, such as vitamin D^[17]. Zimet *et al.* prepared re-assembled casein micelles (50–60 nm) and casein nanoparticles (288.9 nm) as nano-vehicles for encapsulation of ω -3 polyunsaturated fatty acids^[21].

Whey proteins

Beta-lactoglobulin (β -lg), the major whey protein in cow milk, is a small globular protein, folded up into an eight-stranded antiparallel β -barrel with a three-turn α -helix on its outer surface. It has 2 disulfide bridges and a free thiol. It is mainly found as dimers at milk pH (*ca.* 6.7). Alpha-lactalbumin (α -la), the second most prevalent whey protein in cow milk, is a smaller globular metaloprotein with 4 disulfide bridges, which is structurally homologous to lysozyme and requires calcium to assume its functional fold. Bovine serum albumin (BSA) is found both in blood serum and in milk. It is a larger globular protein, with a mainly alpha-helical structure, 17 disulfide bridges and a free thiol. The function of BSA in the blood serum is binding and delivery of various small ligands^[22]. Although much of the BSA used in research is isolated from bovine blood, it may still be obtained from milk. Lactoferrin is a monomeric globular glycoprotein, which belongs to the transferrin family, and presents anti-microbial activity.

β -lg reveals multi potentials in preparation of oral delivery systems for food nutraceuticals. β -lg is able to bind small hydrophobic molecules in the internal cavity. It has been suggested that the surface hydrophobic pocket in the groove between the α -helix and the β -barrel, the outer surface near Trp19-Arg124, and the monomer-monomer interface of the β -lg dimer are the potential sites among the external portions of β -lg for ligand binding^[23]. Whey proteins are generally resistant to some proteolytic enzymes and therefore can possibly serve for enteric delivery^[24]. Previous reports revealed that bovine β -lg is not easily hydrolyzed by gastric pepsin, and partly for that reason is a potential allergen^[25]. However, these authors reported that preheated β -lg (80 °C, 1 h) was hydrolyzed to shorter peptides at gastric conditions, *i.e.* heat treatment makes it more susceptible to gastric digestion, which may also decrease its allergenicity. The relative stability of the β -lg to both pepsin and trypsin leads to a suggestion of using it for intestinal targeted delivery of nutraceuticals. Recently, Liang and Subirade used folic acid, resveratrol and α -tocopherol as the hydrophilic, amphiphilic and hydrophobic compounds respectively to study their interactions between β -lg at neutral and acidic pH and after heating to 85 °C^[26]. Folic acid binds to hydrophobic pocket in the groove between the α -helix and the β -barrel, which is disturbed by decreasing the pH from 7.0 to 2.0. Outer surface of β -lg near Trp19-Arg124 is the binding site for resveratrol to form complexes that are stable at acidic pH. The release of α -tocopherol bound to the internal cavity was caused by acidification that had no influence on that bound to a site at the surface of β -lg. The β -lg/folic acid complex was thermally stable. The affinity of the protein for resveratrol was improved by thermal denaturing, which but decreased somewhat its affinity for α -tocopherol. Shpigelman *et al.*^[27] recently reported that heat-denatured β -lg forms nanoparticles (< 50 nm) with (-)-epigallocatechin-3-gallate (EGCG), which can protect EGCG against oxidative degradation, useful for clear beverage enrichment.

α -la can apparently bind retinol and palmitic acid. BSA, a natural carrier of small molecules in the blood, contains three domains specified for metal ion binding, lipid binding and nucleotide binding^[22]. The binding of sulfamethoxazole to BSA was investigated by spectrofluorescence, FTIR and UV-Vis absorption techniques. The thermodynamic parameters obtained indicated that the hydrogen bonding and weak van der Waals forces played a major role in the interaction^[28].

Peptides

A very interesting discovery was reported by Graveland-Bikker *et al.*, whereby novel nanotubes (Fig. 1) were formed by self assembly of enzymatically hydrolyzed α -lactalbumin induced by calcium. The α -lactalbumin was hydrolyzed by a protease from *Bacillus licheniformis*^[29, 30]. The authors suggested that these α -lactalbumin peptides nanotubes could be utilized for many applications including delivery of nutraceuticals. Due to their high aspect ratio (*i.e.* large surface area) and stiffness, they have higher viscosity compared with same concentration of proteins^[31, 32]. In addition, these high protein-density nanotubes could also be used as thickener alternatives^[32]. Furthermore, these α -lactalbumin peptide nanotubes have cavities of 8 nm in diameter, which might enable the binding of food components, such as vitamins or enzymes. These cavities could also be used to encapsulate and protect nutraceuticals or to mask undesirable flavor or aroma compounds^[32]. Consisting of milk peptides, these nanotubes are considered food-grade materials, which should make their introduction into the market place relatively easy and might facilitate widespread applications in nanoencapsulating of nutrients, supplements and pharmaceuticals.

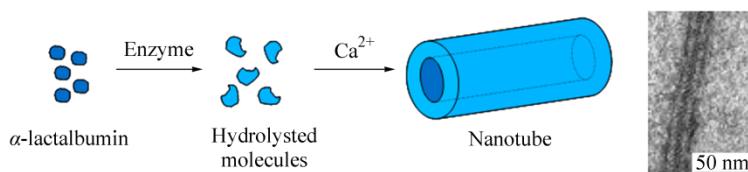


Fig. 1 Schematic presentation of the self-assembly of partially hydrolysed α -lactalbumin into nanotubes in the presence of Ca²⁺^[31]

Gelatin

Gelatin is a denatured protein that is obtained from collagen by acid and alkaline hydrolysis. It is considered as GRAS material by the FDA and has been safely used for a long history in pharmaceuticals, cosmetics, as well as food products. Various chemical modifications of gelatin can be achieved due to the accessibility of its functional groups after denaturation, which may be especially useful in developing targeted drug delivery vehicles^[33]. Furthermore, gelatin is a poly-ampholyte having both cationic and anionic groups along with hydrophobic groups. Gelatin nanoparticles have been reported to be prepared by various methods such as desolvation^[34], coacervation-phase separation^[35], emulsification-solvent evaporation^[36], reverse phase evaporation^[37] and nanoprecipitation^[38] gelatin nanoparticles. Shutava *et al.*^[39] found that EGCG encapsulated in gelatin-based nanoparticles consisting of a soft gel-like interior with or without a surrounding lay-by-lay shell of polyelectrolytes retained its biological activity for blocking hepatocyte growth factor (HGF)-induced intracellular signaling in the breast cancer cell line MBA-MD-231 as potently as free EGCG.

POLYSACCHARIDES BASED NANO-DELIVERY SYSTEMS

Polysaccharides are a significant fraction of various foods, accounting for many of their texture, sensory properties and caloric value. Polysaccharides are composed with monosaccharides joined by glycosidic bonds. Certain polysaccharides (dietary fibers) have been shown to have potential health benefits, such as cholesterol reduction, cancer prevention, or colonic health improvement. Due to their structural versatility and site-specific digestion properties, polysaccharides are suitable carriers for the targeted and controlled release of drugs or nutraceuticals along the human gastrointestinal tract (GIT)^[40]. They are non-toxic, biocompatible, structure stable, low cost, hydrophilic and available of reactive sites for chemical modifications^[40, 41]. Another advantage

of polysaccharides is bioadhesion, especially for mucosal surfaces, which has been used for targeting specific organs or cells and prolonging the drug residence time in intestine. It has shown that release of the bioactive in the intestine can be controlled by pH changes, transit time, the pressure created by peristaltic motion and even enzymatic degradation. Commonly used polysaccharides include plant-derived carbohydrates (*e.g.* starch, pectin and guar gum), animal-derived carbohydrates (*e.g.* chitosan, chondroitin sulfate) and carbohydrates derived from other sources (*e.g.* alginate derived from algae)^[42, 43].

The Major Methods to Prepare Polysaccharides Nanoparticles

Cross-linking methods

Polysaccharide nanoparticles can be prepared through the interconnection of the polymeric chains by crosslinkers, leading to the formation of a 3D network^[44]. During this process, cross-linking density that is determined by the molar ratio between the crosslinker and the polymer repeating units is the main factor, determining the properties of a crosslinked nanoparticle such as drug release and mechanical strength. According to the nature of the cross-linking agents, crosslinked nanoparticles can be classified as: covalently crosslinked nanoparticles and ionically crosslinked nanoparticles.

In a covalently cross-linked nanoparticle, the network structure is permanent since irreversible chemical links are formed unless biodegradable or stimuli-responsive crosslinkers are employed^[44]. The rigid network allows absorption of water and bioactive compounds without dissolution of the nanoparticle even when the pH drastically changes. A covalently crosslinked nanoparticle can contain more than one type of polysaccharide. The covalent bonds are the main interactions that form the 3D network although secondary interactions such as hydrogen bonds and hydrophobic interactions also exist^[44]. Covalent crosslinkers are molecules with at least two reactive functional groups that allow the formation of bridges between the polymeric chains^[45]. The most common covalent crosslinkers used with polysaccharides are dialdehydes such as glutaraldehyde. However, dialdehydes are highly toxic and therefore biocompatible alternatives have been tested. Genipin is a natural biocompatible crosslinker isolated from the fruits of *Gardenia jasminoides* Ellis^[44].

Charged polysaccharides can form ionic crosslinked nanoparticles with oppositely charged ions or small ionic molecules. For example, the polyanion tripolyphosphate (TPP) has been widely used to crosslink the positively charged chitosan, and divalent cations such as Ca^{2+} have been used to crosslink negatively charged alginate^[46]. This method enables the preparation of nanoparticles through reversible physical cross-linking by electrostatic interaction, instead of chemical crosslinking, making it possible to prevent the toxicity of reagents and other undesirable effects^[47, 48]. The nanoparticles formed through ionic cross-linking mechanism are generally considered much more biocompatible than those prepared through covalent cross-linking^[44]. The ionic cross-linking reaction is influenced by several factors and the most crucial are the size of the crosslinker and the global charge of the crosslinker and the polysaccharide^[44]. Unlike covalently crosslinked nanoparticles, ionic crosslinked particles are generally pH sensitive, a welcome trait for stimuli-respond drug delivery purposes. However, this pH sensitivity can also lead to the instability of the ionic crosslinked network^[44].

Polyelectrolyte complexes

The oppositely charged polyelectrolytes can interact with each other through direct electrostatic interaction, leading to the formation of polyelectrolyte complexes. Polyelectrolyte complexes are another group of biocompatible materials for nutraceutical delivery since non-toxic covalent crosslinkers and no harsh reaction environment are used. These complexes resemble ionic cross-linking since non-permanent networks are formed that are more sensitive to changes in environmental conditions^[49]. However, in polyelectrolyte complexes the interaction is between the polyelectrolyte and large molecules with broad M_w range rather than the reaction between ions or ionic molecules with the polyelectrolyte in ionic cross-linking^[44]. The degree of interaction between the polyelectrolytes is the major factor, which determines the formation and stability of polyelectrolyte complexes^[45]. The chemical environment is also crucial: the pH of the solution, the ionic strength, the temperature, and the duration and mixing order. Secondary factors are the M_w of the polyelectrolytes and their

flexibility^[45, 50]. Ionic cross-linking can reinforce the formed interaction. Positively charged polysaccharides, namely chitosan, can form polyelectrolyte complex with a variety of negatively charged polymers such as the polysaccharides alginate, dextran sulfate, chondroitin sulfate, hyaluronan, carboxymethyl cellulose, carrageenan and heparin. In addition, peptides such as poly-g-glutamic acid, nucleic acids and synthetic polymers could also be used^[45, 50].

Self-assembly

An amphiphilic copolymer can be created upon grafting hydrophobic moieties onto a hydrophilic polysaccharide. Amphiphilic copolymers in aqueous solutions tend to self-assemble into nanoparticles in which the inner core is hydrophobic and the shell is hydrophilic. The hydrophilic shell serves as a stabilizing interface between the hydrophobic core and the external aqueous environment^[51]. This self-assembly process is driven by hydrophobic interactions, mainly in order to reduce the interfacial free energy^[50, 52]. The formed nanoparticles have the characteristics of prolonged circulation, thermodynamic stability^[53], and the ability for the delivery of hydrophobic drugs/nutraceuticals due to the hydrophobic property of the inner core. Several properties such as size, surface charge, loading efficiency, stability and biodistribution can be altered through changing the chemical structure of the polymers. Polysaccharides can be modified with a wide range of hydrophobic moieties, among them are bile acids (*e.g.*, 5b-cholanic acid, cholic acid and deoxycholic acid), fatty acids (*e.g.*, palmitoyl acid, stearic acid and oleic acid)^[54], cholesterol and hydrophobic drugs or nutraceuticals.

Chitosan Based Nano-delivery Systems

Chitosan is the deacetylated form of chitin and composed of glucosamine, known as 2-amino-2-deoxy-(1→4)- β -D-glucopyranan. It is considered to be the most widely distributed biopolymer as a cationic, non-toxic, biodegradable and biocompatible polyelectrolyte with an oral LD50 in mice of over 16 g/kg^[54], which has been approved for dietary applications in Japan, Italy and Finland. It is considered beneficial for improving the intestinal absorption of active ingredients, especially for compounds, such as EGCG, that are soluble in water but having low permeability in small intestine. Chitosan has been extensively investigated for its potential applications in the food, cosmetics, biomedical and pharmaceutical fields^[55]. Due to their subcellular and submicron size, chitosan nanoparticles can penetrate deep into tissues through fine capillaries, and cross the fenestration present in the epithelial lining. This allows efficient delivery of therapeutic agents to target sites in the body^[56, 57]. Under the acidic conditions, the $-\text{NH}_3^+$ protonized from $-\text{NH}_2$ of chitosan can interact with an anion such as tripolyphosphate to form microgel particles^[58, 59]. Ionic cross-linking of chitosan and TPP represents an advantageous method for preparing of drug-loaded nanoparticles, because of its mild process of obtaining nanoparticles with a controlled size and satisfactory encapsulation capacity for macromolecules and drugs. Hu *et al.*^[60] investigated the polyanion initiated gelation process in fabricating chitosan-tripolyphosphate (chitosan-TPP) nanoparticles intended to be used as carriers for delivering tea catechins. It was found that the particle size, surface charge of the chitosan-TPP nanoparticles, the encapsulation efficiency and release profile of tea catechins from the nanoparticles can be controlled by fabrication conditions, including contact time between chitosan and tea catechins, chitosan molecular weight, chitosan concentration, chitosan-TPP mass ratio, initial pH value of chitosan solution and concentration of tea catechins. The controlled release of tea catechins was achieved after encapsulation of tea catechins in the chitosan-TPP nanoparticles. In order to deliver curcumin to cancer cells, Bora *et al.*^[61] encapsulated it in the composite nanoparticles that were prepared by using three biocompatible polymers — alginate (ALG), chitosan, and pluronic — by ionotropic pre-gelation followed by polycationic cross-linking. A cytotoxicity assay showed that composite nanoparticles at a concentration of 500 $\mu\text{g/mL}$ were nontoxic to HeLa cells. Cellular internalization of curcumin-loaded composite nanoparticles was confirmed from green fluorescence inside the HeLa cells. The half-maximal inhibitory concentrations for free curcumin and encapsulated curcumin were found to be 13.28 $\mu\text{mol/L}$ and 14.34 $\mu\text{mol/L}$, respectively. In previous study, we prepared a new chitosan-based amphiphile, octanoyl-chitosan-polyethylene glycol monomethyl ether (acylChitoMPEG), using both hydrophobic octanoyl and hydrophilic polyethylene glycol monomethyl ether (MPEG) substitutions^[62]. The critical aggregation concentration (CAC) and hydrodynamic

diameter were found to be 0.066 mg/mL and 24.4 nm, respectively. It is worth mentioning that we characterized the detailed 3D network structure of the self-assembly nanoparticles with small-angle X-ray scattering (SAXS). SAXS results suggested a coiled structure of the triple helical acylChitoMPEG backbone with the hydrophobic moieties hiding in the center of the backbone, and the hydrophilic MPEG chains surrounding the acylChitoMPEG backbone in a random Gaussian chain conformation (Fig. 2).

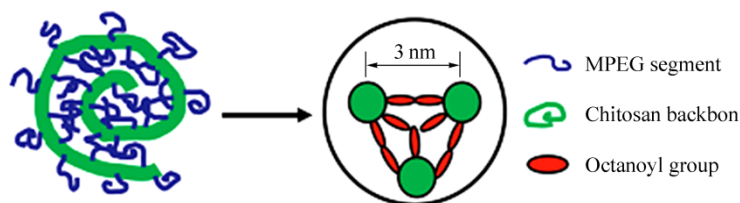


Fig. 2 Scheme of the coiled structure of the triple helical AcylChitoMPEG backbone^[62]

POLYSACCHARIDES-PROTEIN (PEPTIDES) COMPLEX NANO-DELIVERY SYSTEMS

Proteins of food origin, such as casein, gelatin, whey proteins (mainly β -lactoglobulin), casein phosphopeptides (CPP), are considered promising candidates in development of nanocarriers mainly due to their biodegradability, non-antigenicity, high nutritional value and their binding capacity of various drugs, including chemopreventive phytochemicals^[63]. However, the drawback of protein nanocarriers for oral delivery is that they are easily hydrolysed by digestive proteases. For oral delivery, non-starch polysaccharides, including alginate, pectin, dextran and chitosan, possess many favourable characteristics such as stability in the harsh gastric environment, resistance to digestive enzymes and mucoadhesiveness to intestinal mucosal surface^[64]. Biopolymer-based nanoparticles can be rationally engineered through controlled self-assembly, mainly through electrostatic interactions^[65]. One of the most commonly used methods of assembling proteins and polysaccharides into functional biopolymer particles is based on electrostatic attraction between oppositely charged groups under appropriate solution conditions^[66, 67]. Proteins possess a net negative charge above their isoelectric point (pI) and a net positive charge below this pH. At their isoelectric point they have no net charge, but there are localized regions on the protein's surface that are either positively or negatively charged, which has important consequences for their interaction with ionic polysaccharides^[68]. Protein-polysaccharide complexes may be formed from an anionic polysaccharide and cationic protein surface groups or vice versa. For example, consider a system containing an anionic polysaccharide and a globular protein. At $\text{pH} > \text{pI}$, the protein possesses a high net negative charge and so there is an electrostatic repulsive force between it and the anionic polysaccharide. At $\text{pH} = \text{pI}$, localized cationic regions on the protein surface interact with anionic groups on the polysaccharide chain leading to weak electrostatic complexation and the formation of soluble complexes^[69]. Further pH reduction increases the number of cationic groups on the proteins surface, which induces greater electrostatic attraction between these groups and anionic polysaccharide groups, eventually resulting in charge neutralization of the protein-polysaccharide complex formed and phase separation, which is usually referred to as coacervation^[70]. At lower pH values, the electrostatic interaction between the protein and polysaccharide molecules may lead to the formation of precipitates that tend to sediment. If the pH is reduced below the pK_a value of the anionic groups on the polysaccharide chain, then the attractive interactions between the protein and polysaccharide molecules may weaken and the complex may dissociate.

Hydrogel particles can be obtained by heating the mixed solutions of β -lg and chitosan, which formed through the interactions between them. Responding to different pH stimulations, the hydrogel particles exhibited swelling and aggregation behavior with the particle size ranging from nano to micro sizes, and were proposed as potential vehicles for various delivery applications^[71]. Similarly, heat induced hydrogel particles were formed from β -lg and beet-pectin at pH range 3–7^[72, 73]. Another approach to prepare the hydrogel particles was to first form heat induced β -lg nanoparticles, then to coat them with beet pectin^[74]. The characteristics of the biopolymer particles formed were influenced by biopolymer type, protein-polysaccharide ratio, pH, ionic strength and

thermal history. Using the similar method, Livney *et al.* have prepared clear systems by adding excess of pectin to β -lg that were binding with hydrophobic nutraceuticals and decreasing the solution pH below the protein pI, which was very useful for delivery of these water insoluble sensitive bioactive (*e.g.* vitamin D, DHA and conjugated linoleic acid) in clear liquid systems^[75]. Complexes of chitosan with α -la or with β -lg were studied as potential vehicles for bioactives^[76].

Polysaccharides-Peptides Nanoparticles

However, the drawback of using proteins in formulation of the nano-complexes is that the proteins could cause allergy. Replacing the protein with bioactive peptides is a potential approach to solve this problem. In our previous study^[77], we prepared a novel nano-complex that assembled from caseinophosphopeptide (CPP), a bioactive peptide isolated from milk casein protein and chitosan^[77]. CPP, an anionic polyelectrolyte containing clusters of phosphorylated seryl residues, is released from the N-terminus polar region during the tryptic digestion of milk casein proteins^[78]. CPP reveals multi potentials in preparation of oral delivery system, such as strong resistance to gastrointestinal digestion^[79], inhibition of gastric secretion^[80], enhancement of the paracellular absorption in small intestine^[81] and protection against oxidative stress in human intestinal epithelial Caco-2 cells^[82]. The chemical structures of the CPPs arising from the binding to chitosan were identified using LC-MS-MS combined with database searching. The binding mechanism between chitosan and caseinophosphopeptides was investigated systematically in our previous study. Thermodynamic properties associated with complex formation including surface charge (Fig. 3a), particle size (Fig. 3b), complex morphology (Fig. 4) and binding constant were measured. Based on the experimental results, an interaction model between chitosan and CPP was proposed to explain the physical chemistry insights of the structure of the novel nanocomplexes formed. At low chitosan/CPP mass ratio, bioactive peptides (CPPs) and chitosan assembled to form new hierarchical nanocomplexes, in which negatively charged CPPs bound to the positively charged chitosan molecules to form spherical intrapolymer nanocomplexes saturated with CPPs (CPP nanoparticles). Subsequently, the negatively charged CPP nanoparticles were bridged by the added positively charged chitosan to form significantly larger associative polymer complexes. Further increase in chitosan/CPPs ratios caused the reversal of surface charges of CPP nanoparticles from negatively charged to positively charged, and the repulsion between the positively charged CPP nanoparticles resulted in the breakdown of the interpolymer bridges and the formation of isolated positively charged spherical nanocomplexes. The interactions between the peptides and chitosan were mainly driven by electrostatic interactions with the binding constant. Hydrophobic driven also occurred during the interaction between chitosan and CPPs. The phosphorylated groups, Asp and Glu in the CPPs might be the dominant sites for interaction with $-\text{NH}_3^+$ on the chitosan molecular chains. These newly developed nanocomplexes can be used as non-toxic delivery systems for drugs and functional food ingredients.

CPP is a well-known substance to reversibly react with phytochemicals, especially EGCG, which can provide direct drive for entrapping phytochemicals and overcome the general drawback of low drug content in polymeric drug delivery system. Our strategy is the application of caseinophosphopeptides (CPP) not only to crosslink chitosan forming mono-dispersed nanoparticles but also bind with EGCG for trapping it inside the nanoparticles. Therefore, a novel type of nanochemoprevention from encapsulation of EGCG with food grade bioactive peptides/chitosan (chitosan) nanoparticles was formed^[83]. The interaction among chitosan, CPP and EGCG was confirmed and measured according to the frequency and dissipation changes during the deposition process of chitosan, EGCG and CPP in QCMD. The encapsulation efficiency of EGCG in chitosan-CPP nanoparticles (70.5%–81.7%) was about one hundred percent higher than that with chitosan-TPP nanoparticle (25.8%–47.4%). Compared with the release profile of EGCG in previous chitosan-TPP nanoparticles (around 45%–60% at 12 h), the burst release of EGCG in chitosan-CPP nanoparticles (around 23%–40% at 12 h) was slowed down in a much more controllable manner. It might be attributed to the binding between EGCG and the peptides.

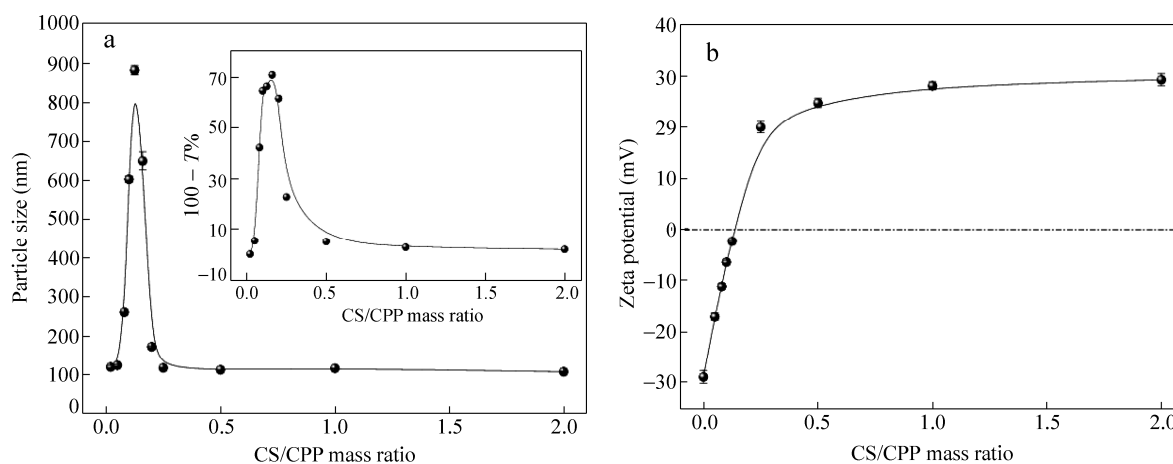


Fig. 3 Change in (a) mean particle size and (b) zeta potential (ζ -potential) of casein phosphopeptide/chitosan nanoparticles with increasing the chitosan/casein phosphopeptide mass ratio. Both the pH values of casein phosphopeptide and chitosan are 6. Insert of (a) is the turbidity ($100 - T\%$) as a function of different chitosan-casein phosphopeptide mass ratios at pH 6^[77].

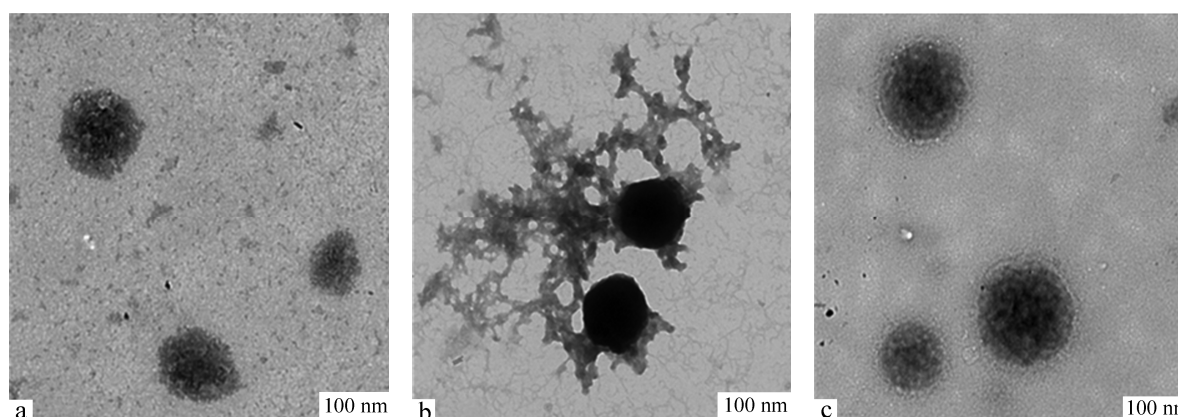


Fig. 4 Transmission electron microscopy (TEM) images of the nanocomplexes at the chitosan (CS)-casein phosphopeptides (CPPs) mass ratios of: 0.02 (a), 0.125 (b) and 1 (c). The concentration of CPPs is fixed at 1 mg/mL, and the pH value is 6^[77].

CYTOTOXICITY OF FOOD POLYMER NANOPARTICLES

Although composed with both food-grade polymers, the toxicity of the polymeric nanoparticles is still the concern in developing nutraceuticals delivery systems. In general, physicochemical properties including particle size and size distribution, agglomeration state, shape, crystal structure, chemical composition, surface area, surface chemistry, surface charge and porosity may all be important for understanding the toxic effects of nanomaterials. The cytotoxicity of nanoparticles can be expressed by the cell viability after incubation of cells with nanoparticles that was evaluated by MTT-based colorimetric method. In previous study, we determined and compared the cytotoxicity of the chitosan-CPP nanoparticles with that of the chitosan-TPP nanoparticles^[83]. Our result showed that cross-linking chitosan with the bioactive peptide could decrease the cytotoxicity of nanoparticles significantly ($p < 0.05$) compared with that with TPP at the same concentration of chitosan, with similar physicochemical properties (Fig. 5). The IC₅₀ values for chitosan-CPP and chitosan-TPP nanoparticles were 0.95 mg/mL and 0.35 mg/mL, respectively. It was reported that cytotoxicity of chitosan nanoparticles is directly proportional to the surface charge (zeta potential)^[84]. Meanwhile, surface charge is an important determinant in the stability, mucoadhesiveness and permeation enhancing effect of nanoparticles^[85]. Here, we

have been successful in making a balance between the two sides of the coin, maintaining the surface charge as high as +32.2 mV, cross-linking chitosan with CPP increased the biocompatibility of chitosan nanoparticles significantly. We also tested the cytotoxicity of the chitosan-based amphiphile. The results showed that acylChitoMPEG exhibited negligible cytotoxicity even at concentrations as high as 1.0 mg/mL. It implies that acylChitoMPEG has the potential to be used as delivery systems for nutraceuticals^[62].

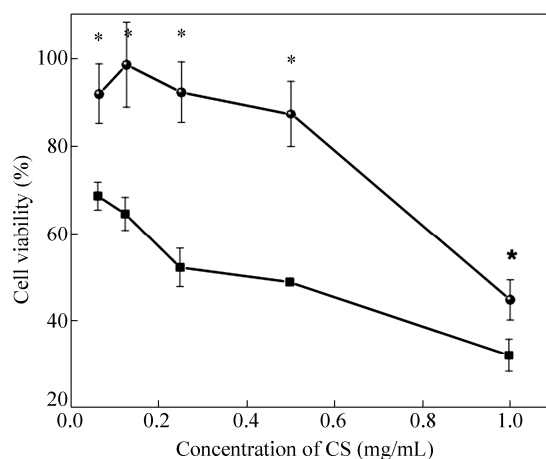


Fig. 5 Cell viabilities of CS-CPP nanoparticles (●) and CS-TPP nanoparticles (■) on cells. Cell viabilities are expressed as mean $\pm$ standard deviation ($n = 3$)^[83]

*represents that difference is significant ($p < 0.05$)

EFFECTS OF POLYMERIC NANOPARTICLES ON THE INTESTINAL ABSORPTION OF NUTRACEUTICALS

The oral bioavailability of nutraceuticals mainly relies on their absorption in small intestine. The human colon carcinoma cell line Caco-2 cells grown on permeable filters have become the golden standard for *in vitro* prediction of intestinal drug permeation and absorption^[86]. In culture, this cell line slowly differentiates into monolayers with a differentiated phenotype with many functions of the small intestinal villus epithelium. The permeability *in vitro* of a compound in Caco-2 cell monolayers can be used to predict the absorption in humans^[87], and the *in vitro* absorption is mainly predicted from the apparent permeation rate (P_{app}) across the Caco-2 cell monolayers from apical to basolateral side. Although the P_{app} obtained from different laboratories are different, there is a general trend that a high P_{app} implies a high absorption. Generally, $P_{app} > 1 \times 10^{-6}$ cm/s means high permeation, while $P_{app} < 1 \times 10^{-7}$ cm/s implies low permeation.

In previous study, we studied the effect of polymeric nanoparticles in the intestinal absorption rate of EGCG across the Caco-2 cell monolayers^[83, 87]. According to previous reports, the P_{app} values for catechins including EGCG ranged from 0.8×10^{-7} cm/s to 3.5×10^{-7} cm/s^[88, 89], which are exceptionally low^[90]. In our experiment, we found that the maximum P_{app} of non-encapsulated EGCG was 3.5×10^{-7} cm/s, which appeared at 30 min after incubation of the nanoparticles with the Caco-2 cell monolayers. After encapsulated with chitosan-CPP nanoparticles, the P_{app} of EGCG increased significantly in accordance with the increase of nanoparticle concentration, and the appearing time of the maximum P_{app} delayed to 90 min followed with a smooth decrease (Fig. 6). The maximum P_{app} values of EGCG encapsulated with 0.0625 mg/mL, 0.125 mg/mL and 0.25 mg/mL chitosan-CPP nanoparticles were 6.2×10^{-7} cm/s, 9.5×10^{-7} cm/s and 1.3×10^{-6} cm/s. These results indicated that EGCG has been successfully promoted to be a compound having high intestinal permeation. Moreover, controlled permeation of EGCG through Caco-2 cell monolayers at constant high rates has also been successively achieved. Several aspects might contribute to the enhanced penetration of EGCG through Caco-2 cell monolayers. First, encapsulation of EGCG with chitosan nanoparticles was confirmed to

stabilize EGCG^[91], avoiding its auto-oxidization, which could be further enhanced by CPP that possesses the antioxidant activity in Caco-2 cell lines^[92]; Secondly, the nanoparticles could open the cellular tight junctions with a width less than 20 nm^[93]. Consequently, the chitosan-CPP nanoparticles must become unstable and disintegrated on their way through the paracellular pathway; thus the loaded EGCG can be released and permeated into the receiver compartment. Thirdly, EGCG-loaded chitosan-CPP nanoparticles could enhance transportation of EGCG *via* intracellular pathways through direct cellular uptake of the nanoparticles, which was also determined in our study, which will be discussed in the late of this review.

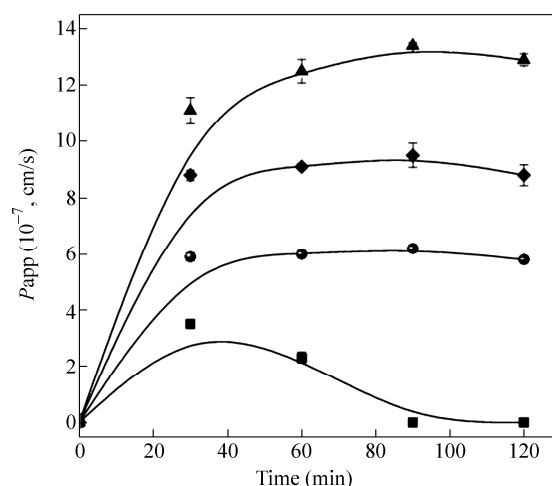


Fig. 6 The P_{app} of EGCG through Caco-2 cell monolayers incubated with EGCG solution (■), or EGCG-loaded CS-CPP nanoparticle suspension with different nanoparticle concentrations (●, 0.063 mg/mL; ◆, 0.125 mg/mL and ▲, 0.250 mg/mL) as a function of time at pH 6.2^[83]

CELLULAR UPTAKE OF THE POLYMERIC NANOPARTICLES

To date, the internalization of nanoparticles into the cells was achieved through cellular pinocytosis^[94]. Internalization of particles by pinocytosis can occur by different mechanisms: macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME) and clathrin- and caveolae-independent endocytosis^[94, 95].

Recently, several reports have discussed the pinocytosis of chitosan nanoparticles into cells by basic mechanisms such as clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, macropinocytosis and lipid raft-mediated endocytosis^[96, 97]. Among these mechanisms, caveolae-mediated endocytosis and lipid raft-mediated endocytosis have been confirmed to be the dominant ones. After entering cells, the chitosan nanoparticles were first intracellular trafficked to endosomes, and finally were entrapped in lysosomes^[96].

We investigated the cellular uptake fate of the chitosan-CPP nanoparticles loaded with EGCG^[87]. In order to visualize the uptake, chitosan was fluorescently labeled by treating with 0.2% of fluorescein isothiocyanate (FITC) in methanol. Cellular internalization of EGCG-loaded chitosan-CPP nanoparticles was confirmed from green fluorescence inside the Caco-2 cells. The process of nanoparticle uptake was dose and time dependent in the range of time and concentration studied. The cellular uptake of the chitosan-CPP nanoparticles might contribute to the enhancement of the intestinal absorption and bioactivity of the encapsulated EGCG.

PERSPECTIVES

The harsh digestive environment and barrier properties of the intestinal mucosa are big challenges for oral delivery of nanocarriers. Although the transport of nanoparticles across intestinal mucosa has been studied, little information is known about the behaviour of nanoparticle structure during digestion and the structure-function

relationships. Particle size, surface charge and hydrophobicity are important but the mechanisms are not clear. The main challenges in developing effective oral delivery systems are to: strengthen nanocarrier structure, control digestibility, program the release of the bioactive ingredients at the desired sites and promote bioavailability through direct endocytosis by the epithelium.

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