Chitosan-based materials for bio-separation and controlled delivery of proteins

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**Chitosan-based Materials for Bio-separation and
Controlled Delivery of Proteins**

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ABSTRACT

Chitosan (CS), a versatile biomaterial derived from the naturally abundant biopolymer of chitin, finds numerous applications in biomedical and chemical industries. This project developed three novel applications with the derivatives of this material. They are: (1) the adsorption of a model protein in fixed bed column, (2) the separation of polypeptide of glycomacropeptide (GMP) from milk whey and (3) the chitosan- κ -carrageenan polyelectrolyte complex for the oral delivery of protein drugs.

For (1): The adsorption of the model protein (bovine serum albumin (BSA)) on the native/cross-linked CS resin was studied in a batch adsorber as well as in a fixed bed column for the potential industrial application. The acidic stability and mechanical strength of native CS beads were improved by cross-linking process. It was found that pH, cross-linking, and ion strength can significantly affect BSA adsorption and the electrostatic interactions between BSA and amine groups of CS was the major adsorption mechanism in single BSA aqueous solution. An optimal pH of ~ 5.5 and a lower mass ratio of 0.2 (cross-linking agent/CS) were found in favor of BSA adsorption. The batch kinetics roughly follows the pseudo-second-order rate equation while the Bohart & Adams and Clark models can better simulate the breakthrough kinetics in fixed bed column.

For (2): the nano scale cavity structure of β -cyclodextrin (BCD) was grafted onto CS matrix successfully by two-step method to selectively adsorb GMP molecules from milk

whey proteins. The modified CS adsorbent beads (CS-HMDI-BCD) have a surface area of 38.43 m²/g that is not much different from native CS beads and EPI cross-linked CS beads. The selective adsorption mechanisms on the adsorbent were complicated, generally the electrostatic interactions and hydrophobic interactions were involved, but the formation of inclusion complex between cavities of BCD and glycosylated part of GMP was the major mechanism for the selective adsorption towards GMP. In the absence of any precipitation process using TCA/ethanol, the resultant modified CS beads presented superior adsorption affinity and capacity towards GMP. A simple acidic ninhydrin assay specific to sialic acid existing in GMP was used for the rapid and sensitive analysis of GMP in whey proteins mixtures. The contents of α -lactalbumin, β -lactoglobulin var. A, and β -lactoglobulin var. B in whey protein powder were ~21.1 %, ~24.6 % and ~46.6 %, respectively. At pH 3.0, 88.63 % of total GMP was adsorbed with a maximum adsorption capacity corresponding to 12.87 mg of sialic acid/g-adsorbent, while minimum, 6.25 % of other whey proteins was adsorbed. Most GMP and other whey proteins adsorbed can be eluted using 1 M sodium chloride solution in 2 hours. Desorption experiments showed that the modified beads could be regenerated and used in many cycles without significant decreases in the capacity and selectivity towards GMP.

For (3): chitosan- κ -carrageenan polyelectrolyte complex (PEC) was prepared by salt induced impeding of polyplex formation method and encapsulated with a model protein of BSA. The FTIR spectra showed the successful formation of the target PEC under the

experimental conditions. The surface area and average pore diameter of prepared PEC particulate were found to be $4.09 \text{ m}^2/\text{g}$ and 17.66 nm , respectively. The release kinetics of BSA from the PEC was studied in the simulated gastrointestinal fluids with and without digestive enzymes (pepsin and pancreatin), respectively. A typical controlled release of BSA from the prepared PEC that showed the nature of strong pH-sensitivity was obtained in the simulated intestinal fluid (SIF, pH 7.5), but only insignificant amount of BSA was released in the simulated gastric fluid (SGF, pH 1.2), confirming acidic stability of the prepared PEC. Little amount of BSA was released in SGF ($11 \mu\text{g}$ of BSA from 3 mg of PEC) while a large amount of BSA was released in SIF ($180 \mu\text{g}$ of BSA from 3 mg of PEC) due to the significant swelling and disintegration of PEC. The presence of digestive enzymes was found both not to affect the response of PEC to the ambient pH value, and to speed up the release of BSA from the prepared PEC particulate.

In conclusion, this project enhanced our fundamental knowledge of CS materials in bio-separation, provided a low cost and environmentally benign solution for the separation of GMP from milk whey proteins and the oral controlled release of protein drugs.

List of Publications

Papers resulted from this thesis

1. Li CB, Hein S, Wang K. Biosorption of chitin and chitosan. Mater. Sci. Tech.-Lond., 2008, 24, 1088-1099.
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3. Li CB, Hein S, Song XH, Wang K. Chitosan-carrageenan polyelectrolyte complex for oral delivery of proteins (submitted to International Journal of Pharmaceutics).
4. Li CB, Hein S, Wang K. Fixed bed study of BSA adsorption on native and cross-linked chitosan (in preparation).

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Conferences

Li CB, Hein S, Wang K. Chitosan Based Adsorbents for the Separation of GMP From Milk Whey, Symposium of Advanced Technology for Pharmaceutical Engineering, 2009, Beijing.

List of Abbreviation

α -la	α -lactalbumin	IgM	immunoglobulin M
β -lg	β -lactoglobulin	LPL	low density lipoprotein
AMG	amyloglucosidases	MIPs	molecular imprinted polymers
BCD	b-cyclodextrin	NeuAc	N-acetylneuraminic acid
BSA	bovine serum albumin	PAOMA	polyalkylenoxide-maleic acid
CB	Cibacron Blue F3GA	PEC	polyelectrolyte complex
CMP	caseinomacropeptide	PEG	polyethylene glycol
CNBr	cyanogens bromide	Ser	serine
CS	chitosan	TCA	trichloroacetic acid
CS-HMDI	chitosan beads bound HMDI	TFA	trifluoroacetic acid
CS-HMDI-BCD	b-cyclodextrin grafted chitosan beads	Thr	threonine
CS-SiO ₂	chitosan coated silica beads		
DEAE	diethylaminoethyl cellulose		
DMF	dimethylformamide	DD	degree of deacetylation
EPI	epichlorohydrin	MW	molecular weight
Gal	galactose	pI	isoelectric point
GalNAc	N-acetyl galactosamine	pK _a	acid dissociation constant
GI	gastrointestinal tract		
GlcN	D-glucosamine		
GlcNAc	N-acetyl glucosamine	ATR	attenuated total reflectance
GLU	glutaraldehyde	FESEM	Field emission Scanning Electron Microscope
GMP	glycomacropeptide	FTIR	Fourier transform infrared spectroscopy
HAS	human serum albumin	IMAG	immobilized-metal affinity chromatography
HMDI	1,6-hexamethylene diisocyanate	MCT	mercury cadmium tellurium
Ig	immunoglobulin	RP-HPLC	reverse-phase high-performance liquid chromatography
IgG	immunoglobulin G	SEM	scanning electron microscope

List of Symbols

α_L	the Langmuir isotherm constant (ml/ μ g)
β_a	the kinetic coefficient of the external mass transfer in Wolborska model (mm/min)
c_0	the initial concentration of adsorbate (μ g/ml)
c_{0m}	the highest initial concentration of adsorbate (μ g/ml)
c_e	the equilibrium concentration of adsorbate in liquid phase (μ g/ml)
C_0	the feed concentration of protein in fixed bed (mg/L)
C_t	the effluent concentration of protein in fixed bed (mg/L)
D	the axial diffusion coefficient
k_1	the rate constant for the pseudo-first-order kinetic model (1/min)
k_2	the rate constant for the pseudo-second-order kinetic model (g/(μ g min))
K_{BA}	the adsorption rate coefficient in Bohart and Adams model (L/(mg min))
K_C	the adsorption rate constant in Clark model (L/(mg min))
K_F	the Freundlich isotherm constant (ug/g (ug/ml) ⁿ)
K_L	the Langmuir isotherm constant (ml/g)
K_W	the adsorption rate coefficient in Wheeler-Jonas model (min ⁻¹)
M	the mass of the adsorbent in fixed-bed column (g)
$1/n$	the heterogeneity factor for Freundlich isotherm
N_0	the adsorption capacity in fixed bed column (mg/L)
ρ_b	the bulk density of the fixed-bed column (g/L)
P_G	adsorption percentage of GMP (%)
P_W	adsorption percentage of other whey proteins (%)
Q_e	the equilibrium concentration of adsorbate in solid phase (μ g/g)
Q_m	the theoretical maximum monolayer adsorption capacity (mg/g)
q_e	the equilibrium concentration of adsorbate in solid phase in kinetics model (μ g/g)
$q_{e,cal}$	the calculated equilibrium concentration of adsorbate in solid phase for normalized standard deviation (μ g/g)

$q_{e,exp}$	the experimental equilibrium concentration of adsorbate in solid phase for normalized standard deviation ($\mu\text{g/g}$)
q_t	the adsorbate concentration in solid phase at time t ($\mu\text{g/g}$)
$\Delta q_e\%$	the normalized standard deviation
R^2	the dimensionless equilibrium parameter for Langmuir isotherm
t	time (min)
U_0	the linear velocity of influent (mm/min)
v	the volumetric flow rate (L/min)
W_e	the equilibrium adsorption capacity in fixed bed column (mg/g)
Z	the bed height in fixed bed column (mm)

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CHAPTER 1 INTRODUCTION

1.1 Chitosan

Chitosan (CS) is a kind of production from *N*-deacetylation of chitin, and consists of D-glucosamine (GlcN) and *N*-acetyl glucosamine (GlcNAc) repeat units through a β -(1-4) linkage (1). Generally, the degree of deacetylation (DD) to amine group is high in chitosan and low in chitin, because the *N*-deacetylation of chitin cannot be totally completed (2). The chemical structure of chitin and chitosan are shown in Fig. 1.1 (3).

Fig.1. 1 The chemical structure of chitin (GlcNAc) and chitosan (GlcN) (3)

Compared with synthetic polymers, CS possesses a number of intrinsic advantages, such as low cost, biodegradability, biocompatibility, bioactivity, non-toxicity, and excellent adsorption properties. Moreover physical/chemical modifications can be readily carried out on CS for specific application because of the reactive amine and hydroxyl groups.

CS and its derivatives have been developed for many versatile applications. They were

extensively applied in biomedical field such as tissue engineering, bone substitute, drug delivery, wound dressing and so on (4-6). They also have been used as the enzyme immobilization matrix, antibacterial agents, cell-stimulating materials and haemostatic materials etc (7-9) in biotechnology. In water treatment, they are low cost and effective adsorbents for the removal of dyes, heavy metals and other contaminants (10-12). Besides, in the fields of food & beverage (13), cosmetics (14), agriculture (15), they also played an important roles. In the peptides/proteins separation and purification, they have been widely used as adsorbents or ligands support materials.

Therefore, CS is proposed as a starting material in our study that addresses bio-separation in batch adsorber/fixed bed column and controlled delivery of proteins, and extends the applications of CS-based materials in such area. The details are introduced in the following sections.

1.2 Milk whey and glycomacropeptide

A large amount of milk whey is generated from cheese production every year in the world. Whey is not a polluting for itself, but when being disposed in water without any treatment, it causes great polluting effects because it has a high biological oxygen demand of 30000 to 50000 mg of oxygen per liter of whey. Milk whey has great economical interest in food industry as a proteins source containing β -lactoglobulin (β -lg), α -lactalbumin (α -la), immunoglobulin, glycomacropeptide, lactoferrin and

lactoperoxidase. Meanwhile it also offers many functional natures of solubility, viscosity, emulsification, and gelification in actual applications.

Glycomacropeptide (GMP) or caseinomacropeptide (CMP), found in milk whey and formed by the κ -casein cleavage during cheese making, is a C-terminal and heterogeneous peptide containing 64 amino acid residues (16). The terms *GMP* and *CMP* can represent the whole peptide but sometimes they refer to the glycosylated and non-glycosylated forms of the peptide, respectively. In this thesis, GMP is used to represent the whole peptide unless otherwise indicated.

Following β -lg and α -la, GMP is the third most abundant component in the whey. It possesses a number of bioactivities mainly attributed to its glycosylated form (17). Its ability of protection against toxins, viruses, bacteria, and immune system regulation has been reported, such as inhibiting the binding of cholera toxin to its oligosaccharide receptor on cell walls (18-19), inhibiting the influenza virus hemagglutination (20), inhibiting the adhesion of cariogenic bacteria to the oral cavity (21) and inhibiting the proliferation of lymphocytes by mitogens (22). The digestion and gastrointestinal mobility also can be affected by GMP (23-24).

Besides these biological properties, GMP also possesses some nutritional properties. Being rich in branched-chain amino acids and poor in methionine, GMP can be an ingredient in diets for hepatic patients (25). There are no aromatic amino acids such as

phenylalanine, tyrosine and tryptophan in GMP sequence. Therefore, it is an ideal ingredient in food for phenylketonuric patients as well (26). GMP can also promote the absorption of some minerals (calcium, iron and zinc), which is important for infant formula (27).

Due to those remarkable biological activities and nutritional properties, GMP has been considered to be a potential ingredient for food and pharmaceutical product. More and more attentions have been given to develop technologies to isolate and purify GMP from the whey. Among them, ion-exchange chromatography is one of the most widely used methods. However, the current adsorbents are either low in efficiency or high in cost. The low cost, environmental-friendly and high throughput adsorbent is highly expected for the bulk production of GMP.

Based on this principle, one of the studies presented in this thesis is to apply CS and its derivatives as the novel adsorbents in the isolation and purification of GMP and to find out a kind of ‘greener’ technology with low cost, high purity and high yield.

1.3 Polyelectrolyte complexes in oral drug delivery

Polyelectrolytes are the polymers positively or negatively charged at near neutral pH value. When two oppositely charged polyelectrolytes (polyanion and polycation) bind each other in aqueous solution, a complex is formed by the electrostatic interactions as ionically cross-linked networks, termed as polyelectrolyte complex (PEC) (28). The

absence of chemical cross-linking agents makes PEC non-toxic and no harmful effects of reagents. As PEC is highly stimuli-responsive to the changes of pH, temperature, electric and magnetic field, it is used in the application of drug delivery as drug carriers.

Oral route is preferred for drug administration due to its high level of patient acceptance and long term compliance (29). Moreover, the dosage forms can be formulated with relative ease and are manufactured without sterile conditions (30). However, the peptide and protein drugs in oral delivery route face low bioavailability because the drugs can be easily hydrolyzed by the extreme acidity in the stomach and proteolytic enzymes in the gastrointestinal (GI) tract. The penetration barrier also prevents them from crossing the intestinal and then basal membranes to enter the blood (31). Therefore, improving the oral bioavailability of polypeptide drugs delivered through GI tract has attracted intensive researches.

The net charge on the PEC, which is an important factor determining the swelling and induced volume change, is affected by the pH value of ambient solution due to the variation in the degree of ionization of functional groups (32). Thus, the nature of highly pH-sensitive swelling makes the PEC an ideal candidate for oral drug delivery (33-34) because the pH varies at each organ or the diseased part of human body. CS is the ideal candidate to form PEC for oral drug delivery, due to its biocompatibility, reactivity, hydrophilicity and cationic nature.

Therefore one of the studies presented in this thesis is to develop a kind of CS-based PEC for the controlled delivery of proteins as a matrix in simulated gastrointestinal fluids of human body. The ideal matrix could potentially protect proteins in simulated gastric fluid and release proteins in simulated intestinal fluid.

1.4 The objectives and scopes of the thesis research

As introduced above, the general target of this project is to develop novel CS-based materials and to extend the applications of CS and these derivatives in bio-separation (adsorption of proteins in batch adsorber/fixed bed column and separation of GMP from milk whey) and controlled delivery of proteins (the pH-sensitive delivery of protein drugs with PEC). The specific targets are:

- 1) To synthesize CS hydrogel beads from the commercial CS powders.
- 2) To cross-link the native CS hydrogel beads with various spacers to increase the acidic stability and mechanical strength of CS beads.
- 3) To characterize the structure and the morphology of the native/modified CS beads and to evaluate their performance in the adsorption of a model protein in batch adsorber/fixed bed column under various operation conditions.
- 4) To graft nano scale cavity structure of β -cyclodextrin onto the CS matrix to enhance the adsorption capacity and selectivity towards GMP.
- 5) To develop a simple and easy to use assay for the quick determination of the GMP concentrations in whey protein solutions.

- 6) To develop a reverse-phase high-performance liquid chromatography gradient for the simultaneous analysis of GMP and other whey proteins.
- 7) To develop GMP desorption and regeneration technologies for the modified CS resins.
- 8) To develop CS-carrageenan PEC for the delivery of a model protein in the simulated gastrointestinal fluids of human body.
- 9) To study the operation conditions in the PEC prepared and the effect of enzyme in the controlled delivery of proteins under specific pH value.

1.5 Overview of the thesis

The thesis consists of 7 chapters:

Chapter 1 outlines the background, motivation, and what is addressed of this project.

Chapter 2 reviews the current state of the following research areas that are involved in my proposed study and shows the gaps that are related to the study.

I the CS-based materials applied in the adsorption of peptides, proteins, and lipids in batch adsorber,

II the adsorption of proteins in fixed bed column,

III the CS-based materials used in the isolation and purification of GMP from milk whey,

IV the CS-based PEC used as the media for controlled drug delivery.

Chapter 3 describes the general experimental works and principles of this project followed by some specific experimental conditions and procedures as examples.

Chapter 4 investigates the batch /fixed bed adsorption of a model protein (bovine serum albumin) on native/cross-linked CS adsorbents. Both the adsorption mechanism and the effect of pH value, functional groups, and ion strength will also be studied to enhance our basic knowledge on proteins adsorption on such adsorbents and help us better understand the selective adsorption mechanism in the proteins solution, such as the whey proteins solution. This chapter is the basis for Chapter 5 and has good potential in industrial applications.

Chapter 5 presents the isolation and purification of GMP from milk whey using the native and modified CS beads including the chemical modification of CS beads, the characterization of structure, morphology, and function groups of the adsorbents prepared, and the analytical assay (methods) for the GMP and other proteins existing in the whey.

Chapter 6 focuses on another application of CS-based materials (the pH-sensitive delivery of protein drugs) besides the bio-sorption processes that are presented in both Chapters 4 and 5. It includes the preparation, characterization and the evaluation of

CS-carrageenan PEC for the delivery of a model protein. The factors such as enzyme and pH value, etc. will be investigated.

Chapter 7 draws the conclusions and gives future recommendations for this project finally.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

This chapter will review the current state of the relevant research including the CS-based materials in peptides, proteins and lipids adsorption in batch adsorber and fixed bed column, the CS-based materials in isolation and purification of GMP from the milk whey, and the CS-based PEC as the carrying matrix for controlled proteins delivery, and show the gaps that are related to my proposed study.

2.2 CS-based materials in the separation of peptides/proteins

Many proteins are negatively charged while CS is positively charged in the mild acidic and near neutral pH conditions. Therefore, this property is exploited in the separation and purification of protein using CS powder/hydrogel beads as anion exchange chromatographic media. Further modifications of CS with different ligands lead to development of affinity chromatographic media.

2.2.1 Ion exchange chromatography

As an ion exchange chromatographic media, cross-linked CS beads are more frequently used than native CS beads because they are insoluble in acidic solution and not compressed by column pressure. The system was successfully verified by the sorption

of bovine serum albumin (BSA) as a model protein (35-37). To improve the sorption capacity with permanent positive charge, didodecyldimethyl ammonium chloride with quaternary ammonium was used to react with amine groups of CS (37). Although the concentration of reactive amine groups from CS is decreased due to the cross-linking, the presence of quaternary ammonium group helps to boost the sorption capacity. Epichlorohydrin (EPI) is a cross-linker that is capable of reacting with hydroxyl or amine group to form cross-linked beads that was used to purify the GMP from sweet whey (38). Grafted polymers of CS have also been investigated for protein adsorption (39-42). In this application, CS is grafted with anionic polymers (e.g., methacrylic acid, or acrylic acid) to become negatively charged beads. Because of the surface negative charge, these beads have been investigated for cationic exchange chromatographic media. These media are particularly useful for the sorption of positively charged protein like low density lipoprotein (LDL) (40). Table 2-1 summarized the application of CS ion exchange chromatography.

Table 2-1 Examples of CS based material used in ion exchange chromatography

Adsorbent	Adsorbate	Sorption property	Eluate	Ref.
Cross-linked CS beads	BSA	Adsorption capacity: 165gl ⁻¹ wet resin	NA	(35)
Cross-linked CS gel	BSA	Yield: 70.2%	0.05M NaCl in tris-HCl at pH 9.05	(36)
Cross-linked CS gel	Protease	Yield: 48%	0.05M NaCl in tris-HCl at pH 9.05	(36)
Cross-linked CS gel	BSA	adsorption capacity: 190 gL ⁻¹ wet resin	0.2 M NaCl	(37)
Epichlorohydrin-treated CS resin	GMP	Adsorption capacity: 2.18gg ⁻¹ dry resin; yield: 19.8% of dry weight of nondialyzable fraction of SW	2 M NaCl	(38)
CS-g-PMMA beads	Lysozyme	Adsorption capacity: 65.7 mgg ⁻¹ ; yield: 94%	1.0M KSCN at pH 8.0	(39)
CS-g-PAA beads	LDL	Adsorption percentage: 64.3%	NA	(40)

CS=chitosan, PAA=poly (acrylic acid), PMAA=poly (methacrylic acid), BSA=bovine serum albumin, GMP=glycomacropeptide, LDL=low density lipoprotein, SW=sweet whey

2.2.2 Affinity chromatography

The affinity chromatography, which is based on the highly specific strong interaction between the affinity ligand and the target protein, is widely used in protein separation. Because the separation is not pH dependent as in ion exchange chromatography, the biological activity of the target protein is preserved and the isolation of protein from very dilute solution is possible. The affinity ligands attached onto CS include dyes, metal ions, amino acids, proteins, and other chemical moieties. They are attached to

amino or hydroxyl groups of CS directly or indirectly via spacers (43-44). Some ligands and spacers are summarized in Table 2-2 (2) with their target protein for separation.

Because certain dyes have specific affinity towards specific proteins, dyes are attached to CS beads for the specific protein sorption. For instance, Cibacron Blue F3GA (CB), attached to CS microspheres can adsorb catalase, BSA, and human serum albumin (HAS) (45-47). The maximum sorption capacity is observed at isoelectric point (pI) of proteins. Therefore, it is clear that the dominant force contributing to dye-protein interaction is hydrophilic rather than electrostatic.

Chemical moieties specific to certain types of proteins are attached to CS beads via spacers to enhance protein separation. Some bifunctional chemical molecules, such as GLU, EPI, carbodiimide and diepoxide, are usually used as spacers (48). CS beads are attached with N-butylamine, isobutylamine and N-octylamine via 1,4-butanediol diglycidyl ether as spacer was studied for the purification of HAS from mock-serum (49). Similarly, 4-amino-4-oxobutanoic acid is attached to CS beads via 1,4-butanediol diglycidyl ether to separate human IgG from cell culture supernatants (50-51). The total yield of the target protein was found comparable with the commercial column for immunoprotein purification. The majority protein-ligand interaction is electrostatic forces in this category. The β -cyclodextrin-CS affinity support has also been reported for the specific adsorption of amyloglucosidases (AMG) (52-54).

Another category exploits the specific interaction between two specific proteins in which one protein is immobilized on CS matrix to purify another protein. For instance, chicken ovomucoid was immobilized on CS support to separate trypsin (55). Although the interactions between two proteins are highly specific, these bio-ligands are macromolecular, fragile, expensive, and preferential to gentle elution condition. Their activities can easily be lost in their attachment to matrix. Therefore, the use of synthetic smaller molecules (e.g., dyes, chemical moieties) immobilized onto CS beads is preferred.

The terminal groups at the distal ends of the spacers also can react with the amino groups or hydroxyl groups of CS. The stability of such cross-linked CS beads under acidic condition and their mechanical property are improved (49). Therefore, they can withstand high pressure and high flow rate in chromatography in addition to the specific attraction with proteins. Moreover, the excellent stability of ligands attached onto CS overcomes the ligands leakage. Therefore, the cross-linked CS beads are more frequently used than native CS beads. Although, synthetic polymers such as acrylates, polyamide and derivatized polystyrene are more rigid, but they are difficult to immobilized bio-ligand due to their less compatibility with bio-ligands and high non-specific integration.

Silica is a popular porous inorganic chromatographic support with excellent mechanical properties. Its pore size distribution can easily be controlled to prefer the transfer of

macromolecules inside the matrix. However, it is difficult to be used directly in protein purification because its hydrophobic surface causes non-specific sorption of protein and it is not stable at alkaline pH ($\text{pH} > 8.0$). CS coated silica beads, therefore, were developed. The silica in the core of the beads act as the rigid support, while the macro-porous CS layer coated on the silica surface provide reactive sites for ligands and hydrophilic surface for specific sorption. Thus, the advantage of CS and the excellent mechanical property of silica are combined. Trypsin immobilized CS coated silica beads (CS-SiO_2) are reported for the purification of trypsin inhibitor (56). CS-SiO_2 beads were prepared by phase inversion and polyethylene glycol (PEG) molecular imprinting methods. The functional group of epoxy, diazo and aldehyde can serve as spacer for trypsin attachment (57). Trypsin inhibitor was efficiently separated with 9.1 of purification fold from crude egg white (56).

CS sulfoderivatives, such as CS-O-sulfate, N-succinylated CS-O-sulfate, and CS N-succinyl sulfate, were also coated on silica beads and to acted as ligands for the purification of lipoprotein because sulfopolysaccharides can form biospecific complex with LDL in plasmas (58). Among these sulfoderivatives, CS N-succinyl sulfate exhibits comparable complexing ability (removal efficiency $\sim 53\%$) in spite of low sulfur content because the carboxyl groups of CS N-succinyl sulfate are closed to the sulfo groups and can form a chelating structure.

2.2.2.1 Immunoaffinity chromatography

Immunoaffinity chromatography uses the specific binding of antibody to the target

protein to selectively purify the protein. CS or cross-linked CS beads also can be used as matrix. DNA is conjugated to the hydroxyethyl cross-linked CS beads via the cyanogens bromide (CNBr) activation. The resultant beads can be used to efficiently reduce the anti-DNA antibody levels in serum (59). Anti-lisinopril IgG is covalently bound to alginate-CS gel beads via GLU and the system can be applied for immunoaffinity purification (60). Apart from the specific antigen-antibody interaction, phenylalanine and tryptophan are immobilized covalently on CS beads using N_2 -plasma, carbodiimide or GLU to purify γ -globulin, IgG and IgM (61).

2.2.2.2 Metal binding affinity chromatography

Immobilized-metal affinity chromatography (IMAC), which was proposed in 1974 (62), represents a separation technique based on the coordination between immobilized metal ion and electron donor groups from the protein surface. The electron-pair acceptor such as Cu (II), Ni (II), Zn (II), Co (II) and Fe (III) are most commonly used. The native histidine residues of proteins and engineered histidine tags or histidine clusters of recombinant proteins serve as electron donor groups (63). Many surface modification techniques have been developed to increase surface hydrophilicity and loading capacity to eliminate the non-specific adsorption of matrix (64-68).

The use of cross-linked CS coated silica beads (CS-SiO₂) as matrix (64, 69-71) is one of the resolutions due to the metal chelating ability, hydrophilic surface, and inexpensiveness of CS. The need of organic ligands can also be completely eliminated.

Normally, CS-SiO₂ adsorbents are prepared by a three-step procedure consisting of CS deposition (PEG molecular imprinting methods) (56), cross-linking and metal ion immobilization. The cross-linking ratio plays an important role. The increase in cross-linking ratio is known to enhance the stability of CS film and to reduce the residual non-specific sorption of proteins with protonated amino groups in CS via electrostatic interactions. However, very high cross-linking ratio also caused the lower metal chelating capacity and subsequently lowers the protein separation. Therefore, an optimal cross-linking ratio is necessary for the efficient separation. The binding of BSA on CS-SiO₂ was found optimal at 3.4 of cross-linking ratio and 5.0 of pH value, close to the pI of BSA (69). The formation of the complex between protein and Cu (II) was found easier due to the absence of electrostatic repulsive forces (69-70). The affinity of a protein also strongly depends on the choice of the metal ions. The maximum sorption capacity of trypsin on CS-SiO₂ was achieved in the following order: Cu (II) > Zn (II) > Ni (II) (71).

IMAC suffers from some disadvantages as moderate sorption selectivity compared with the specific bio-ligands (63), and the metal toxicity (72). The target proteins have to contain histidine residues. If not, the proteins have to be engineered histidine tags or histidine clusters ahead, which is technically more complicated (73-75). However, IMAC also has a number of advantages, such as ligand stability, high protein loading, moderate elution condition, and inexpensiveness (76). Such factors are of crucial

importance in the development of large-scale protein purification processes (63).

2.2.2.3 Molecular imprinted affinity chromatography

Similar principle as in the ion imprinting technique has been employed in the development of CS based molecular imprinted adsorbent for protein separation (77-80). The additional step is to entrap a soft polymer (e.g., polyacrylamide) inside the pores of cross-linked CS hydrogel beads together with imprinting proteins (template molecule) instead of direct mixing with CS solution. Although this method is started with physical entrapment of polyacrylamide gel in the CS beads (77), the chemical modification of cross-linked CS hydrogel beads are introduced to ensure the stable entrapment of the soft polymer inside the beads (79-81). The process is illustrated in Fig. 2.1(2). The chemical modification include the preparation of active double bonds bearing cross-linked CS beads (78-79) and the grafting of acrylamide onto CS (80). The grafted polymer allows the direct mixing of template molecules and therefore it is not necessary to do the additional step to entrap the soft polymer inside the pores of CS beads. Instead of polyacrylamide, the possible use of polymethacrylic acid gel was also discussed for separation of quercetin (81). All the resultant molecular imprinted polymers (MIPs) exhibited high sorption capacity for target protein [Table 2-3] (2).

2.2.2.4 Fluidized-bed affinity chromatography

Fluidized-bed chromatography used in the separation of proteins/peptides is of more

recent origin, and the adsorbents are freely suspended in a flowing liquid stream. This process can deal with crude feedstock directly because particulate matters contained in crude feedstock, which could clog a packed column, can readily pass through a fluidized-bed column in the flow direction from the bottom to top. Consequently, the fluidized-bed column can combine the procedures of reducing initial volume, concentrating the sample with clarification and selectively capture in a single unit process. However, the protein binding principles are the same as the standard packed column chromatography and common adsorbents can be used (82). CS beads are also exploited in this process, and affinity between CS beads and *Aspergillus niger* cellulose was founded (83-84) [Table 2-3] (2). CS seems to act as a macroaffinity ligand.

2.2.3 Affinity separation with CS-magnetite composite particles

The extraction of target protein from a mixture requires pre-purification steps to eliminate insoluble particles to avoid column clogging. The association of magnetite particles with CS allows the skipping of these pre-purification steps because it is possible to separate the target protein attached to CS-magnetite composite particles by an external magnet (85-89). These composite particles are prepared by directly mixing CS solutions with magnetite particles to form hydrogel particles (85-86), by co-precipitating or water-in-oil micromulsion with magnetite nanoparticles (87-88) or by conjugating covalently with magnetite nanoparticles via EDC (1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride) cross-linker (89).

CS-magnetite composites nanoparticles exhibit a rapid adsorption rate because of the large surface area to volume ratio. These magnetite composite particles have been studied for the separation of lysozyme (85), solanum tuberosum lectin (86) and BSA (89). Moreover, aprotinin is attached to the CS-magnetite composite particles to purify trypsin (90) because of the exchange characteristics of aprotinin and trypsin (91) [Table 2-3] (2).

2.3 CS-based materials in the separation of lipids

The hypolipidemic activity of CS makes it a dietary supplement to reduce obesity and to lower cholesterol. The binding of bile acid and salts to CS through electrostatic interaction in the small intestine has been proposed as a mechanism for the reduction of blood cholesterol (92-95). The binding capacity is increased in deoxycholic acid than in cholic acid, which indicates the presence of additional hydroxyl groups on bile acids enhances their adsorption on CS (94). The hydrophobic interactions play an important role in the sorption of bile acids. CS and CS-based materials directly bind neutral lipids such as cholesterol and other sterols (96) through hydrogen bonding and hydrophobic interaction. CS-based materials also bind fat efficiently from cheese whey, serum and palm oil effluent (97-98) [Table 2-4] (2). Hydrophobic modification of CS and the immobilization of β -cyclodextrin on CS are the major methods of increasing binding capacity of cholesterol (99-101) and fat (97-98). No convincing data presents a straightforward correlation between lipid-binding capacity and %DA and molecular

weight of CS (102). Although the in vitro efficient bio-sorption of lipids, cholesterol and fats to CS is unanimously accepted, its efficacy as weight reducing dietary supplement is still under serious debate (103-104)

In the literatures reviewed above, various adsorbents based on CS have been prepared using different methods as well as different materials. Mechanisms, such as electrostatic interactions, hydrogen bonding and hydrophobic interactions, are involved in the sorption of peptides, proteins, and lipids depending on the experimental conditions. Because of the high efficiency of CS-based materials in the bio-sorption of dyes and metal ions, the preparation of CS beads affinity matrix such as ligands attached affinity chromatography and IMAC becomes feasible for industrial scale separation of proteins. The biocompatibility of the biopolymer contributes the stability of the immobilized bio-ligands on the CS beads matrix.

The bio-sorption processes actually are mainly operated in fixed bed columns. Since particles and flakes can cause column clogging, CS beads are preferred in fixed bed columns. However, hydrogel beads have a drawback of high water content and poor mechanical stability. The dried beads with low water content will improve volumetric adsorption and diffusion limitation. The biodegradability of CS is also a limitation for long-time sorption process. Freeze-drying, cross-linking, coating on rigid supports are the techniques used to prevent structure collapse and biodegradability while allowing high pressure drop and high flow rate.

Fluidized bed chromatography and affinity separation using CS-magnetite composite particles can give an alternative resolution especially when cells and cell debris particles are mixed with target bio-macromolecules. If these two methods are integrated with current methods of affinity separation using MIPs, there is a great potential to develop novel affinity separation processes for bio-macromolecules for industrial scale. Fig.2.2 illustrates such as particles (2). Magnetite nanoparticles can be prepared with silica shell and coated with protein imprinted CS layer for affinity separation of protein in solution containing cell debris.

Because of the low cost, comparable affinity with bio-ligands and compatibility of crude elution, small molecules ligands, such as metal ions, dyes and chemical moieties, bound with CS beads possess a favorable tendency towards the affinity separation for several bio-macromolecules. However, the leakage of ligands and the attenuation of binding capacity should be carefully evaluated.

Another promising method is the molecular imprinting techniques. It shows high selectivity in the separation of peptides/proteins. This process can rapidly extend to affinity separation of other bio-macromolecules to develop CS-based microfluidics devices for lab-on-chip investigation.

Finally, no matter what kind of adsorbents was reported, most adsorption experiments

were only carried out in batch adsorber at the laboratory level to evaluate the performance of adsorbents. Few adsorbents were evaluated and adsorption kinetic was rarely studied in fixed bed column. However, in order to scale up for the industrial purpose, the knowledge of operation conditions in fixed bed column are essential. Therefore, the studies of operation conditions, adsorption kinetics as well as the modeling in fixed bed column might be another hot issue for the bio-sorption of protein using CS-based materials as the adsorbents.

Fig.2. 1 Schematic illustration for the preparation of CS MIP for protein bio-sorption

1: CS solution, 2: CS porous hydrogel beads, 3: mixture containing cross-linked CS beads, and target protein; 4: one CS hydrogel bead containing soft polymer and target protein, 5: cross-linked CS interconnect pores filled with soft polymer and target protein, 6: imprinted CS hydrogel.

I: pores filled with soft polymer, II: protein inside the soft polymer, III: interconnected pores of CS.

a: phase inversion with sodium hydroxide or ionic gelation with TPP, b: cross-linking, c: imprinting [(i) diffusion of monomer and protein into interconnected pores of CS, (ii) polymerization, (iii) removal of soft polymer attached on the surface of CS beads] (2).

Fig.2. 2 Molecular imprinted CS coated iron oxide/silica core-shell nanoparticle (2)

Table 2-2 Examples of CS-based materials used in affinity and immunoaffinity chromatography (2)

Adsorbent	Ligand	Spacer	Adsorbate	Sorption property	Eluate	Ref.
Cross-linked CS microspheres	Cibacron blue F3GA	NA	HSA	Adsorption capacity: 15.35 mgg ⁻¹ ; desorption ratio: 86%	0.5 M KSCN at pH 8.0	(45)
Cross-linked CS microsphere	Cibacron blue F3GA	NA	Catalase	Adsorption capacity: 28.4 mgg ⁻¹ Desorption ratio: 90.6%	0.5M NaSCN at pH 8.0	(46)
Cross-linked CS microspheres	Cibacron blue F3GA	NA	BSA	Desorption ratio: 99%	0.1 M Tris-HCl buffer containing 1.0M NaSCN at pH 8.0	(47)
Cross-linked CS beads	n-butylamine	1,4-butanediol diglycidyl ether	BSA	Adsorption capacity: 6.5 mgg ⁻¹ desorption ratio: 90%	0.5 M NaCl	(49)
Cross-linked CS beads	4-amino-4-oxobutanoic acid	1,4-butanediol diglycidyl ether	Human IgG	Yield: 78-83%	10Mm KHPO ₄ , 1.0M NaCl at pH 5.5	(50)
β-CD-CS matrix	β-cyclodextrin	NA	AMG	Yield: 72%	2M NaCl at pH 4.0(l), borate buffer at pH 8.0(lI)	(54)
Cross-linked CS beads	Chicken ovomucoid	Glutaraldehyde	Trypsin	Yield: 70%	0.05M Tris-HCl at pH 7.5, with 0.2M CaCl ₂ (l), 0.1M glycine-HCl at pH 2.4(lI)	(55)
CS-SiO ₂	Trypsin	1,2-ethylene diglycidyl ether	Trypsin inhibitor	Purification time: 9.1	4 M urea	(56)
Cross-linked CS beads	DNA	2-chloroethanol	Anti-DNA antibody	Adsorption percentage: 65.33%	NA	(59)

CS= chitosan, CD=cyclodextrin, HSA=human serum albumin, BSA=bovine serum albumin, IgG=immunoglobulin G, AMG=amylglucosidase

Table 2-3 Examples of CS based-materials used in metal binding, molecular imprinted, fluidized-bed affinity chromatography, and CS-magnetic composite particles (2)

Adsorbent	Ligand	Adsorbate	Sorption property	Eluate	Ref.
Metal CS-SiO ₂	Cu(II)	BSA	Adsorption capacity: 192mgg ⁻¹ chitoan; recovery ratio: 85%	0.1M imidazole solution	(70)
Metal CS-SiO ₂	Cu(II)	Trypsin	4980±135 IUg ⁻¹	Acidic solution with pH below 2 or EDTA, imidazole	(71)
Metal CS-SiO ₂	Zn(II)	Trypsin	3762±681 IUg ⁻¹	Acidic solution with pH below 2 or EDTA, imidazole	(71)
Metal CS-SiO ₂	Ni(II)	Trypsin	2636±531 IUg ⁻¹	Acidic solution with pH below 2 or EDTA, imidazole	(71)
CS-MIPs	NA	hemoglobin	Adsorption capacity: 35.7mgg ⁻¹	10% (v/v) acetic acid containing 10% (w/v) SDS	(78)
CS-MIPs	NA	BSA	Adsorption capacity: 36.25mgg ⁻¹	NA	(80)
CS-MIPs	NA	Quercetin	Adsorption capacity: 318ugg ⁻¹	Acetonitrile/methanol 50:50, v/v	(81)
CS beads	NA	<i>Aspergillus niger</i> cellulase	Adsorption capacity: 50Uml ⁻¹ wet beads; purification time: 30	1 M phosphate buffer containing 20% ethylene	(84)
Magnetic acetylated CS	NA	Lysozyme	Activity: 10 fold higher than crude egg white	0.01 M HCl	(85)
Magnetic cross-linked CS	NA	Solanum tuberosum lectin	Yield: 50; purification time: 12.6	Glycin/HCl buffer at pH 2.2	(86)
CS-MNPs	NA	BSA	Adsorption capacity: 94.45 mgg ⁻¹ ; desorption ratio: 91.5%	0.36 M NaH ₂ PO ₄	(89)
Cross-linked magnetic CS particles	Aprotinin	Trypsin	Yield: 70.6% Purification time: 1.73	0.1 M borax buffer at pH 2.7	(90)
Cross-linked magnetic CS particles	Trypsin	Aprotinin	Purification time: 3.71	0.01 M acetic acid and 0.5 M potassium chloride at pH 2.7	(91)

CS= chitosan, MNPs=magnetic nanoparticles, MIPs=molecular imprinted polymers, BSA=bovine serum albumin, SDS=sodium dodecyl sulfa

Table 2-4 Examples of CS used as lipid-binder (2)

Adsorbent	Adsorbate	Maximum adsorption capacity	Ref.
Chitosan	Taurocholate	1.8mmolg ⁻¹	(98)
Chitosan	Cholic acid	0.61umolg ⁻¹	(94)
Chitosan	Deoxycholic acid	1.63umolg ⁻¹	(94)
Chitosan	Chendeoxycholic acid	1.61umolg ⁻¹	(94)
CTS in acetate buffer	Sodium taurocholate	4mmolg ⁻¹	(93)
chitosan	Sodium glycocholate	1.42mmolg ⁻¹	(95)
Diethylaminoethyl Chitosan	Sodium glycocholate	3.12mmolg ⁻¹	(95)
Quaternized diethylaminoethyl chitosan	Sodium glycocholate	4.06mmolg ⁻¹	(95)
Chitosan chloride	Cholesterol	23mgg ⁻¹	(96)
Chitosan	Cholesterol	127mgg ⁻¹	(96)
CS-BCD powder	Cholesterol	20.68% bound	(99)
CS-BCD beads	Cholesterol	430mgg ⁻¹	(100)
Chitosan	Olive oil	1239mgg ⁻¹	(94)
Chitosan powder	Oil in palm oil mill effluent	3960mgg ⁻¹	(97)
chitosan taurocholate	Olive oil	22gg ⁻¹	(98)
Chitosan glycocholate	Olive oil	60gg ⁻¹	(98)
Chitosan deoxycholate	Olive oil	27gg ⁻¹	(98)
Chitosan taurocholate	Butter oil	22.1gg ⁻¹	(98)
Chitosan taurocholate	Corn oil	22.1gg ⁻¹	(98)

CS= chitosan, BCD=β-cyclodextrin

2.4 Protein adsorption in fixed bed column

The fixed bed is a popular separation technology in chemical plant. Generally, a cylindrical column is filled with suitable packing materials and the feed passes through the column. In the industrial application, a fixed bed column is advantageous to a batch adsorber because of low cost, large contact surface area and the ability to process large amount of feed continuously (105). The performance of a fixed bed column is generally described by its breakthrough curve, which is plotted as the concentration versus time profile at the outlet. The breakthrough curve is an important characteristics that determines the response and the process parameters because it affects the adsorption capacity and, in actual application, the economics and feasibility of the adsorption operation. Therefore, a number of mathematical models have been developed to simulate/predict the breakthrough curves of fixed bed columns, which are essential for the design and optimization of the operation conditions. Some of those models were widely used in the adsorption of organic vapors (106-107), dye (108-110) and heavy metal ion (111-116), etc.

The protein separation chromatography is basically a fixed bed column process. Many research efforts were devoted to this application to investigate the influence of operation conditions, such as column length, feed concentration, flow rate, protein size and temperature. For example, the adsorption of HAS onto DEAE Trisacryl M and DEAE Sepharose resins were investigated in a fixed bed column to study the shape of breakthrough curve and kinetics parameters (117-118). As protein adsorption processes are more complicated than the adsorption of small hydrocarbon molecules, some traditional mathematical models cannot be used to simulate the experimental data. Thus, various mathematical models have been formulated/improved for specific bio-system.

Some models are local equilibrium model that neglected all the transport resistance (119-124). But when real mass transfer resistances were taken into account, these models also cannot work correctly (125). Therefore, models taking into account axial dispersion and all the mass transfer resistances for the non-equilibrium conditions were developed, such as the general rate model (126-127), the lumped pore diffusion model (128-129), the transport-dispersive model (130).

Another category of mathematical models was specially developed for the inert core adsorbents. Due to high density in the core the inert core adsorbents had the short diffusion path that was capable of reducing the high intraparticle diffusion resistance for biological macromolecules (131-132). In addition, this kind of adsorbents was capable of recovering biological macromolecules fast and maintaining the biological activity, which is essentially important in the pharmaceutical and biotechnology application. This property made the inert core adsorbents, such as the Poroshell 5- μm particle with a thin layer of porous silica on a solid core, suitable for fast, high-resolution protein separation (133). The previous analytical solutions of breakthrough curve for the porous packing materials were not suitable for the inert core adsorbents. Therefore, a model was specially developed for the inert core spherical particles without the consideration of axial dispersion and external mass transfer resistance (134). On the contrary, with axial dispersion, external mass transfer resistance and intraparticle diffusion considered, the general rate model was also extended and analytical solution, parabolic-profile approximate solution and Q-LND approximate solution was derived under linear isotherm kinetic for this kind of adsorbents (135-137).

However, those models mentioned above are too mathematical and numerical complicated. As a result, many computational calculations are extremely needed to solve the equations. Alternatively, semi-empirical or simplified models, such as Thomas model (138) and Bohart-Adams model (139), are more common and convenient for the study of chromatography process (140-142), predicting or correlating breakthrough curve as well (143-148). Those models are briefly introduced here.

Bohart and Adams model

This model, established by Bohart and Adams for the adsorption of chlorine on charcoal (139), has been applied successfully in the adsorption of protein in fixed bed column (118). After solving the differential equations, the following equation was obtained:

$$\ln \left(\frac{C_0}{C_t} - 1 \right) = \frac{K_{BA} N_0 Z}{U_0} - K_{BA} C_0 t \quad (1)$$

Where C_t is the effluent concentration (mg/L), C_0 is the feed concentration (mg/L), K_{BA} is the adsorption rate coefficient (L/(mg min)), N_0 is the adsorption capacity (mg/L), Z is the bed height (mm), U_0 is the linear velocity (mm/min) and t is the time (min).

Wheeler-Jonas model

The Wheeler-Jonas model (149-150) can be used to describe any type of adsorption of a single vapor by a suitable adsorbent and estimate the breakthrough time of fixed-bed, based solely on measurable and readily available macroscopic parameters. The expression of the equation is as follows (151):

$$t = \frac{MW_e}{vC_0} - \frac{W_e \rho_b}{K_W C_0} \ln \left(\frac{C_0 - C_t}{C_t} \right) \quad (2)$$

Where K_W is the overall adsorption rate coefficient (min^{-1}), M is the mass of the adsorbents in fixed bed column (g), v is the volumetric flow rate (L/min), W_e is the equilibrium adsorption capacity (mg/g), and ρ_b is the bulk density of the fixed bed column (g/g).

Wolborska model

This model was specially established to predict the breakthrough curve in low-concentration based on general mass transfer mechanisms. With some assumptions, the solution can be obtained as:

$$\ln\left(\frac{C_t}{C_0}\right) = \frac{\beta_a C_0}{N_0} t - \frac{\beta_a Z}{U_0} \quad (3)$$

$$\beta_a = \frac{U_0^2}{2D} \left(\sqrt{1 + \frac{4\beta_0 D}{U_0^2}} - 1 \right) \quad (4)$$

Where β_a is the kinetic coefficient of the external mass transfer (mm/min), D is the axial diffusion coefficient that can be negligible in short bed or at high flow rate of influent where β_a is equal to β_0 .

Clark model

This model combined the mass transfer mechanisms and the Freundlich isotherm:

$$\left(\frac{C_0}{C_t}\right)^{n-1} - 1 = Ae^{-rt} \quad (5)$$

$$A = \exp\left(\frac{K_C N_0 Z}{U_0}\right) \quad (6)$$

and

$$r = K_C C_0 \quad (7)$$

Where K_C is the rate constant (L/(mg min)), N_0 is the adsorption capacity (mg/L) and $(1/n)$ is heterogeneity factor in Freundlich isotherm model.

A number of progresses have been made in the field of protein adsorption in the fixed bed column and chromatography. Various commercial packing resins, such as TSK-GEL DEAE-5PW, Resource-Q, SP Sepharose FF, DEAE Trisacryl M and DEAE Sepharose, coupled with specific mathematical models have been developed. However, the packing resins based on CS were rarely used. In Section 2.2 and 2.3, it could be seen that a number of CS-based adsorbents have been developed for the adsorption of peptides, protein, and lipids in the batch adsorber. Future efforts are needed to explore the application of these materials in the field of the fixed bed column or chromatographic separation of peptides, proteins, and lipids for the industrial application.

2.5 Structure and composition of GMP

During the cheese making, chymosins break the Phe₁₀₅-Met₁₀₆ bond of κ -casein and form two peptides, para- κ -casein in the cheese and GMP in the whey. GMP contains 14 kinds of amino acids, which are the residues 106 to 169 in κ -casein (152-153). The sequence and heterogeneity of GMP have been extensively studied and well known. The sequence of bovine GMP and the chemical structure of amino acid contained in GMP are shown in Fig. 2.3 (26, 154) and Fig. 2.5, respectively.

Fig.2. 3 The sequence of bovine glycomacropeptide (GMP) variant A and B (26, 154)

Fig.2. 4 The chemical structures of Gal, GalNAc, and NeuAc; the polysaccharide chains identified in GMP (26)

The genetic variance and post-translational modification (phosphorylation, glycosylation) are found to make GMP heterogeneous. As shown in Fig. 2.3, different amino acids appearing at position 136 and position 148 result in the two genetic variants (GMP_A, GMP_B) (155-156). Phosphorylation occurs at Ser 127 and 149, while in glycosylation, N-acetylgalactosamine (GalNAc) might covalently attach at positions of Thr 121, 131, 133, 135, 136, 142, 165 and Ser 141, 142 through an *O*-glycosidic linkage

(157-161). The other two saccharides, galactose (Gal) and N-acetylneuraminic acid (NeuAc), as shown in Fig. 2.4 (26), further attach to GalNAc in five different way (160), those make the composition of GMP more complicated with 14 glycosylated forms in GMP_A (162). Usually 50%-60% of bovine GMP is glycosylated (156), but it is essentially dependent on the whey source (163). In addition, GMP contains approximately 80 % of total sialic acid (NeuAc) in the whey (164). This is a very important property for the determination of GMP through the content of sialic acid.

Fig.2. 5 The chemical structures of amino acids contained in GMP

2.6 Isolation and purification of GMP

GMP has been isolated from whey by exploiting differences in molecular properties from other whey proteins, such as differences in trichloroacetic acid (TCA) solubility, isoelectric point, polarity, and pH-dependent molecular weight. The various approaches for GMP isolation and purification consist of single process or a combination of different processes and can be divided into three main categories.

2.6.1 Membrane filtration

Membrane filtration is one of the common methods used in separating GMP from other whey proteins. It is mainly based on the difference in membrane pore size and molecular weight (MW) of whey proteins. The experimental MW of GMP varies due to its self-association and dissociation under different pH value. It was reported that the MW ranges from 10 to 30 kDa at pH 3.5 and from 45 to 50 kDa at pH higher than 4 (165-166). They are both higher than the theoretical MW (7~9 kDa) (165) of GMP and smaller than the MW of other majority whey proteins.

In a reported process, the peptide solution was adjusted to pH<4 and ultrafiltered by a

membrane with cutoff of 10-50 kDa. The filtrate then was concentrated by a membrane of cutoff lower than 50 kDa (167-168). The purity GMP product was 81% with 0.6% W/W of phenylalanine content (165). Cold ultrafiltration also can replace ultrafiltration after pH adjustment to preserve the membranes (169). In order to increase the recovery and purity of final GMP product, some pretreatments were conducted before ultrafiltration. The raw materials can be treated with an ion-exchanger first. The non-absorbable fraction was then dealt with the same processes described above. The purity was between 55 and 88% (170). Due to the excellent heat stability of GMP compared to other whey proteins, heating whey at acidic pH can cause protein denaturation and precipitation, after that, the supernatant containing GMP was concentrated with a membrane of 10-20 kDa cutoff at pH 7. The maximum purity of GMP could be 90% (171). Besides ion-exchanger and heating treatment, whey protein concentrate was pre-treated with enzyme transglutaminase, and then was microfiltered (172). The transglutaminase is capable of binding GMP selectively due to the residues of glutamine and lysine in GMP sequence.

2.6.2 Selective precipitation

Selective precipitation is a method that can cause GMP remaining in the supernatant and other whey proteins to aggregate/precipitate by the means of heating, adding ethanol, sodium acetate, acetic acid and TCA (173).

In an ethanol method, cold precipitation using ethanol (50% V/V) was carried out at 4°C after the whey solution was heated at pH 6.0 and 98°C for one hour accounting for the high heat stability of GMP. The supernatant containing GMP was pumped through a DEAE (diethylaminoethyl cellulose)-Toyoperal 650M column. The yield was 1.1% (1.1

g of GMP for 100 g of whey powder) (174). The ethanol precipitation also was used after ultrafiltration to precipitate the residual soluble proteins. The final GMP product contained 10.9% of sialic acid and 8.4% of other whey proteins (175). CS dissolved in acetic acid was also used to fractionate aqueous solution of GMP. The formation of complex between positively charged CS and negatively charged GMP molecules resulted in their flocculation. At pH 5.0, 0.08 mg/ml of CS removed the glycosylated GMP whereas 70% of non-glycosylated GMP remained in solution (176). Precipitation of whey proteins using TCA was more frequently used before other process (177-178). In 2% TCA (W/V), most whey proteins are insoluble while all GMPs are soluble (179). When the concentration of TCA is above 8%, all the whey proteins and some non-glycosylated GMP_B are insoluble (180). Continuing to increasing the concentration of TCA to 12%, only the glycosylated GMP is soluble (179). Therefore, the content of GMP (glycosylated and non-glycosylated) might be approximately estimated by using TCA precipitation due to the different sensitivity of GMP fractions to TCA.

TCA precipitation, ethanol precipitation and ultrafiltration of whey protein isolate have been compared. TCA precipitation only recovered glycosylated GMP and eliminated all other proteins with low recovery of 6.7%. Ethanol recovered both non-glycosylated and glycosylated GMP with 20.4% of recovery and 75.7% of purity of glycosylated GMP. Ultrafiltration had 33.9% of recovery and 81.6% of purity of glycosylated GMP. Therefore, a single selective precipitation process cannot isolate GMP efficiently and it usually combines with membrane filtration or chromatography techniques as pre-treatment.

2.6.3 Chromatographic techniques

Because GMP has relatively low pI compared with other whey proteins, it has been isolated and purified by ion-exchange chromatography. In an anion exchanger, GMP can be adsorbed on the anion materials whereas the other whey proteins are not adsorbed.

The working pH value in selective adsorption varies with ion-exchange columns used. A commercial QA GiboGel™ ion exchange, manufactured by Life Technologies Ltd, was used in the GMP purification process. At pH 5.1, around 91% of GMP was eluted (181). The performance of pH 3.0 and 6.4 in purification of GMP from non-dialyzable fraction of whey were compared by using a DEAE-Sephacel anion column. At pH 3.0, higher purity and yield were obtained (182). In another case, a Sephacryl S-200 column was used to adsorb GMP at pH 3.5 after precipitation (153, 183). IRA93 resin and Amicon YM100 membrane were also used to separate immunoglobulin (Ig) and GMP from milk whey with the IRA93 resin adsorbing negatively charged GMP at pH 4.7 (166). The procedure of dialysis can increase the recovery of GMP. For example, the recovery of GMP from goat whey increased from 50% to 97% by using a DEAE-cellulose column (184). Since CS had similar chemical structure to cellulose and it is very easily to be protonated, it was also applied for GMP purification. It was found that the primary amine groups of CS were major binding sites for GMP uptake (38). Besides these separation columns and resins, there were also other columns and resins used such as styrene-based anion column (185), DEAE-Sephadex column (186) and Q-Sepharose column (187-188).

In addition to ion-exchange chromatography, other chromatographic methods were applied to isolate and purify GMP, such as exclusion chromatography, affinity

chromatography (186) and hydrophobic interaction chromatography in a phenyl-agarose column (189).

In the literatures reviewed above, most separation processes reported are complicated and/or combine two or three separation methods, such as precipitation followed by ultrafiltration and precipitation followed by chromatography, using commercial separation membranes or chromatography columns. These processes are largely of laboratory scale and are difficult to scale up for the industrial application, because those materials are expensive, not environmental-friendly, or the procedures generate too much wastewater. As one of the most widely used methods in the lab scale, ion-exchange chromatography also has some disadvantages. For example, some packing resins either have low binding capacity or cannot withstand high flow rate, which cause a low throughput. Those expensive adsorbents incur high cost as well. Therefore, a low cost, environmental-friendly and high throughput method is urgently needed for the GMP bulk production.

2.7 CS-based PEC in drug delivery

Because of its biocompatibility, reactivity, hydrophilicity and cationic nature, CS is an ideal candidate to be used as carrier substance in the application of drug delivery. But CS dissolves quickly in the stomach due to the low pH value, which limits the application of CS as the drug carriers for oral administration. The formation of PEC is one of physical modifications to overcome this shortcoming (190). Up to now, many different drug delivery systems have been developed using various PECs which were formed between CS and natural polymers, synthetic polymers, polyphosphoric acid and

poly (L-lactide) (191).

2.7.1 PECs between CS and natural polymers

Natural polymers containing carboxylic groups include alginate, pectin, xanthan gum, carboxymethyl cellulose, hyaluronic acid, poly- γ -glutamic acid, etc. The CS-alginate PEC in the form of beads with micropores showed stronger mechanical stability at low pH value and the ability of α -amylase entrapment (192-193). The PECs formed were also biodegradable, biocompatible, and can withstand the proteolysis of lysozyme (194). The CS-alginate PEC in forms of fibers and tablets were also prepared and studied for the controlled drug release with good results (195-196). The CS-pectin PEC, formed at pH 5.5, could be used for the colon-specific delivery of vancomycin, since the tablet of this PEC was pH-sensitive swelling and partially degraded by β -glucosidase (197-198). The CS-xanthan PEC was obtained in the form of powders after freezing-dry followed by mechanical grinding. It showed the ability of pH-sensitive swelling (199). Therefore, it can be used to immobilize enzymes as a matrix (200-201). It also can make insoluble drugs such as fenofibrate, ursodeoxycholic acid, nifedipine and indomethacin dissolving into water (202). The CS-carboxymethyl cellulose PEC was prepared in the form of nanoparticles and coated with plasmid DNA. These nanoparticles were stable against serum and could be used for genetic immunization (203). The CS-hyaluronic acid PEC was prepared in the forms of beads and nanoparticles. The study of heparin release using the CS-hyaluronic acid PEC nanoparticles indicated these nanoparticles could be used as the delivery system for pulmonary administration (204). The CS-hyaluronic acid PEC beads incorporating with poly (*N*-isopropylacrylamide), which showed temperature-sensitivity, were also applied for the controlled release of nalbuphine *in vitro* studies (205). In the way of ionic-gelation, the CS-poly- γ -glutamic acid PEC was

prepared in the form of nanoparticles as well (206). These PEC nanoparticles can penetrate into the cancer cells and mouse skin efficiently (207). In addition, being loaded in these nanoparticles, the bioavailability of insulin in oral administration can increase by 15.1-20% (208-209).

Natural polymers containing sulfate groups instead of carboxylic groups include carrageenan and dextran sulfate. The CS-carrageenan PEC is stimuli-responsive to both temperature and pH value (210-211). This kind of PEC has been investigated in the controlled release of glucose oxidase. The results showed that the glucose oxidase can be efficiently encapsulated, released rather slowly and stable even at pH 1.2, in pepsin solution as well as in chitosanase solution with the CS-carrageenan PEC (212). The CS-dextran sulfate PEC in the form of nanoparticles was prepared by various methods and was applied to test the association efficiency and release profiles of anti-angiogenesis peptide (213), amphotericin B (214) and insulin (215). Another natural polymer is gelatin, a heterogeneous polypeptide. The CS-gelatin PEC in the form of sponge was formed only in the pH range of 4.7 to 6.2 (216).

2.7.2 PECs between CS and synthetic polymers

Polycarbophil and carbopol are both cross-linked poly (acrylic acid) by different cross-linking agents. The mechanism of PECs formed between CS and cross-linked poly (acrylic acid) was the same to that of nature polymers containing carboxylic acid groups. As a matrix, the CS-carbopol PEC in the form of tablets released theophylline at pH 6.8 and pH 1.2 with diffusional release and relaxational release mechanism, respectively (217). The CS-polycarbophil PEC in the form of tablets was also reported to encapsulate and release diltiazem, ibuprofen and insulin at a lower release rate (218).

The same PEC in the form of films was prepared as formulations for topical and transdermal drug delivery due to the excellent flexibility, resistance and water vapor transmission rate of these films (219). The drug release results *in vitro* showed that the CS-polycarbophil could be an ideal matrix for modified release dosage forms. The CS-polymethacrylic acid PEC in the forms of nanoparticles was prepared and showed high association efficiency with insulin and BSA, and pH-sensitive release property *in vitro* for oral drug delivery (220). The CS-polyacrylic acid in the form of nanoparticles was also reported to entrap plasmid DNA very well for the gene delivery (221).

Polymethacrylate copolymers were generally used to coat capsules or tablets for the oral drug administration. Some of them, which contain carboxylate groups, are polyanions and can complex with CS (222). The CS-polymethacrylate copolymer PEC in the form of tablets loaded with sodium diclofenac was studied in various aspects of copolymer types, swelling, molecular weight of CS and oral drug release (223). The same drugs, encapsulated in the CS-polymethacrylate copolymer PEC were also reported in colon targeted release (224). Another synthetic polyanion is polyalkylenoxide-maleic acid (PAOMA). The CS-PAOMA PEC in the form of films was prepared to investigate the encapsulation and release of salicylic acid and phenol. The results showed that drug release behavior of the CS-PAOMA PEC films was not only dependent on pH but also dependent on temperature (225).

In conclusion, PECs, which combine the properties of various polymers, become more and more important in pharmaceutical industry. Among them, CS-based PECs have been prepared in various dosage forms while many studies of drug release have been conducted using these dosage forms *in vitro*. However, further work on *in vivo* studies

of the drug delivery is needed to tell whether these CS-based PECs are still effective as carrier substance *in vivo*.

2.8 Summary

There are three key factors, surface area, affinity and stability, which dictate the development and application of CS-based materials in the adsorption of peptides, proteins and lipids. The raw CS powder and flake were used in the first place, to obtain adsorbents with higher adsorption capacities. This can be achieved by treating the crystallite site of CS to get more amorphous and porous structures such as those in decrystallization and hydrogel beads. From hydrogel beads to nanoparticle, smaller particle size is designed and obtained in order to obtain higher surface area. From the point of view of affinity, various ligands, such as small molecules, bio-macromolecules, and metals that present the affinity towards specific peptide/protein, are attached onto CS to obtain the high purity/yield in the isolation and purification process. As a result the relevant technologies of immunoaffinity chromatography, metal binding affinity chromatography, molecular imprinted chromatography, and fluidized-bed affinity chromatography are developed as well. From the point of view of stability, CS is physically and chemically cross-linked by various cross-linking agents and coated on the rigid supports to prevent structure collapse and biodegradability under crude conditions. Take a closer look at these three stories and they have a number of intersections in the application. For instant, the ligands are attached on CS layer coating on the silicon beads and the physical cross-linking by TPP is the way to prepare CS nanoparticles.

However, no matter what kind of CS-based materials is reported, most adsorption

experiments are only carried out in batch adsorber at the laboratory level to evaluate the performance of adsorbent materials. Few materials are evaluated and adsorption kinetic is rarely studied in fixed bed column, although operation conditions in fixed bed column are essential in order to scale up the process for the industrial purpose. Meanwhile, in the study of protein adsorption in fixed bed column, people usually use the commercial column packed with traditional adsorbents and focus on the study on modeling. Therefore we believe there should be some intersections worthy of studying as a lot of work has been done in the two areas. That is the originality that we carry out the basic studies on operation conditions and adsorption kinetics of protein adsorption in fixed bed column using CS-based adsorbents with subsequent modeling. This work could be useful to extend the application of CS-based materials in peptide/protein adsorption and separation in fixed bed column. This part will be presented in chapter 4.

GMP, existing in milk whey, is a C-terminal and heterogeneous polypeptide containing 64 amino acid residues. The sequence and heterogeneity of GMP have been extensively studied and well known. But the composition of GMP is quite complicated because the saccharides attach at different amino acid residue in five orders. Therefore the glycosylated fraction of GMP is essentially dependent on the milk source.

The approaches of GMP isolation and purification can be divided into three main categories, membrane filtration, selective precipitation, and chromatographic techniques based on the different properties of whey proteins. However, up to now, most separation processes reported in paper and patents have three kinds of disadvantages. Firstly they are not readily to operate. They are complicated and/or combine two or three separation methods, such as precipitation followed by ultrafiltration and precipitation followed by

chromatography. The second disadvantage is low throughput or high cost. The commercial separation membranes and one or more than one commercial chromatography columns are usually used in these reported processes. For the commercial ion exchange chromatography columns that are most widely used, some packing resins either have low binding capacity or cannot withstand high flow rate, which cause a low throughput. Those expensive adsorbents incur high cost as well. Thirdly, TCA precipitation is almost involved in every process including that use CS-based materials as adsorbents. But this procedure could generate too much wastewater with negative effects on environment. Therefore our proposed study is to prepare a kind of low cost CS-based material as adsorbents for isolation and purification process of GMP with relatively high purity and yield in a simply way without precipitation procedure. This part will be presented in chapter 5.

CS-based PECs have been prepared in various dosage forms while many studies of drug release have been conducted using these dosage forms *in vitro*. However, further work on *in vivo* studies of the drug delivery is needed to tell whether these CS-based PECs are still effective as carrier substance *in vivo*. For CS- κ -carrageenan PEC, its nature of highly pH-sensitive swelling in solution has been evidenced and reported, but to our knowledge, there is no relevant report on the application of CS- κ -carrageenan PEC in oral delivery of protein drugs until now, and our proposed study is the first time to explore that the CS- κ -carrageenan PEC particles can be tailored to the pH responsive oral delivery of protein drugs. This part will be presented in chapter 6.

CHAPTER 3 EXPERIMENTAL METHODS

This chapter describes the general experimental methods and principle involved in this project, including the fabrication of CS hydrogel beads, fabrication of cross-linked CS beads, characterization methods, procedures of protein adsorption experiments and analytical methods, etc. Following the general descriptions, some specific experimental conditions and procedures that are related in adsorption isotherm and kinetic experiments are also given for a clear view.

3.1 Fabrication of CS hydrogel beads

Compared with the commercial CS flakes and powders, CS hydrogel beads possess larger surface area, which corresponds to higher adsorption capacity. Moreover, the solubilization of CS can destroy the crystallinity of CS and make CS more porous. More amine groups are exposed as adsorption sites. Another advantage that CS beads have over powders and flakes is the fix bed application, where beads lead to lower pressure drop. In this project, phase inversion method was used to fabricate CS hydrogel beads from commercial CS powders. The detail procedures are as follows and are illustrated in Fig. 3.1:

2.0 g of CS powder (90% DD, Bio-line Co., Ltd., Thailand) were dissolved in 100 ml, 1% (v/v) of acetic acid solution in water. The aqueous solution was stirred over night and filtered. This solution was pumped through a syringe needle (27G) drop-wisely into 1 M sodium hydroxide solution under stirring to form spherical

gels beads (with the diameter ~ 2 mm). The hydrogel beads were removed by filtration and rinsed with tap water until neutral. Then the beads were washed with ethanol followed by deionized (DI) water and then preserved in DI water. Finally, the beads were dried in a freeze dryer (Christ Alpha 1-2 LD) for further application.

Fig.3. 1 The illustration of the fabrication of CS hydrogel beads

1: CS solution, 2: peristaltic pump, 3: syringe needle, 4: tank containing NaOH, 5: freezing dryer

3.2 Fabrication of cross-linked CS beads

The wet native CS hydrogel beads prepared previously (e.g. containing 0.4 g of dry CS) and 50 ml of DI water were mixed together in a flask. After adding GLU into the flask, the flask was shaken for 6 h at 50°C in an incubator (EPI was used in 1M NaOH solution, instead of DI water.). Different amount of cross-linking agents were used to obtain the different mass ratio. After 6 h, the cross-linked CS beads were filtered out, washed with DI water to remove unreacted chemical agents, and finally preserved in DI water for future use. GLU and EPI, which are based on different cross-linking mechanisms, are popularly used agents for CS modification. The structures of the GLU and EPI cross-linked CSs are presented in Fig. 3.2a and 3.2b, respectively.

Fig.3. 2 The chemical structures of cross-linked CS with (a) GLU and (b) EPI

3.3 Characterization methods

3.3.1 Electron microscope (SEM, FESEM)

Scanning Electron Microscope (SEM) and Field Emission Scanning Electron Microscope (FESEM) were used to characterize the morphology characteristics of the derived CS-based adsorbents and the prepared CS- κ -carrageenan PEC. The samples were firstly coated with platinum under vacuum for 60 seconds using an auto fine coater (JEOL JFC-1600). The micrographs of sample were collected using SEM (JEOL JSM-6390LA) and FESEM (JEOL JSM-6500F), respectively.

3.3.2 N₂ adsorption

The pore structures of native/modified CS beads and CS- κ -carrageenan PEC particles were characterized with the nitrogen isotherm at 77 K, which was measured with a

commercial pore and surface analyzer (PSA, Quantachrome Inc, Autosorb-6B). Prior to nitrogen adsorption isotherm measurements, the samples were degassed overnight under vacuum by Autosorb degasser (PSA, Quantachrome Inc.) to remove contaminants, such as water, CO₂, hydrocarbons, etc. Normally, the degassing temperature is < 375 K and the vacuum is below 1.0×10^{-4} torr.

3.3.3 Fourier transform infrared spectroscopy (FTIR)

The Fourier transform infrared (FTIR) spectra of the derived CS beads were directly recorded in attenuated total reflectance (ATR) mode by a FTIR spectrometer (NICOLET 5700, Thermo) with a mercury cadmium tellurium (MCT) detector. The FTIR spectra of the prepared CS- κ -carrageenan PEC were recorded with KBr pellet in a Perkin Elemer Spectrum One FTIR. The samples were finely ground together with KBr. All the FTIR spectra were recorded in the range of 4000-500 cm⁻¹. The number of scan was set at 64 and the spectra resolution was 4 cm⁻¹.

3.4 Analytical methods

3.4.1 Low pressure chromatography

The Biologic low-pressure chromatography was used to conduct the experiments of adsorption in the fixed bed column. It is equipped with a conductivity meter and a UV detector with one channel of 280 nm. Therefore, the data of conductivity and UV absorbance in the effluent can be acquired continuously and analyzed in real time.

3.4.2 Assay for proteins

Besides the UV detector equipped in low pressure chromatography, the concentration of BSA was also determined with Bradford reagent using a UV/Vis spectrophotometer (Nicolet Evolution 500, Thermo). The specific assay for GMP and other whey proteins will be described in Section 5.2.4.

3.4.3 Reverse phase high-performance liquid chromatography (RP-HPLC)

The quantitative and simultaneous analysis of GMP and other whey proteins (β -lg and α -la) was also carried out in a reverse-phase high-performance liquid chromatography (RP-HPLC) on a Agilent 1100 series liquid chromatography equipped with a Analyt-FC (G1364C) UV detector, an auto sampler (G1313A), a degasser (G1379A), a Quat Pump (G1311A) and a Vydac 201TP5414 column (C_{18} on polymer, $5\mu\text{m}$ particle size, $300\text{ \AA}$ pore size, $4.6 \times 150\text{ mm}$; W. R. Grace & Co.–Conn.).

Prior to the experimental samples test, the standard samples of GMP, β -lg and α -la were injected and analyzed, respectively. The retention time of fractions of GMP, β -lg and α -la was identified and the peak profiles of each protein standard were calibrated. After that, the diluted whey proteins solution prepared from the raw whey protein isolate was injected and analyzed, and then the content of fractions of GMP, β -lg and α -la in raw whey protein isolate powder was calculated according to the calibration curve. Finally, the quantitative and simultaneous analysis of experimental samples was carried out, the adsorption percentage and yield of GMP, β -lg and α -la were calculated, respectively.

A gradient of solvent A containing 0.05 % (v/v) trifluoroacetic acid (TFA) dissolved in

DI water and solvent B containing 0.05 % (v/v) TFA in acetonitrile were applied. The gradient for the simultaneous determination of GMP and other whey proteins started with 8 % of solvent B followed by 10-minute equilibration. Then the solvent B was increased to 20 % within 0.5 minutes, to 39.2 % with 16.5 minutes, to 41.6 % in 5 minutes, to 44 % in 3.5 minutes, to 80 % in 0.5 minutes and then kept at 80 % for 5 minutes before returning to 8 %. Column effluent was monitored at 214 nm for the detection of proteins.

3.5 Protein adsorption experiments

3.5.1 Batch equilibrium experiments

Batch adsorption experiments were conducted to measure the adsorption isotherm, adsorption rate (kinetics), as well as to study the effect of pH and mass ratio (cross-linking agents/CS), etc. The pH value was adjusted by using phosphate buffer and acetate buffer with a buffer strength of 10 mM for BSA solution as well as HCl and NaOH solution for whey protein solution. The adsorption equilibriums were measured in batch adsorber with the following steps.

- (1) A fixed amount of adsorbents was put into conical flask together with protein solution with a fixed volume. The initial concentration of protein solution varied at the determined pH value.
- (2) The flasks were shaken at a rate of 200 rpm and room temperature in the incubator.

(3) After 24 hours, the supernatants were extracted and analyzed. The adsorbents were freezing dried for the SEM analysis.

(4) The equilibrium concentrations of adsorbate in liquid phase were calculated and the equilibrium concentration of adsorbate in solid phase, Q_e , was calculated using Eq. (8):

$$Q_e = (c_0 - c_e)V/W \quad (8)$$

where c_0 and c_e is the initial and equilibrium concentrations of adsorbate in liquid phase, respectively. V is volume of the protein solution, and W is the weight of adsorbents.

Specifically, for example, in the adsorption experiments of BSA in batch adsorber at pH 4.0 on GLU cross-linked CS beads with a mass ratio of 0.2 (GLU/CS), 0.1 g of adsorbent beads (blot dried) were added into 20 ml of BSA solution with eight different initial concentrations in the range of 25 ppm to 1000 ppm, which were 25, 50, 125, 250, 500, 750, 800, and 1000 ppm, respectively. The systems were stirred at a rate of 200 rpm at 298.15 K. After 24 hours, the supernatant was collected for analysis. The same experimental conditions were used for the other adsorption experiments of BSA in batch adsorber. However, the procedures in the adsorption experiments of GMP in batch adsorber are a little bit different and are described specifically as follows:

0.05 g of CS-HMDI-BCD beads was put in contact with the mixture of 0.02 ml of concentrated whey proteins solution and 10 ml of DI water. The system was stirred at a rate of 200 rpm and 298.15 K. After 24 hours, 0.5 ml of supernatant was extracted for analysis and more 0.02 ml of concentrated whey proteins solution was

added into the system. Repeat this step every 24 hours until 340 ml of concentrated whey proteins solution totally added.

Each experiment above was triplicate under the same conditions and the average was used for the isotherm model. Two adsorption isotherm models were used in this project to describe the bio-sorption isotherms.

Langmuir isotherm

The Langmuir equation is for monolayer adsorption and is expressed as:

$$Q_e = \frac{K_L c_e}{1 + \alpha_L c_e} \quad (9)$$

where Q_e is the equilibrium concentration of adsorbate in solid phase ($\mu\text{g/g}$), c_e is the equilibrium concentration of adsorbate in liquid phase ($\mu\text{g/ml}$), K_L (ml/g) and α_L ($\text{ml}/\mu\text{g}$) are the Langmuir isotherm constants. K_L/α_L (Q_m) gives the theoretical monolayer saturation capacity.

The effect of Langmuir isotherm shape can be used to predict whether an adsorption system is favourable or unfavourable both in fixed bed column as well as in batch adsorber (226) according to a dimensionless constant separation factor or equilibrium parameter R_L that was first defined in fixed bed system by Hall et al. (227). In fixed bed system, the Langmuir equation can be written as:

$$\frac{q^*}{Q_m} = \frac{\alpha_L c}{1 + \alpha_L c} \quad (10)$$

where q is the solid-phase concentration of the solute being adsorbed, the asterisk (*)

denotes an equilibrium value, Q_m is the ultimate sorptive capacity at high concentration, and c is the liquid-phase concentration. Equation (10) includes the description of the equilibrium between the feed concentration, c_0 , and the saturation level, q_0^* , which may be the adsorbed phase in the column. The relation for saturation conditions can be divided into the general relation, we have:

$$\frac{q^*}{q_0^*} = \frac{c(1 + \alpha_L c_0)}{c_0(1 + \alpha_L c)} \quad (11)$$

The R_L is defined as:

$$R_L = \frac{1}{1 + \alpha_L c_{0m}} \quad (12)$$

where c_{0m} is the highest initial concentration of the adsorbate. Dimensionless concentrations, $Y=q/q_0$ and $X=c/c_0$, are defined, each bounded by the values 0 and 1.

Equation (11) then becomes

$$Y^* = \frac{X}{R_L + X(1 - R_L)} \quad (13)$$

$$R_L = \left[\frac{X(1 - Y)}{Y(1 - X)} \right]_{equil} \quad (14)$$

The mean concentrations at any given cross section in a fixed bed column will be designated Y and X , and equation (13) and (14) apply to these values as well as to the point functions. $R_L=0$ for the irreversible case, $0 < R_L < 1$ for favorable equilibrium, $R_L=1$ for the linear case, and $R_L > 1$ for unfavorable equilibrium. These equilibrium considerations have a particularly effect on the shapes of breakthrough curves. For values of R_L smaller than 1, the curves tend to attain a constant pattern and thus to become relatively self-sharpening as they advance through an increasing length of column. For a given mass transfer mechanism, a single theoretical curve for

breakthrough represent all operating conditions. With $R_L > 1$, a proportionate pattern is set up which shows no tendency to sharpen but instead stretches out continually as the utilized column length increases.

In batch adsorption system, the value of R_L indicates the type of the isotherm to be unfavorable ($R_L > 1$), linear ($R_L = 1$), favorable ($0 < R_L < 1$) or irreversible ($R_L = 0$).

Freundlich isotherm

The Freundlich isotherm equation is an empirical equation and frequently used to describe heterogeneous systems. It is characterized by the heterogeneity factor $1/n$, and is expressed as:

$$Q_e = K_F c_e^{1/n} \tag{15}$$

where K_F ($\mu\text{g/g } (\mu\text{g/ml})^n$) is roughly an indicator of the adsorption capacity and $(1/n)$ denotes the adsorption intensity.

In order to compare validity of isotherm equations, a normalized standard deviation, $\Delta q_e \%$, is calculated as follows:

$$\Delta q_e (\%) = \sqrt{\frac{\sum [(q_{e,\text{exp}} - q_{e,\text{cal}})/q_{e,\text{exp}}]^2}{N - 1}} \times 100 \tag{16}$$

where, $q_{e,\text{exp}}$ and $q_{e,\text{cal}}$ are experimental and calculated equilibrium concentration of the adsorbate in solid phase, and N is the number of data points.

3.5.2 Batch kinetic experiments

The kinetics of protein adsorption onto CS-based adsorbents was also studied in the

batch adsorber with the similar procedures described in Section 3.5.1. The system was sampled at different time intervals, and from which the uptake of protein can be calculated and plotted against equilibrium time.

Specifically, for instance, the adsorption of BSA in batch adsorber at pH 6.0 and using native CS beads, around 1.0 g of native CS beads after blot dry were added into 100 ml of BSA solution with an initial concentration of 1000 ppm. The system was stirred at a rate of 200 rpm at 298.15 K. After different time intervals, ranging from 10 minutes to 24 hours, 0.5 ml of supernatant was collected for analysis. For another instance, the adsorption of GMP in batch adsorber at pH 3.0, around 0.05 g of CS-HMDI-BCD beads was mixed with 0.1 ml of concentrated whey proteins solution and 10 ml of DI water. The system was stirred at a rate of 200 rpm and 298.15 K. After different time intervals, ranging from 10 minutes to 48 hours, 0.05 ml of supernatant was extracted for analysis. Each experiment above was triplicate under the same conditions and the average was applied to the different kinetic models.

In order to investigate the mechanism of adsorption, two different kinetic models, the pseudo-first-order and the pseudo-second-order kinetic models were used to investigate the experimental data in this project.

The pseudo-first-order kinetic model of Lagergren is expressed as (228):

$$\log(q_e - q_t) = \log q_e - \frac{k_1}{2.303} t \quad (17)$$

where q_e and q_t are the adsorbate concentration in solid phase at equilibrium and at time t , respectively. k_1 (1/min) is the rate constant of first-order adsorption. In many cases, the first-order equation of Lagergren does not fit the data quite well over the whole

range of contact time, but generally applicable over the initial stage of the adsorption processes (229).

The pseudo-second-order kinetic model is expressed as:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{1}{q_e} t \quad (18)$$

Where k_2 (g/(μ g min)) is the rate constant of second-order adsorption. This model equation is more applicable for the whole range of adsorption time in which the chemical sorption is the rate controlling step (230). The fitting goodness of each kinetic model was compared with the normalized standard deviation $\Delta q_e\%$ in the equation (11).

3.5.3 Fixed bed adsorption experiments

The fixed-bed adsorption experiments were carried out in an analytical scale column, which has a diameter of 1.5 cm, a cross section area of 1.77 cm², and a pack length of 15 cm. The temperature of the column was maintained at room temperature. The adsorption of BSA onto native/cross-linked CS beads were studied at various flow rates and feed concentrations. The detailed experimental procedures are as follows.

- (1) The column packed with adsorbents was placed in the Biologic low pressure chromatography system (BIO-RAD), as shown in Fig. 3.3 below.
- (2) DI water passed through the column until the conductivity in effluent unchanged.
- (3) Phosphate buffer at desired pH was directed through the column until the conductivity in effluent was constant and was circulated in the column for another 12 hours to equilibrate the column.

(4) BSA solution at desired pH was pumped through the column at a desired flow rate. At the same time, the effluent samples were collected by an auto fraction collector and analyzed using UV/Vis spectrophotometer. The experiment was stopped when the effluent concentration reached the feed concentration.

Fig.3. 3 The low pressure chromatography system with fixed bed column

CHAPTER 4 BATCH/FIXED BED

ADSORPTION OF A PROTEIN ON CS

4.1 Introduction

This chapter will investigate the adsorption properties of native CS hydrogel beads and cross-linked CS beads for a model protein, bovine serum albumin (BSA, MW=66.4 kDa, pI=4.7), in a batch adsorber as well as in a fixed bed column. To enhance the acidic and mechanical stability of the native CS hydrogel beads, Glutaraldehyde (GLU) and Epichlorohydrin (EPI) were used to modify CS beads at different mass ratios.

As mentioned in Section 2.7, most peptide/protein adsorption experiments those are related to CS-based materials were only carried out in batch adsorber at the laboratory level. Few materials were evaluated and adsorption kinetic is rarely studied in fixed bed rig, although operation conditions in fixed bed rig are essential in order to scale up the process for the industrial purpose. Meanwhile, in the study of protein adsorption in fixed bed column, people usually use the commercial column packed with traditional adsorbents and focus on the study on modeling. Therefore we believe there should be some intersections worthy of studying as a lot of work has been done in the two areas. That is the originality that we carry out the basic studies of operation conditions and adsorption kinetics of the model protein, BSA, in fixed bed column using native/cross-linked CS beads and reported models. This work could be a useful exploration to extend the application of CS-based materials in peptide/protein adsorption and separation in fixed bed column.

On the other hand, both the adsorption mechanism and the effect of pH value, functional groups, and ion strength will also be investigated to enhance our basic knowledge on proteins adsorption and help us to understand the selective adsorption mechanism well in the multiple proteins solution, such as the whey proteins solution. This chapter is the basis for Chapter 5 and has good potential in industrial applications.

4.2 Experiments

BSA adsorption isotherm and kinetics were measured in a batch adsorber with the methods described in Section 3.5. Then, based on the results of the batch adsorption, the optimum conditions were selected for the fixed bed experiments. The column kinetics were investigated at different operation conditions, according to the procedures described in Section 3.5. The structures of the native and cross-linked CS beads were characterized using standard instruments.

4.3 Results and discussion

4.3.1 SEM micrographs of CS beads

Prior to the scanning electron microscope carried out, all the adsorbent samples were freezing dried in order to maintain the porous structure and remove the water inside. The SEM micrographs of native CS beads before and after BSA adsorption in batch adsorber (pH 5.5 and 48 hours) are presented in Figs. 4.1. Figs. 4.1a is the overall dimension image of native CS bead. Figs. 4.1b is the image of external surface of native CS beads. Figs. 4.1c and 4.1d are the images of the external and internal surface of CS

beads after BSA uptake, respectively. It was observed that in Fig 4.1b the process of freezing dry did not cause the collapse of porous structure of CS. In Figs. 4.1c, we see that the external surface of CS beads became smoother (less porous), which indicates that a large amount of BSA has been adsorbed on the external surface of the adsorbents. As seen from Figs. 4.1d, the cross-section of CS beads was largely porous with some interconnected pores. The less adsorption on the internal surface may be due to the slow intraparticle diffusion, low concentration of BSA in the residual solution, and the large molecular size of BSA.

Fig.4. 1 The SEM micrographs of native CS beads, (a) the overall dimension of clean bead, (b) the surface of clean beads, (c) the external surface of beads after adsorption, (d) the cross-section of beads after adsorption

4.3.2 SEM micrographs of cross-linked CS beads

Prior to the scanning electron microscope carried out, all the adsorbent samples were freezing dried in order to maintain the structure and remove the water inside. The SEM micrographs of cross-linked CS beads with GLU as the cross-linking agent are presented in Figs. 4.2. On the left, Figs. 4.2a, 4.2b, 4.2c, and 4.2d are the images of the external surface of beads with the mass ratio of 0.2, 0.5, 0.7 and 1.0, respectively. On the right, i.e. Figs. 4.2e, 4.2f, 4.2g, and 4.2h are the images of the internal surface of those beads. We see that, as the mass ratio of GLU increased, which means more GLU were reacted with CS, both external and internal surface of those beads became smoother. In the images of each mass ratio, the external surface was less porous than internal surface. This is expected as more cross-linking reactions occurred on the external surface and the cross-linking reactions made final cross-linked CS beads less porous than native CS beads. Same phenomena are also observed on the EPI cross-linked CS beads. The SEM micrographs of EPI cross-linked CS beads are presented in Figs. 4.3. The symbols, a, b, c, d, e, f, g, and h have the same meaning to Figs. 4.2.

Fig.4. 2 The SEM micrographs of cross-linked CS beads (GLU as cross-linking agent)

Fig.4. 3 The SEM micrographs of cross-linked CS beads (EPI as cross-linking agent)

4.3.3 Adsorption of BSA on native CS beads

4.3.3.1 Adsorption equilibrium isotherms

The BSA adsorption on native CS beads was strongly influenced by pH value due to the electrostatic interactions between BSA and CS beads. At selected pH value of, 6.0, 6.5, 6.9 and 8.0, batch experiments were carried out to measure the adsorption isotherms. Figs. 4.3-4.6 show the experimental data (symbols) and the fitting by Langmuir equation (dashed lines) and Freundlich equation (solid lines), while the fitting results are listed in Table 4-1. Judged from R^2 values, Langmuir equation appears to be the better model. Therefore the adsorption equilibrium isotherms that was simulated in Langmuir equation and obtained at different pH values were summarized in Fig.4.7. As pH decreased from 8.0 to 6.0, the amount of BSA adsorbed onto CS beads increased significantly with the largest adsorption capacity (Q_m) of 134.4 (mg/g-wet beads) at pH 6.0.

The pK_a of CS is around 6.5. Therefore, more amine groups of CS will be protonated when pH is lower than 6.5, which correspond to the binding sites for BSA. Furthermore, BSA has a pI of 4.7 and is adsorbed mainly via the electrostatic interactions when pH is higher than 4.7 (35). Therefore, the more adsorption could happen at pH lower than 6.0. However, the native CS beads will dissolved after 24-hour at pH 5.0 and 5.5. Moreover, a swelling ratio of $\geq 30.4\%$ was also observed for CS beads in 24 hours. Cross-linking is the solution to improve mechanical strength and the chemical stability of native CS beads in strong acidic solution. However, cross-linking also can reduce the binding sites. Therefore, the performance of cross-linked CS beads must be evaluated for the pros and cons.

Fig.4. 4 Adsorption isotherms of BSA on CS beads at pH 6.0, dashed line is Langmuir model, solid line is Freundlich model

Fig.4. 5 Adsorption isotherms of BSA on CS beads at pH 6.5, dashed line is Langmuir model, solid line is Freundlich model

Fig.4. 6 Adsorption isotherms of BSA on CS beads at pH 6.9, dashed line is Langmuir model, solid line is Freundlich model

Fig.4. 7 Adsorption isotherms of BSA on CS beads at pH 6.5, dashed line is Langmuir model, solid line is Freundlich model

Fig.4. 8 Summary of adsorption isotherms of BSA on CS beads at pH 6.0, 6.5, 6.9 and 8.0, fitting to Langmuir model

Table 4-1 Isotherm parameters of CS beads with Langmuir and Freundlich models

pH value	Langmuir model		Freundlich model		
	Q_m (mg/g-wet beads)	R^2	K_F	n	R^2
6.0	134.4	0.9768	40.6	1.78	0.9757
6.5	44.3	0.9843	46.1	2.09	0.9127
6.9	30.2	0.9797	11.5	1.74	0.9856
8.0	39.7	0.9643	14.0	1.97	0.8909

4.3.3.2 Adsorption kinetics

Fig. 4.9 shows the adsorption kinetics of BSA measured on CS beads at 298.15 K and with the initial pH 6.0. What is also shown in the same figure is the pH response of the adsorber during the adsorption process. The kinetics data were fitted to the

pseudo-second-order kinetic model (Equation 18) and shown in Fig. 4.10 as the line. We can find that the fitting is good with a high correlation coefficient of 0.9986. This suggests that the present adsorption system may follow the second-order rate model with the overall process being controlled by chemical sorption. In comparison, the fitting to the first-order equation is poor (not shown here). Meanwhile, with the utilization of phosphate buffer (buffer strength: 10 mM), the pH value of adsorber system is seen to change slightly only. This means that the buffer strength is strong enough to keep pH constant during the process of BSA adsorption and therefore the change of pH could be ignored in BSA adsorption.

Fig.4. 9 Adsorption kinetics of BSA and the change of pH in BSA adsorption using CS beads

Fig.4. 10 Adsorption kinetics data and the fitting (line) to pseudo-second-order model

4.3.4 Adsorption of BSA on cross-linked CS beads

GLU and EPI were used to cross-link CS beads with different mass ratio of 0.2, 0.5, 0.7 and 1.0 (cross-linking agent/CS), respectively. The effects of mass ratio, ion strength and pH on BSA uptake were investigated as follows.

At pre-determined pH value, 4.0, 5.0, 5.5, and 6.0, all the cross-linked beads were seen stable in batch experiments. Figs 4.11 and Figs 4.12 show the BSA adsorption isotherm (symbols) onto GLU and EPI cross-linked CS beads, respectively. Also shown in those figures are the fittings from Langmuir isotherm (lines). The fitting results are listed in Table 4-2 and Table 4-3, respectively, for each kind of cross-linking agent. In the case of GLU cross-linked CS beads, we see that the amount of the cross-linking agent affected the BSA adsorption significantly. The BSA adsorption decreased significantly with the increase in the mass ratio at each pH value, and the largest adsorption capacity occurred with the mass ratio of 0.2. As discussed before, this is because the cross-linking reaction

consumed some amine groups of CS beads and resulted in lower capacity of CS beads towards BSA. Moreover, as shown in SEM micrographs of cross-linked CS beads (Figs.4.2), lower porosity of the exterior surface of the cross-linked beads could deter BSA molecules from accessing the internal porous networks of the beads.

Fig.4. 11 Adsorption isotherms of GLU cross-linked CS beads at pH 4.0, 5.0, 5.5, and 6.0, respectively, fitted to Langmuir equation

The performance of EPI cross-linked CS beads is shown in Figs 4.12. A similar relationship between the BSA adsorption capacity and the mass ratio of cross-linking agent to CS was noted, and the EPI cross-linked CS beads with a mass ratio of 0.2 possess the largest adsorption capacity at each pH value too, although it has been

postulated that EPI molecules mainly react with the hydroxyl groups of CS. It might be tempting to suggest that, with the increased mass ratio, which means an increased amount of EPI used, the decreasing porosity of exterior surface exert strong diffusion barriers for BSA molecules to enter the internal beads. Therefore less free amine groups of CS are available for BSA adsorption. In other words, the adsorption process is controlled by the structure and kinetics.

Fig.4. 12 Adsorption isotherms of EPI cross-linked CS beads at pH 4.0, 5.0, 5.5, and 6.0, respectively, fitted to Langmuir equation

The effect of pH was investigated and can be seen in Figs.4.13. By comparing the isotherm parameters within Table 4-2 and 4-3, it is noted that the cross-linked CS beads

(GLU or EPI) can show higher capacity than native CS beads at acidic conditions. The lowest adsorption capacity was seen at $\text{pH} < 5.0$, at which both BSA and cross-linked CS are positively charged so that the repulsive force set in. The highest capacity was obtained at $\text{pH} \sim 5.5$ not ~ 5.0 . That means more BSA molecules are negatively charged at ~ 5.5 than at 5.0 that is too closing to pI value of BSA. Therefore ~ 5.5 is the optimal pH for BSA adsorption on such beads.

Fig.4. 13 Adsorption isotherms of BSA using adsorbents with mass ratio of 0.2, fitting to Langmuir equation

Table 4-2 Saturation adsorption capacity of BSA on GLU cross-linked beads

GLU cross-linked CS beads (mass ratio)								
pH value	0.2		0.5		0.7		1.0	
	Q_m (mg/g)	R^2	Q_m (mg/g)	R^2	Q_m (mg/g)	R^2	Q_m (mg/g)	R^2
4.0	41.9	0.9847	32.1	0.9922	29.3	0.9861	27.4	0.9901
5.0	74.5	0.9808	63.8	0.9721	59.1	0.9842	50.5	0.9726
5.5	96.4	0.9840	67.7	0.9896	61.5	0.9799	52.9	0.9693
6.0	63.4	0.9826	62.0	0.9733	57.7	0.9774	52.9	0.9873

Table 4-3 Saturation adsorption capacity of BSA on EPI cross-linked beads

EPI cross-linked CS beads (mass ratio)								
pH value	0.2		0.5		0.7		1.0	
	Q_m (mg/g)	R^2	Q_m (mg/g)	R^2	Q_m (mg/g)	R^2	Q_m (mg/g)	R^2
4.0	53.5	0.9914	38.8	0.9755	36.4	0.9920	30.0	0.9822
5.0	147.7	0.9812	127.2	0.9846	107.3	0.9801	81.4	0.9764
5.5	193.6	0.9844	141.6	0.9825	93.9	0.9830	71.9	0.9701
6.0	136.5	0.9826	80.1	0.9746	62.1	0.9887	56.5	0.9868

The effect of ionic strength was also investigated. Batch adsorption experiments were conducted using EPI cross-linked beads (mass ratio: 0.2) at pH 5.5 and with different concentration of sodium chloride in the presence of phosphate buffer (buffer strength: 10mM). The experimental data (symbols) and fittings of Langmuir equation (lines) are presented in Fig 4.14. The fittings parameters are listed in Table 4-4. We see that the presence of NaCl in BSA solution significantly affected the BSA adsorption. When the concentration of NaCl increased from 0 to 0.1 M, the saturation adsorption capacity

decreased from 193.6 mg/g to 60.7 mg/g. It further went down to 11.9 mg/g with 1.0 M of NaCl. It indicates that the electrostatic interactions between BSA and adsorbent beads play a major role in the adsorption behavior.

Fig.4. 14 Effect of concentration of NaCl on BSA adsorption at pH 5.5

Table 4-4 Saturation capacity of EPI cross-linked CS beads with various concentration of NaCl

	Concentration of NaCl (M)			
	0	0.1	0.5	1
Q_m (mg/g)	193.6	60.7	19.7	11.9
R^2	0.9701	0.9801	0.9805	0.9791

4.3.5 Adsorption of BSA in fixed bed

4.3.5.1 Breakthrough experiments with CS beads

The fixed bed column, which has a diameter of 1.5 cm, a cross section area of 1.77 cm², and a pack length of 15 cm, was packed with around 13 ml of CS hydrogel beads and

the fixed bed adsorption experiments were conducted at pH 6.0 and room temperature with different flow rates and BSA feed concentrations. The average diameter of CS hydrogel beads is ~1.7 mm and the void fraction of the fixed bed column is ~0.5. The breakthrough data were studied by three mathematical models (Bohart and Adams, Clark, and Wolborska models), respectively. The experimental data (symbols) and model fitting (lines) are shown in Figs. 4.15-4.17, while the derived model parameters are listed in Table 4-5.

Figs. 4.14-4.16 presents the breakthrough data on native CS beads under two sets of operating conditions: (i) BSA feed concentration fixed at 25 ppm while three flow rates are 1.5 ml/min, 3.0 ml/min and 4.0 ml/min, respectively; and (ii) flow rate fixed at 4.0 ml/min while three BSA concentrations are 25 ppm, 50 ppm and 75 ppm, respectively.

It was observed that the Bohart & Adams model and the Clark model gave a good fit with a correlation coefficient of $R^2 > 0.98$ to the experiment data at all flow rates and all feed concentrations. Wolborska model deviated extensively from the experimental data ($R^2 < 0.9$). The latter model cannot predict the whole breakthrough curves at certain experimental conditions.

4.3.5.2 Breakthrough experiments with cross-linked CS beads

The same fixed bed column was then packed with around 14 ml of EPI cross-linked CS beads with a mass ratio of 0.2 and run at pH 5.5 and at room temperature. The average diameter of beads is ~1.7 mm and the void fraction of the fixed bed column is ~0.53. Figs. 4.18-4.20 present the breakthrough data (symbols) on the EPI cross-linked CS beads under two sets of operating conditions: (i) BSA feed concentration fixed at 50

ppm while three flow rates are 1.0 ml/min, 2.0 ml/min and 4.0 ml/min, respectively; and (ii) flow rate fixed at 2.0 ml/min while three BSA feed concentrations are 50 ppm, 75 ppm and 100 ppm, respectively. The derived model parameters are listed in Table 4-6.

Similar to the case of packing native CS beads, the Bohart & Adams and the Clark models fitted the experimental data much better than the Wolborska model. Compared with the calculated adsorption capacity (N_0) presented in Table 4-5 and Table 4-6, under each operation condition and at a proper pH, the EPI cross-linked CS beads adsorbed much more BSA than native CS beads, which is consistent with the results in batch adsorber. The calculate adsorption capacity can be improved approximately by 20-fold dramatically.

Either native CS beads or EPI cross-linked CS beads are used, it seems that the breakthrough time decreased with increasing the flow rate. It is due to the reduced residence time of BSA molecules in the column. However, despite the reduced residence time, the calculated adsorption capacity seems to increase with increasing flow rate, which may be due to the lower mass transfer resistance at higher Re number. At such conditions, more BSA molecules can overcome the mass transfer resistance to diffuse into packing beads. The breakthrough time also showed a decreasing trend with increasing the feed concentration, because the packing beads were saturated more rapidly. The calculated adsorption capacity showed an increasing trend, possibly due to the higher driving force of mass transfer at the initial stage of adsorption.

Fig.4. 15 Breakthrough curves on CS beads at pH 6.0 and fitting of Bohart & Adams model

Fig.4. 16 Breakthrough curves on CS beads at pH 6.0 and fitting of Clark model

Fig.4. 17 Breakthrough curves on CS beads at pH 6.0 and fitting of Wolborska model

Fig.4. 18 Breakthrough curves on EPI cross-linked CS beads at pH 5.5 and fitting of Bohart & Adams model

Fig.4. 19 Breakthrough curves on EPI cross-linked CS beads at pH 5.5 and fitting of Clark model

Fig.4. 20 Breakthrough curves on EPI cross-linked CS beads at pH 5.5 and fitting of Wolborska model

Table 4-5 Parameters obtained in CS beads packing column

flow rate (ml/min)	feed concentration C_0 (ppm)	Z (mm)	Bohart & Adams			Clark			Wolborska		
			U_0 (mm/min)	K_{BA} (L/(mg min))	N_0 (mg/L)	R^2	K_C (L/(mg min))	N_0 (mg/L)	R^2	β_a	N_0 (mg/L)
1.5	25	150	8.5	0.0009	142.4	0.9731	0.0008	118.4	0.9745	0.0903	289.3
3.0	25	150	16.9	0.0018	195.6	0.9849	0.0017	172.5	0.9843	0.2237	343.1
4.0	25	150	22.6	0.0028	163.1	0.9885	0.0026	142.5	0.9846	0.3014	282.2
4.0	50	150	22.6	0.0017	221.9	0.9876	0.0016	187.4	0.9884	0.2230	533.6
4.0	75	150	22.6	0.0079	359.9	0.9678	0.0007	328.4	0.9647	0.2420	856.2

Table 4-6 Parameters obtained in EPI cross-linked CS beads packing column

flow rate (ml/min)	feed concentration C_0 (ppm)	Z (mm)	Bohart & Adams			Clark			Wolborska		
			U_0 (mm/min)	K_{BA} (L/(mg h))	N_0 (g/L)	R^2	K_C (L/(mg h))	N_0 (g/L)	R^2	β_a	N_0 (g/L)
1.0	50	150	5.6	0.0008	6.6	0.9733	0.0007	5.5	0.9743	0.0659	12.8
2.0	50	150	11.3	0.0017	6.4	0.9873	0.0015	5.3	0.9896	0.1131	14.8
4.0	50	150	22.6	0.0085	3.4	0.9912	0.0079	3.0	0.9910	0.2872	6.1
2.0	75	150	11.3	0.0018	7.8	0.9928	0.0017	6.9	0.9921	0.2070	37.3
2.0	100	150	11.3	0.0009	9.7	0.9870	0.0009	7.8	0.9865	0.2054	48.1

4.4 Summary

The adsorption of a model protein, BSA, was investigated on CS hydrogel beads. A number of factors, such as pH value and buffer strength were investigated in a batch adsorber. As pH decreased from 8.0 to 6.0, the amount of BSA adsorbed onto CS beads increased significantly with the largest adsorption capacity (Q_m) of 134.4 (mg/g-wet beads) at pH 6.0. Therefore the pH value affected the adsorption performance significantly because of the electrostatic interactions between amine groups of CS and BSA molecules. The CS hydrogel beads dissolved in acidic solution at pH ≤ 5.5 . It is feasible to use phosphate buffer and acetate buffer with 10 mM of buffer strength to keep the pH constant during adsorption process.

Two kinds of general cross-linking agent, GLU and EPI were used to cross-link CS beads. The acidic stability of the cross-linked beads was much improved. The factors, pH value, mass ratio (cross-linking agent/CS) and ion strength, were investigated in a batch adsorber. It was found that pH value also affected the adsorption performance significantly while either mass ratio or ion strength has a negative effect on the adsorption behavior of cross-linked CS beads. Under same operation conditions, EPI cross-linked CS beads performed better than GLU cross-linked CS beads. For example, EPI cross-linked CS beads with 0.2 of mass ratio are better adsorbents with a maximum adsorption capacity of 193.6 mg/g-adsorbents at pH 5.5. Therefore the electrostatic interactions were still major adsorption mechanism and amine groups of CS were major binding sites. It also found that the adsorption kinetics had a strong influence on the uptake capacity and most adsorption occurred on exterior surface of the cross-linked

adsorbents because lower porosity of the exterior surface could deter BSA molecules from accessing the internal porous networks of the beads.

The adsorption of BSA in a fixed bed column that has a diameter of 1.5 cm, a cross section area of 1.77 cm^2 , and a pack length of 15 cm was investigated. The column was packed with ~13 ml of CS hydrogel beads and ~14 ml of EPI cross-linked CS beads, respectively. Based on the parameters determined in batch adsorption, the column kinetics were investigated at different set of flow rate and feed concentration. The Bohart & Adams, Clark and Wolborska models were used to predict the breakthrough curve and the Bohart & Adams and Clark model were found to be better option in simulating the fixed bed breakthrough curves.

CHAPTER 5 SEPARATION OF GMP FROM MILK WHEY

5.1 Introduction

It has been known that protein adsorption is dependent on the pI of proteins. In the milk whey, GMP has a pI below 3.8 with a MW of 7~9 kD. However, pI of GMP varies with the content of sialic acid ($pK_a=2.6$) in GMP. A higher content of sialic acid in GMP gives a lower pI to GMP and results in tighter binding of GMP to anion exchanger (231). Meanwhile, β -lg, α -la and BSA have pI of 5.3, 5.1 and 4.7 with MW of 18.6, 14.2 and 66.4 kDa, respectively. Immunoglobulins have higher pIs above 5.5 and MW more than 150 kDa (166). These proteins also have higher hydrophobicity than GMP. On the other hand, the pK_a of CS is around 6.5. CS solution has been shown to selectively precipitate GMP from casein hydrolysate (176). Therefore, CS beads could be capable of separating GMP from milk whey as an anion exchanger at a proper acidic pH value theoretically.

Cyclodextrin (CD) is a cyclic oligosaccharide containing six, seven and eight D-glucose units in α -, β - and γ - form, respectively. The chemical structures of cyclodextrin are shown in Fig. 5.1. The torus-like structures give a cavity structure, which has diameter of 5.0 Å, 6.5 Å and 8.5 Å, to α -, β - and γ -cyclodextrin, respectively (232). These nano-scale cavities are hydrophobic and have the ability of forming inclusion complex with various compounds. The β -cyclodextrin (BCD) grafted CS beads has been reported to separate cholesterol from egg yolk containing majority content of lipid successfully

(100). Similarly, the major contamination of whey in the separation of GMP is also the lipid. That is why that we proposed to graft β -cyclodextrin onto CS and it was expected to combine the hydrophobic bucket of BCD as well as the cationic property of CS and enhance the selectivity/capacity towards GMP in the separation process. CS used as supporting materials for BCD was also a way to solve the solubility of BCD in aqueous solution.

Fig.5. 1 The chemical structures of α -, β - and γ -cyclodextrin

Therefore, in this chapter, BCD was grafted onto native CS beads by a two-step method. By omitting any precipitation step, the resultant grafted beads were applied for the separation of GMP from milk whey as adsorbents. The selectivity of native CS beads, EPI cross-linked CS beads, and the BCD grafted CS beads towards GMP in the whey solution at various pH was investigated. The maximum adsorption capacity of GMP onto BCD grafted CS beads was evaluated by fitting batch equilibrium experimental data to Langmuir and Frenudlich models. Last, the reusability of the adsorbents was evaluated with respect to adsorption capacity and selectivity towards GMP.

5.2 Experiments

5.2.1 Fabrication of β -cyclodextrin grafted CS beads

β -cyclodextrin (BCD) was grafted onto dry CS beads by 1,6-hexamethylene diisocyanate (HMDI) reagent that has two isocyanate groups ($-\text{N}=\text{C}=\text{O}$) in two-step reaction. The hydroxyl groups of CS reacted with an isocyanate groups to form a urethane structure in interim beads (CS-HMDI) first. The other isocyanate group of HMDI reacted with hydroxyl groups of BCD to form the same urethane structure and thus formed the final CS-HMDI-BCD beads in the second step. HMDI cannot react with amine groups of CS due to the lower affinity towards amine groups as compared to hydroxyl groups. The above mechanism and the presumed chemical structure of CS-HMDI-BCD beads are shown in Fig. 5.2. The amine groups of CS are retained as the major binding sites for GMP. This two-step method has been reported with a maximum BCD loading of 0.43 g/g-CS (100). The details of the fabrication process are as follows:

In the first step, 0.2 g of CS beads (freezing dried) was placed in 10 ml of dimethylformamide (DMF) containing 2.5 % (v/v) of HMDI. The mixture was magnetically stirred at room temperature after adding drops of stannous 2-ethylhexanoate that catalyzed the grafting reaction. After stirring for 1 hour, the supernatant was discarded and the interim CS beads bound HMDI (CS-HMDI) were dried in vacuum oven.

In the second step, the CS-HMDI beads were placed in 10 ml of DMF solution containing 1 % (g/ml) BCD. The mixture was stirred for 1 hour at room

temperature after adding few drops of the same catalyst. The supernatant was decanted and the final beads (CS-HMDI-BCD) were washed several times with DI water followed by ethanol washing. Next the beads were washed with DI water again before freezing drying.

Fig.5. 2 The illustration of BCD grafting onto CS using HMDI as a spacer

5.2.2 Preparation of whey protein powder

The CS RT-90TM native whey protein isolate is the kind gift of ANF innovations Pty, Australia. First, whey solution was prepared by 10 g of whey protein isolate in 200 ml of water. The non-dialyzable fraction of the whey solution was obtained by dialyzing under running water for 24 hours using a 6000-8000 molecular weight cutoff membrane. The fraction retained in the dialysis tube (non-dialyzable fraction of milk whey) was freeze-dried and stored at 4°C.

5.2.3 Preparation of whey proteins solution

The preparation of concentrated whey proteins solution is described as follows:

0.5 g of whey protein powder that was obtained after dialysis was dissolved well in 10 ml of DI water. The insoluble substance in solution was removed by using the syringe filter with 0.75 μm of pore size. Then the concentration of the final concentrated whey proteins solution was 0.05 g/ml.

The preparation of diluted whey proteins solution for RP-HPLC analysis is described as follows:

0.3 ml of the concentrated whey protein solution was diluted with 10 ml of DI water containing HCl at a pre-determined pH value. Therefore, approximated 0.015 g of whey protein powder was added into each flask for the adsorption at different pH value. After adsorption, the supernatant was filtered and analyzed by RP-HPLC, the adsorbent beads carrying proteins were washed by DI water and then put into 10 ml of NaCl solution with the concentration of 1M for desorption. After that, the supernatant was filtered and analyzed by RP-HPLC in the same whey.

5.2.4 Assay for GMP and other whey proteins

Because GMP does not contain aromatic amino acids, it presents no ultraviolet absorbance at 280 nm and can be detected in the range of 205-271 nm. The difference in the absorbance of 210/280 nm is generally used for the determination of GMP. However, it is difficult to apply this assay in the mixture of proteins as other proteins also give

background absorbance at 210 nm.

In our study, sialic acid contained in GMP is used as an effective indicator to determine the presence of GMP because approximately 80 % of total sialic acid present in the whey is from GMP. Meanwhile, in the non-dialyzable fraction of milk whey that was obtained after dialysis, only sialic acid rich in GMP are there. Asialio-GMP (fraction without sialic acid) has already been got rid of during the dialysis process. That is the second reason that sialic acid was used as an indicator to detect GMP. An assay of thiobarbituric acid reaction related to sialic acid generally used by Nakano et al. (231) to determine GMP needs several sample preparation steps including the sample digestion that makes the assay not readily to operate. Thus, a simple acidic ninhydrin assay specific to sialic acid (233) was used in this project for the rapid and sensitive determination of GMP. The method was also proven to be as effective as the HPLC method in the determination of adulteration of whey in milk (234).

An acidic ninhydrin reagent of Gaitonde was prepared by 5 g of ninhydrin, 120 ml of glacial acetic acid and 80 ml of 37% HCl (w/v). The chemical structures of sialic acid, N-acetylneuraminic acid and ninhydrin are shown in Fig. 5.3. The acidic ninhydrin solution was shown to be specific for sialoglycoproteins with stable color development at 470 nm, at which other asialoproteins cannot be detected. The procedures for the determination of sialic acid are described as follows:

- (1) The reaction mixture containing 1.0 ml of acid ninhydrin reagent, 1.0 ml of glacial acetic acid, and 1.0 ml of a sample solution was heated for 10 min in a boiling water bath.

- (2) The mixture was rapidly chilled in a tap water bath and a stable color (yellowish) was produced.
- (3) The absorbance at 470 nm was measured for the mixture by the UV/Vis spectrophotometer (NICOLET EVOLUTION 500).

Fig.5. 3 The chemical structures of sialic acid, N-acetylneuraminic and ninhydrin

The adsorption percentage of GMP (P_G) was expressed by the adsorption percentage of sialic acid (the ratio of the concentration of sialic acid in solid phase to the initial concentration of sialic acid). The other whey proteins in the milk whey were determined directly at 280 nm, and the adsorption percentage (P_W) was simply expressed by the ratio of their UV absorbance at 280 nm.

5.2.5 Adsorbents selection and effect of pH

The adsorption selectivity of three types of adsorbents, CS beads, EPI cross-linked CS beads (mass ratio: 0.2), and CS-HMDI-BCD beads, towards GMP at various pH varying from 2.5 to 6.5 was evaluated and compared. 0.05 g of each kind of adsorbent beads was mixed with 0.2 ml of concentrated whey proteins solution and 10 ml of DI water

that pH was adjusted by the addition of concentrated hydrochloride acid or sodium hydroxide solutions. The system was stirred at a rate of 200 rpm and 298.15 K. After 24 hours, the supernatant was obtained after filtration and the adsorption percentage of GMP (P_G) and the adsorption percentage of the other whey proteins (P_W) were obtained using the method described in Section 5.2.4. Each experiment was triplicate under the same conditions and the average was used. Therefore, the better adsorbent and pH value was identified, which presented the highest adsorption selectivity for the separation of GMP from milk whey.

5.2.6 Adsorption of GMP onto CS-HMDI-BCD beads

At the pre-determined pH value, batch adsorption experiments were conducted to measure and analyzed the adsorption isotherm and adsorption rate (kinetics) using the methods described in Section 3.5.1 and 3.5.2.

5.2.7 Re-generation of CS-HMDI-BCD beads

For practical applications, it is important that the beads can be well re-generated for further usage. Experiments were conducted in the following way:

Adsorption: 0.05 g of CS-HMDI-BCD beads was mixed with 0.2 ml of concentrated whey proteins solution and 10 ml of DI water. The pH value of the system was adjusted to 3.0. The system was stirred at a rate of 200 rpm for 24 hours at 298.15 K.

Regeneration: the adsorbents beads carrying GMP and other whey proteins were

mixed with 10 ml of 1 M sodium chloride solution and were stirred at a rate of 200 rpm for 2 hours at 298.15 K. After desorption, the adsorbents beads were washed by DI water to eliminate residual sodium chloride. The concentrations of GMP and other whey proteins in liquid phase were determined with the assay described previously.

This sorption cycle was repeated many times to examine the change in the adsorption selectivity and capacity of GMP. All the data presented correspond to the average of triplicate measurements.

5.3 Results and discussion

5.3.1 Characterization of adsorbents

5.3.1.1 Physical properties of adsorbents

The physical properties, such as surface area and dimension of adsorbents (native CS beads, EPI cross-linked CS beads, and CS-HMDI-BCD bead) were listed in Table 5-1. The average diameter of adsorbents was obtained after 100 beads were measured. The surface area of native CS beads, EPI cross-linked CS beads, and CS-HMDI-BCD beads that were determined by N₂ sorption at 77K were found to be similar at 38.43, 36.65, and 35.47 m²/g, respectively.

Table 5-1 Physical properties of three kinds of adsorbents

Type of adsorbents	Surface area (m ² /g)	Average pore size (nm)	Average diameter (mm)
CS beads	38.43	6.8	1.7
EPI cross-linked CS beads	36.65	6.3	1.7
CS-HMDI-BCD beads	35.47	6.7	1.5

5.3.1.2 SEM micrographs

Prior to the SEM and FESEM carried out, all the adsorbent samples were freezing dried in order to maintain the porous structure and remove the water inside. The structures of CS beads and EPI cross-linked CS beads are exemplified in the SEM and shown in Figs. 4.1a Figs. 4.1b, Figs. 4.3a, and Figs. 4.3e, respectively. The SEM and FESEM micrographs of CS-HMDI-BCD beads are presented in Figs. 5.4 and Fig. 5.5, respectively. It is seen that the external surface of the CS-HMDI-BCD beads (Fig. 5.4a and Fig. 5.5) is smoother and less porous than native CS beads (Figs. 4.1a). This might be due to the high loading of BCD at the exterior surface. However, the inner (cross-sectional) structure of the CS-HMDI-BCD beads is highly porous (Figs. 5.4b). The less porous nature of the exterior surface of the CS-HMDI-BCD, similar to EPI cross-linked CS beads (Fig. 4.3a and 4.3e), might present diffusion barriers to larger protein molecules and result in high selectivity for GMP.

After contacting with whey proteins solution at pH 3.0, the SEM micrographs of the CS-HMDI-BCD beads are presented in Figs. 5.6. It is observed that majority of proteins adsorption occurred at the external surface and little adsorption occurred within the internal structures.

Fig.5. 4 The SEM micrographs of (a) external and (b) internal surface of CS-HMDI-BCD beads

Fig.5. 5 The FESEM micrographs of exterior surface of CS-HMDI-BCD beads

Fig.5. 6 The SEM micrographs of (a) external and (b) internal surface of CS-HMDI-BCD beads after contacting with whey proteins

5.3.1.3 FTIR studies

The ATR-FTIR spectra of the native CS beads, interim CS-HMDI beads, and the CS-HMDI-BCD beads were recorded using the method described in Section 3.3.3. They are shown in Figs. 5.7. In Figs. 5.7a native CS beads, there were two peaks at around 3300 cm^{-1} . The one at 3357.0 cm^{-1} is assigned to the stretch vibration band of aliphatic primary amine (N-H), and the other one at 3296.2 cm^{-1} is assigned to the O-H stretch band. The absorption band at 1149.9 cm^{-1} assigned to the alkyl-substituted ether C-O stretch band and at 1061.1 cm^{-1} assigned to the cyclic ether C-O-C stretch band are both characteristic bands of saccharide structure in CS. The peak at absorption band of 895.3 cm^{-1} is also the characteristic peak of the β -pyranyl vibration of CS. The methyne C-H stretch band at 2876.0 cm^{-1} and the methyne C-H bend band at 1376.2 cm^{-1} provide the evidence for the carbon backbone structure of CS. The features of amide group (amide I and amide II band) are present at 1649.9 and 1588.3 cm^{-1} , respectively. They are in the acetylation part of CS. The amide II band may be overlapped by the NH_2 bend band, the presence of this group being confirmed by the adsorption occurring at 1421.0 cm^{-1} . The primary amine C-N stretch band at 1028.2 cm^{-1} also supports the presence of $-\text{NH}_2$ groups. Finally the absorption peaks at 1320.5 cm^{-1} and 1259.7 cm^{-1} are assigned to the primary or secondary hydrogen bond bend band.

In Fig. 5.7b CS-HMDI beads, the presence of the methylene C-H asymmetric stretch band at 2931.8 cm^{-1} , the methylene C-H bend band at 1478.5 and 1461.3 cm^{-1} , the aliphatic polyurethane C=O band at 1253.5 cm^{-1} , and the isocyanate asymmetric stretch band at 2288.7 cm^{-1} confirms the introduction of HMDI, the formation of the CS-HMDI, and the existence of the left $-\text{NCO}$ groups, which will react with the hydroxyl groups of BCD in the second step. At around 3300 cm^{-1} , the two peaks combined to one peak at

3315 cm^{-1} , which is assigned to aliphatic secondary amine stretch or aliphatic primary amine stretch. The O-H bend band at 3296.2 cm^{-1} disappeared. The hydroxyl group bend band becomes much weak and shifts to 1303.1 cm^{-1} . These evidences indicate that most of hydroxyl groups react with -NCO groups. However, the amide I band and amide II band also shifted to lower wave number, 1618 and 1518.3 cm^{-1} , respectively. It would be inferred that some amine groups also react with -NCO groups.

The spectrum of CS-HMDI beads (Figs. 5.7b) is similar to that of final CS-HMDI-BCD beads (Figs. 5.7c) because both BCD and CS have some similar groups. When BCD is grafted onto CS, the similar groups on CS cover those on BCD in the FTIR spectrum. However, in Figs. 5.7c, the characteristic peak of the α -pyranyl vibration of BCD appears at 946.5 cm^{-1} . The absorption band at 1417.4 cm^{-1} being related to -NH_2 confirms the presence of -NH_2 after modification. The disappearance of the isocyanate asymmetric stretch band at 2288.7 cm^{-1} indicates the absence of -NCO groups on the final CS-HMDI-BCD beads.

To sum up, from Figs. 5.7a, b, and c, it is suggested that a large portion of hydroxyl groups and a small portion of amine groups of CS react with -NCO groups of HMDI in the first modification step, after the second step BCD is grafted onto CS successfully and most of amine groups are retained, and there is no -NCO groups on the final modified beads.

Fig.5. 7 FTIR spectra of (a) native CS beads, (b) interim CS-HMDI beads, and (c) CS-HMDI-BCD beads

5.3.2 Adsorbents selection and effect of pH

The effect of pH on the adsorption of GMP and other whey proteins are presented in

Figs. 5.8. GMP, a heterogeneous peptide, may present different pI values depending on its resource and composition. Silva-Hernandez demonstrated that the pI of bovine GMP was below 3.8. Nakano and Ozimek found that the optimum pH value ranged from 2.5-4.0 for GMP purification on anion-exchange chromatography with DEAE-Sephacel. When the pH is between the pI of GMP and 4.7, stronger electrostatic interaction will take place between the positively charged CS and the negatively charged GMP, whereas strong repulsive force are between CS and positively charged other whey proteins, because β -lg, α -la and BSA have pI of 5.3, 5.1 and 4.7, respectively. When the pH is lower than the pI of GMP, repulsive force will set in, as both CS and GMP are now positively charged. As shown in Figs. 5.8, the adsorption percentage of GMP at pH 2.5 was less than that at pH 3.0. It is believed that the pI of GMP existing in our raw material is higher than 2.5 and close to 3.0 as well.

It was observed that the native CS beads (Figs. 5.8a) adsorbed both GMP and the other whey proteins with relatively high adsorption percentage (capacity) from acidic to near neutral conditions (pH 2.5 to 6.5), but with poor adsorption selectivity towards GMP and pH affected the adsorption of GMP and other whey proteins slightly as well. This is in disagreement with the observation of Casal et al (2005), who reported that CS solution could selectively precipitate GMP from casein hydrolysate (176). It might be due to the positive hydrophobic interactions between CS and GMP, β -lg and α -la in aqueous solution besides electrostatic interactions. As a result the high adsorption capacity of whey proteins was obtained even when pH was lower than 4.7 that the whey proteins were all negatively charged.

In Figs. 5.8b, the EPI cross-linked CS beads also adsorbed GMP and other whey

proteins with the similar adsorption percentage and did not show high adsorption selectivity towards GMP. The lowest adsorption percentage occurred at pH ~4.5. That indicated that both hydrophobic interactions and electrostatic interactions possibly affected the adsorption simultaneously, and the less porous external surface of EPI cross-linked beads could not provide the barrier for the selective adsorption based on the size exclusion.

In the case of the CS-HMDI-BCD beads (Figs. 5.8c), a peak value for the adsorption selectivity towards GMP (90.23 % of P_G , while 29.05 % of P_W) at pH 3.0 was observed in the same pH range and pH affected the adsorption of GMP dramatically as well. Obviously, the introduction of BCD made the difference. As introduced in Section 5.1, the nano scale cavity structure of BCD is hydrophobic and has the ability of forming inclusion complex with various compounds. These cavities were grafted on the external surface of CS. It supposed that GalNAc, Gal and NeuAc as branches of GMP entered the cavities and formed the inclusion complex with cavities at low pH. It has generally been accepted that the driving force for the formation is the van der Waals interaction between their hydrophobic moiety and the cavities and the hydrogen bonding between their polar functional groups and the hydroxyl groups of BCD. With the increase of pH from 2.5 to 6.5, GMP monomers aggregated, the molecular weight increased from 7 kDa to 9 kDa at pH ≤ 4 , to 45-50 kDa at pH ~6.5, and the accessibility of GalNAc, Gal and NeuAc for the formation of inclusion complex decreased due to polymer chain coil. That is why the adsorption percentage of GMP decreased from pH 3.0. The hydrophobic interaction also affected positively and caused the adsorption percentage higher 60 % at pH 6.5. On the other hand, other whey proteins, β -lg (18.6 kDa, $55.6 \times 81.92 \times 66.99 \text{ \AA}^3$), α -la (14.2 kDa, $35.47 \times 69.56 \times 45.96 \text{ \AA}^3$) and BSA (66.4 kDa,

$140 \times 40 \times 40 \text{ \AA}^3$) as lipids, have larger molecular weight and molecular size than GMP. They have no glycosylated branches that could associate with cavities of BCD, and cannot enter the cavities by themselves. That has been proved by chiu etc. (100). The adsorption percentage of other whey proteins was in small magnitude of $\sim 30\%$ because hydrophobic interaction possibly affect positively, as indicated in Figs. 5.8c. Overall, the electrostatic interactions and hydrophobic interactions are generally involved in this adsorption, but the formation of inclusion complex between cavities of BCD and glycosylated part of GMP is the major mechanism for the selective adsorption towards GMP. Therefore, the pH value of 3.0, which gave the highest selectivity and good capacity towards GMP, are selected as the separation condition for GMP together with CS-HMDI-BCD beads as adsorbents.

Fig.5. 8 Effect of pH on adsorption of GMP and other whey proteins (a) CS beads, (b) EPI cross-linked CS beads, and (c) CS-HMDI-BCD beads

5.3.3 Quantitative and simultaneous analysis of GMP, α -la and β -lg by RP-HPLC

As α -la, β -lg and GMP are the major contents of milk whey, the quantitative and simultaneous analysis of α -la, β -lg and GMP in adsorption and desorption experimental samples was carried out by RP-HPLC. The equipments and chromatographic gradient are described in Section 3.4.3.

5.3.3.1 Analysis of standard samples

The RP-HPLC chromatograms of the standards of GMP, α -la and β -lg are shown in Fig. 5.9. The standard solution of GMP (caseinoglycopeptide from bovine casein, Sigma-Aldrich Co.), α -la (α -lactalbumin from bovine milk, Type III, calcium depleted, $\geq 85\%$, Sigma-Aldrich Co.), and β -lg (β -lactoglobulin from bovine milk, approx. 90%, Sigma-Aldrich Co.) were prepared in concentration of 1000 ppm. 20 μ l of each standard solution was injected. The buffer B gradient separates GMP, α -la, and β -lg successfully at different retention time. The order of elution of the GMP, α -la, and β -lg is directed by their average hydrophobicity, with the GMP eluting earlier than α -la followed by β -lg variant B and variant A. No interference between the GMP fractions and whey proteins are observed. The multiple peaks in peaks 1, which include different GMP fractions such as glycosylated GMP, non-glycosylated GMP_A, and non-glycosylated GMP_B, proves the heterogeneity of GMP.

The GMP compounds were eluted in the retention interval between 9 and 15 minutes. In zoomed segments of chromatogram of standard GMP (Fig.5.10), the chromatographic profile shows a typical shape and position of peaks according to the different GMP fractions. The overall elution order is: glycosylated GMP < non-glycosylated GMP_A < non-glycosylated GMP_B. The glycosylated fraction of GMP eluted in 7 overlapping

peaks between $t_R = 9$ min and $t_R = 12.5$ min followed by the two major non-glycosylated components of variant A ($t_R = 12.7$ min) and variant B ($t_R = 14$ min), each eluted as a single, almost baseline-resolved peak.

Fig.5. 9 Superposition of RP-HPLC chromatograms of standard β -Ig (a), α -Ia (b) and GMP (c), Peaks 1 and 3: glycosylated GMP gen. var. A and B, peak 2: non-glycosylated GMP gen. var. A, peak 4: non-glycosylated GMP gen. var. B, peak 5: α -Ia, peak 6: β -Ig gen. var. B, peak 7: β -Ig gen. var. A. Chromatographic conditions and a gradient as described in Section 3.4.3

Fig.5. 10 Zoomed segments of RP-HPLC chromatogram of standard GMP, *= glycol GMP A+B

5.3.3.2 Analysis of whey proteins powder

The diluted whey proteins solution was prepared from whey proteins powder in the way described in Section 5.2.3. 20 μ l of diluted whey proteins solution at pH 3.0 was injected and the resultant RP-HPLC chromatogram is presented in Fig. 5.11. It is observed that GMP, α -la, and β -lg eluted at similar retention time with standard samples. The intensity of α -la and β -lg (var. A and var. B) is much higher than that of GMP. The means there are much α -la, and β -lg than GMP in our whey protein powder. Compared with RP-HPLC chromatogram (c) in Fig. 5.9, it is found that there is much non-glycosylated GMP gen. var. B in our whey protein powder than in commercial standard GMP bought from Sigma-Aldrich Co. The contents of α -la, β -lg var. A and β -lg var. B in our whey protein powder are ~21.1 %, ~24.6 % and ~46.6 %, respectively. The content of GMP is not available due to the overlapped peaks.

Fig.5. 11 RP-HPLC chromatogram of original whey proteins solution, Peaks 1 and 3: glycosylated GMP gen. var. A and B, peak 2: non-glycosylated GMP gen. var. A, peak 4: non-glycosylated GMP gen. var. B, peak 5: α -la, peak 6: β -lg gen. var. B, peak 7: β -lg gen. var. A

5.3.3.3 Analysis of experimental samples

To verify the experimental results presented in Section 5.2.5 (adsorbents selection and effect of pH) as well as the accuracy of the acidic ninhydrin assay for GMP, the original whey proteins solution, the supernatant after adsorption, and the supernatant after desorption were collected and analyzed quantitatively by RP-HPLC. The adsorption-desorption process followed the methods described in Section 5.2.7 using CS beads, EPI cross-linked beads and CS-HMDI-BCD beads. The respective amount of injection was 20 μ l, 40 μ l, and 20 μ l. The RP-HPLC chromatograms of the samples are shown in Fig. 5.12 (CS beads), Fig. 5.13(EPI cross-linked CS beads) and Fig. 5.14 (CS-HMDI-BCD beads). The RP-HPLC chromatographic results of the samples are

presented in Table 5-2 (CS beads), Table 5-3 (EPI cross-linked CS beads) and Table 5-4 (CS-HMDI-BCD beads), respectively. However, the results of GMP fractions are not quite accurate due to the overlapped peaks.

It is observed that at pH 3.0, CS beads adsorbed each fraction of GMP, α -la and β -lg with adsorption percentage higher than 90, but with poor adsorption selectivity towards GMP. That is in agreement with the results obtained via UV/Vis spectrophotometer. 1 M sodium chloride solution is capable of eluting the fractions GMP, α -la and β -lg efficiently, and the final yield is high. The results in Fig. 5.13 and Table 5-3 show that EPI cross-linked CS beads also adsorbed fractions of GMP, α -la and β -lg with the high efficiency and did not show high adsorption selectivity towards GMP. On the contrary, the results in Fig. 5.14 and Table 5-4 indicate that CS-HMDI-BCD beads possess extremely high adsorption selectivity towards GMP. 87.9 % of glycosylated $\text{GMP}_{(A+B)}$, 96.9 % of non-glycosylated GMP_A and 81.1 % of non-glycosylated GMP_B were adsorbed approximately. That is a little bit less than adsorption percentage of 90.23 % obtained via the acidic ninhydrin assay. 9.8 % of α -la and 2.7 % of β -lg $_{(A+B)}$ were adsorbed. That is much less than adsorption percentage of 29.05 % obtained via UV-Vis test at 280 nm. Overall, the results of RP-HPLC analysis are agreement with the results of UV/Vis spectrophotometer analysis, and the acidic ninhydrin assay for GMP is proved to be reliable and feasible.

Fig.5. 12 RP-HPLC chromatograms of original whey proteins solution (a), supernatant after adsorption (b), and supernatant after desorption (c), Peaks 1 and 3: glycosylated GMP gen. var. A and B, peak 2: non-glycosylated GMP gen. var. A, peak 4: non-glycosylated GMP gen. var. B, peak 5: α -la, peak 6: β -lg gen. var. B, peak 7: β -lg gen. var. A

Table 5-2 The RP-HPLC chromatographic results of α -la, β -lg and fractions of GMP in sorption process, CS beads

	Adsorption percentage (%)	Desorption percentage (%)	Yield (%)
glycosylated GMP (A+B)	96.3	64.9	62.5
non-glycosylated GMP A	96.9	73.7	71.4
non-glycosylated GMP B	96.8	80.7	79.7
α -la	93.4	96.3	90
β -lg (A+B)	76.4	67.1	51.3

Fig.5. 13 RP-HPLC chromatograms of original whey proteins solution (a), supernatant after adsorption (b), and supernatant after desorption (c), peaks identified as Fig.5.12

Table 5-3 The RP-HPLC chromatographic results of α -la, β -lg and fractions of GMP in sorption process, EPI cross-linked CS beads

	Adsorption percentage (%)	Desorption percentage (%)	Yield (%)
glycosylated GMP (A+B)	99.1	86.7	85.7
non-glycosylated GMP A	~100	81	81
non-glycosylated GMP B	~100	90.2	90.2
α -la	92.8	50.1	46.6
β -lg (A+B)	97.5	62.1	60.6

Fig.5. 14 RP-HPLC chromatograms of original whey proteins solution (a), supernatant after adsorption (b), and supernatant after desorption (c), peaks identified as Fig.5.12

Table 5-4 The RP-HPLC chromatographic results of α -la, β -lg and fractions of GMP in sorption process, CS-HMDI-CS beads

	Adsorption percentage (%)	Desorption percentage (%)	Yield (%)
glycosylated GMP (A+B)	87.9	89.6	78.8
non-glycosylated GMP A	96.9	74.9	72.6
non-glycosylated GMP B	81.1	85.9	69.7
α -la	9.8	82.2	8
β -lg (A+B)	2.7	85.5	2.3

5.3.4 Equilibrium adsorption isotherm

Fig. 5.15 shows the apparent adsorption isotherm of GMP (dots) on the CS-HMDI-BCD beads and the fitting (line) of Langmuir isotherm. It was termed as “apparent” isotherm because the system consisted of many other components. The batch experiments were carried out at pH 3.0 and 298.15 K. Repeated experiments found that 24 hours were enough for the system to reach adsorption equilibrium. The amount of GMP adsorbed was corresponding to that of sialic acid adsorbed, which was determined using the assay described in Section 5.2.4. The fitting results are listed in Table 5-5, with the R^2 of 0.9541 and R_L of 0.094. The maximum monolayer adsorption capacity, Q_m , was found to be 12.87 mg/g (corresponding to sialic acid).

Fig.5. 15 The apparent GMP adsorption isotherm on the CS-HMDI-BCD beads and the fitting of Langmuir equation

Table 5-5 The Langmuir parameters for the apparent GMP isotherm on CS-HMDI-BCD beads

$q_{e,exp}$ (mg/g) (sialic acid)	Langmuir isotherm			
	Q_m (sialic acid)(mg/g)	R_L	R^2	$\Delta q_e\%$
8.37	12.87	0.094	0.9541	5.74

5.3.5 Adsorption kinetics

The GMP adsorption kinetics on the CS-HMDI-BCD beads (pH 3.0, 298.15 K, 200 rpm) was investigated with the pseudo-first-order and pseudo-second-order kinetic models. Fig. 5.16 shows the experimental data (symbols) and the fittings of pseudo-firs-order equation (dashed lines) and pseudo-second-order equation (solid lines).

Fig.5. 16 Adsorption kinetics data, fitted to pseudo-first-order and pseudo-second-order models

Table 5-6 The kinetics parameters of different rate equations for the adsorption of GMP

Model	$q_{e, \text{exp}}$ (sialic acid, mg/g)	$q_{e, \text{cal}}$ (sialic acid, mg/g)	k	R^2	$\Delta q_e \%$
1 st order	4.6	2	4.0×10^{-3}	0.9732	6.23
2 nd order	4.6	4.1	9.8×10^{-6}	0.9997	1.66

Table 5-6 presents the derived optimal fitting parameters from the two kinetics models. Judging from the R^2 and $\Delta q_e \%$, the pseudo-second-order model gave a better fit. However, both models should deem as being adequate for the fitting of the kinetics. As

the system is multicomponent in nature, no further effort was devoted to further explore the true nature of the kinetics of the system.

5.3.6 Re-generation of the CS-HMDI-BCD beads

For the practical separation process, the adsorbents must be re-generated easily and present a long life (or recycles numbers). The effect of (re-generation) cycles on the adsorption and desorption of GMP on the CS-HMDI-BCD beads are shown in Fig. 5.17. It was observed that the adsorption percentage and the desorption percentage of GMP decreased only slightly with the increased cycle number. After ten cycles, the percentage of adsorption for GMP decreased from 91.3% to 83.6% and the percentage of desorption for GMP decreased from 85.3% to 81.9%, respectively. This proves that the CS-HMDI-BCD beads present good re-generation performance with the proposed re-generation method (1 M NaCl solution, 2 hours).

Fig.5. 17 The re-generation cycles of GMP adsorption/desorption on the CS-HMDI-BCD beads

5.4 Summary

The cationic property of CS was used to adsorb GMP from milk whey proteins, The FTIR spectra demonstrated that β -cyclodextrin was grafted to native CS beads with 1,6-hexamethylene diisocyanate as spacer successfully. The BCD grafted CS beads (CS-HMDI-BCD) has a surface area of $38.43 \text{ m}^2/\text{g}$ that is not much different from CS beads and EPI cross-linked CS beads. In selective adsorption towards GMP, the nano scale cavity structure of BCD had the capability of forming inclusion complex with GalNAc, Gal and NeuAc that were as branches of GMP and excluding other whey proteins, while the electrostatic interactions and hydrophobic interactions were involved generally in the adsorption of GMP and other whey proteins as well. The quantitative and simultaneous RP-HPLC analysis of α -la, β -lg and fractions of GMP were carried out. The results of RP-HPLC analysis were agreement with the results of UV/Vis spectrophotometer analysis, and the acidic ninhydrin assay for GMP was proved to be reliable and feasible. The CS-HMDI-BCD beads presented superior adsorption affinity and capacity towards GMP in the whey proteins solution. According to the RP-HPLC analysis, the contents of α -la, β -lg var. A and β -lg var. B in whey protein powder prepared were $\sim 21.1 \%$, $\sim 24.6 \%$ and $\sim 46.6 \%$, respectively. At pH 3.0, approximately 88.63% of total GMP was adsorbed with a maximum adsorption capacity corresponding to 12.87 mg of sialic acid/g-adsorbent, while minimum, 6.25% of other whey proteins were adsorbed approximately. Desorption experiments showed that the CS-HMDI-BCD beads could be regenerated and used in many cycles without significant decreases in the capacity and selectivity towards GMP.

CHAPTER 6 CS-CARRAGEENAN PEC FOR ORAL DELIVERY OF PROTEIN DRUGS

6.1 Introduction

In this chapter, a CS- κ -carrageenan polyelectrolyte complex was prepared by salt induced impeding of polyplex formation method and encapsulated with bovine serum albumin as a model protein drug to study its potential to be tailored to the pH responsive oral delivery of protein drugs. The simulated gastric fluid (with and without pepsin, pH 1.2) and the simulated intestinal fluid (with and without pancreatin, pH 7.5) were prepared and used to mimic the gastrointestinal tract in human body. The release kinetics of BSA from the CS- κ -carrageenan PEC was studied in these simulated gastrointestinal fluids with and without digestive enzymes.

6.2 Experiments

6.2.1 Preparation of BSA loaded CS- κ -carrageenan PEC

It is known in the prior art that pH-sensitive chitosan- κ -carrageenan PEC and encapsulation of drug can be prepared by simple mixing of two aqueous solutions of polyelectrolytes together with drug solutions under optimal conditions based on the electrostatic interaction between protonated chitosan and negatively charged κ -carrageen (as shown in Fig. 6.1).

Fig.6. 1 the chemical structure of κ -carrageen

However, limited success has been reported on the stability of CS- κ -carrageenan PEC in acidic gastric pH (195, 235). It has been found that the solution properties of carrageenan differ upon degree of sulfation, the presence of salts, and its concentration (236). Moreover, being a polyelectrolyte, rheology of CS solution are affected by the presence of salt (237). In this project, the CS- κ -carrageenan PEC was prepared by mixing aqueous solutions of CS and κ -carrageenan in the presence of NaCl. It is expected that, due to the presence of electrolyte counterions, the interaction between two polyelectrolytes will be delayed and the characteristic of PEC formed will have different properties. This process is named as the 'salt induced impeding of polyplex formation' method. The detail chemicals and procedures are as follows:

500 ml of 0.3 % (w/v) CS solution was prepared by dissolving 1.5 g of CS in 1 % acetic acid. 400 ml of κ -carrageenan solution was prepared by dissolving 1.5 g of κ -carrageenan in DI water with 25 g of sodium chloride at 90°C and cooled to room temperature. 100 ml of BSA solution was prepared by dissolving 0.5 g of BSA in DI water. The κ -carrageenan solution and BSA solution were mixed gently to obtain 0.3 % (w/v) κ -carrageenan solution with BSA of 1000 $\mu\text{g/ml}$. Subsequently, CS solution was added dropwise to the κ -carrageenan solution at the rate of 3 ml/min under stirring. When the resultant particles settled, the supernatant was decanted

and 0.01% acetic acid solution was added to the particles to wash away the unreacted free polymer and sodium chloride in the suspension. This step was repeated everyday for a week. After that, the suspension was freeze-dried and ground to obtain the final product.

6.2.2 Preparation of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

Simulated gastric fluid (SGF pH 1.2), which contains 7 ml of hydrochloric acid, 2 g of sodium chloride in 1 L of DI water, was prepared with and without 3.2 g of pepsin. Similarly, simulated intestinal fluid (SIF pH 7.5), which contains 6.8 g of potassium dihydrogen phosphate and 190 ml of 0.2 M sodium hydroxide in 1 L of DI water, was prepared with and without 10 g of pancreatin (US pharmacopoeia, 1995).

6.2.3 Protein release in SGF and SIF

The freeze-dried PEC agglomerates were ground into powders for the study of release of BSA. Firstly, the release profiles of BSA from the encapsulated particles in SGF and SIF were investigated, respectively. Each sample containing 3 mg of BSA encapsulated particles and 1 ml of simulated fluids was incubated at 37°C with a shaking rate of 200 rpm. After the predetermined time intervals, particles were filtered with 0.45 µm membrane and the BSA concentration in the filtrate was determined with Bradford reagent using a Nicolet Evolution 500 UV spectrophotometer.

Next, the release profiles of BSA from the encapsulated particles were investigated in SGF then followed by in SIF. After the encapsulated particles were incubated in SGF at

37°C with a shaking rate of 200 rpm for 4 hours, the particles were centrifuged at 20000g for 10 minutes before transferred to SIF and incubated at the same conditions. The release medium in SIF was taken out for the analysis using the method described above.

Finally, the release profiles of BSA encapsulated particles in simulated GI tract were investigated with digestive enzymes. All the experiments were conducted in triplicates and the average was used.

6.3 Results and discussions

6.3.1 BSA encapsulated PEC particles

The presence of electrolyte counterions from NaCl (Na^+ and Cl^-) can effectively prevent the immediate interaction of the oppositely charged CS and κ -carrageenan due to the weaker ionic attraction induced by the counterion atmosphere around the polyelectrolytes. With this principle, a homogeneous CS- κ -carrageenan PEC hydrogel was prepared and found to be stable in acidic pH with substantial swelling (238). In this project, the BSA encapsulated CS- κ -carrageenan particles were formed in suspension in the presence of NaCl. The concentrations of CS, κ -carrageenan, the ratio between the two solutions, and the flow rate of CS solution were optimized (with a number of trials) to get homogeneous particle suspension under visual judgment, shown in Figs. 6.2a. After washing away the unreacted components and freeze-drying concentrated particle suspension, dried particle agglomerates were obtained. To facilitate the BSA release study, the particle clusters were ground into particulate form, as shown in Figs. 6.2b. The particulate form has a diameter of 1-2 mm in irregular shapes

Fig.6. 2 Photos of CS-κ-carrageenan particles, (a) CS-κ-carrageenan particles obtained in suspension forms and (b) CS-κ-carrageenan particles in white particulate form

6.3.2 Characteristics of the PEC

The SEM image of the BSA encapsulated CS-κ-carrageenan agglomerates is shown in Fig. 6.3. It is seen that the agglomerates present interconnected pores of micrometer scale with some lumpy areas.

Fig.6. 3 The SEM micrograph of the BSA encapsulated CS-κ-carrageenan agglomerate

The pore properties of BSA encapsulated CS-κ-carrageenan PEC particulate were

characterized with the nitrogen isotherm at 77 K. The surface area and average pore diameter of PEC particulate were found to be 4.09 m²/g and 17.66 nm, respectively. The pore size distribution is shown in Fig. 6.4. It is observed that the pore size distributed in four ranges and the diameter of most pores was smaller than 20 nm.

Fig.6. 4 The pore size distribution of BSA encapsulated CS-κ-carrageenan PEC particulatee

FTIR spectra of κ-carrageenan (a), CS (b), and polyelectrolyte complex of CS and κ-carrageenan (c) are shown in Fig. 6.5 (wavelength 1800-600 cm⁻¹). The sulfate groups of κ-carrageenan attributed to the peaks at 1261, 924 and 851 cm⁻¹. The amide I peak of chitosan appeared at 1655 cm⁻¹. The broad bands around 1200-1000 cm⁻¹ in both CS and κ-carrageenan are from stretching of C-O-C. After the formation of CS-κ-carrageenan polyelectrolyte complex, a new absorption band at 1525 cm⁻¹ due to -NH₃⁺ groups was appeared. Moreover, the amide I peak from CS at 1655 cm⁻¹ was shifted to 1641 cm⁻¹ and the reduction of sulfate peak intensity at 1267 cm⁻¹ was observed. It indicates the interaction between protonated amine groups of CS and

sulfates groups of κ -carrageenan in the successful formation of CS- κ -carrageenan PEC.

Fig.6. 5 FTIR spectra of (a) κ -carrageenan, (b) CS, and (c) PEC of CS and κ -carrageenan from 1800 to 600 cm⁻¹

6.3.3 Release of BSA in SGF and SIF as the separated medium

The freeze-dried PEC agglomerates were ground into powders for the study of release of BSA. The release behaviors of BSA from the PEC in SGF and SIF without digestive enzymes were measured and shown in Fig. 6.6. The typical fast first-order followed by zero-order release kinetics was observed in both SGF and SIF media. However, the initial burst release observed in SIF is much faster than that in SGF. Within first 5 min, the release rate in SIF was 14 times faster than that in acidic SGF. Although no dramatic increase in BSA release was found in SGF after 5 min, BSA was further released in SIF for another one hour. Only 11 μ g of BSA was released in SGF, while 180 μ g of BSA was released in SIF.

Fig.6. 6 The release profile of BSA in SGF and SIF

Gan and Wang had proposed the following protein release mechanisms: (a) desorption of protein molecules from the surface of particles, (b) diffusion through the swollen PEC and (c) disintegration of the PEC (239). The release of BSA from CS- κ -carrageenan PEC is illustrated in Fig. 6.7. In acidic SGF medium (pH 1.2), CS and κ -carrageenan were oppositely charged and bound tightly. Therefore, further release was limited because of the absence of swelling or disintegration of PEC. The little amount of BSA released in SGF might be due to desorption of loosely bound BSA from the surface of particle. In SIF (pH 7.5), the ionic attraction between CS and κ -carrageenan became weaker because the pKa of CS is around 6.5 and little amino groups of CS got protonated. This will lead to significant swelling and disintegration of PEC, which allows large amount of encapsulated BSA to be released. As a result, the amount of BSA released in SIF was much higher than that in SGF. Those experimental results demonstrated that the CS- κ -carrageenan complex can protect the loaded protein

drug in acidic gastric condition and release it in higher pH of intestinal environment.

Fig.6. 7 The illustration of the release of BSA from CS-κ-carrageenan PEC

The release kinetics of BSA loaded PEC was also investigated in SGF and SIF in the presence of digestive enzymes. SGF was supplemented with pepsin and SIF with pancreatin. The results are shown in Fig. 6.8. The trends of release kinetics were similar to those observed as in SGF and SIF without enzymes. In SGF with pepsin, maximum amount (23 μg) of BSA released was detected at 5 minutes, and then it decreased gradually to 8 μg at the end of 4 hours. In SIF with pancreatin, the maximum BSA release (170 μg) occurred at 5 minutes, and then it decreased to 95 μg after 4 hours. The release of BSA in SIF was again significantly higher than in SGF.

Compared with the BSA release profiles in SGF and SIF without digestive enzymes, more BSA is released in both SGF and SIF with enzymes in initial release period. It indicates that the digestive enzymes help to disintegrate the CS-κ-carrageenan PEC and speed up the release from the carriers. The decreasing trends of protein concentration after the maximum release are attributed to the degradation of BSA by the digestive

enzymes in the release media. Although there is no significant protein degradation was observed in plain SGF and SIF media, most of BSA was degraded in SGF with pepsin and up to 20% degradation of BSA was observed in SIF with pancreatin after 1h. (as shown in Fig. 6.8)

Fig.6. 8 The release profile of BSA in SGF and SIF with digestive enzymes

6.3.4 Release of BSA in SGF and followed by in SIF as a consecutive release medium

In order to mimic the physiological gastrointestinal conditions, the release study of the BSA loaded PEC was conducted in SGF for the first 4 hours and then the particles were transferred to SIF for another 4 hours. Similarly, the release was conducted in the media with and without digestive enzymes for comparison. The results are shown in Fig. 6.9.

In the absence of enzymes, around 40 μg of BSA was released after 4 h of incubation in SGF. After transferring to SIF, about 65 μg of BSA was abruptly released in 5 minutes. It continued to increase with incubation time and reached 185 μg of cumulative release in a total of 8 hours incubation time. This amount was similar to the one released in 4

hours in SIF without pancreatin.

Fig.6. 9 The cumulative release profile of BSA in SGF (4 hrs) and followed in SIF

In the presence of digestive enzymes, only 5 μg of BSA was released after 4 hours of incubation in SGF with pepsin. After transferring to SIF with pancreatin, no increase of BSA concentration was detected for the next 15 minutes. Then, the amount of BSA released slowly increased to the maximum of about 35 μg in 2 hours and remained the same for the next 4 hours of incubation. It was believed that much more BSA was actually released in both SGF and SIF, but was digested by enzymes. When the particles were first incubated in SGF with pepsin, the enzymes could immobilize on the surface of the particles through electrostatic interaction. When the PEC was put in SIF with pancreatin, not only the loaded BSA protein but also the pepsin immobilized on the surface of the PEC would release into the release medium of SIF. Apparently, the combined action of pepsin and pancreatin has synergistically degraded the released BSA protein. This would lead to the low amount of BSA detected in SIF although no

significant amount of BSA was released in SGF in the presence of digestive enzymes.

Overall, only little amount of BSA was released from CS- κ -carrageenan PEC in acidic SGF (with and without pepsin), but significant amount of BSA was released after the release medium is switched to SIF (with or without pancreatin).

The trends of the results are encouraging in response to pH value. However, the presence of digestive enzymes in the gastrointestinal tract is still detrimental to the activity of protein drugs because the enzymes could digest the released protein or peptide. The longer the residence time of drugs in the gastrointestinal tract, the higher the degree of the in-situ degradation. Therefore, the released protein drug from the PEC should pass gastrointestinal mucosa as quickly as possible to reach the blood circulation to get the desired bioactivity. In the real life scenario, the presence of CS could potentially help the faster passage of the protein drug from the intestinal mucosa to the blood circulation because of the mucoadhesive properties of CS. CS is known to interact with cystein-rich mucus to delay the mucus turnover rate and transiently open the tight junction of the intestinal wall. This process would help the released protein drug to transport across the intestinal barrier to reach higher bioavailability. In vivo studies are needed in the future to prove this speculation.

6.4 Summary

The pH responsive CS- κ -carrageenan PEC was successfully prepared by salt induced impeding of polyplex formation method and was encapsulated with BSA. The resultant CS- κ -carrageenan PEC was stable under acidic conditions. The surface area and average

pore diameter of PEC particulate were found to be 4.09 μm and 17.66 nm, respectively. In the simulated gastric fluid (pH 1.2), only 11 μg of BSA was released from 3 mg of PEC, while in the simulated intestinal fluid (pH 7.5), 180 μg of BSA was released from 3 mg of PEC in four hours. In simulated gastrointestinal media (SGF followed by SIF), 185 μg of BSA was released from 3 mg of PEC, which is similar to the one released in SIF. However, In the presence of digestive enzyme the amount of released BSA increased in the first period, and decreased with the time going on significantly. Most of BSA was degraded in SGF with pepsin and up to 20% degradation of BSA was observed in SIF with pancreatin after 1 hour. These results showed that the PEC matrix based on CS is suitable for oral delivery of protein and peptide drugs. The digestive enzymes present in the gastrointestinal tract could be potentially detrimental to the released protein or peptide drugs depending on the residence time in the intestinal tract. However, mucoadhesive and mucopermeation properties of CS would offer the particles to adhere in intestinal mucosa and release the drug in sustained manner to increase bioavailability of protein or peptide drugs.

CHAPTER 7 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE STUDIES

7.1 Conclusions

In this project, a model protein (bovine serum albumin) adsorption was studied on native/cross-linked CS beads in a batch adsorber and a fixed bed column under various operation conditions, respectively. This project also successfully developed chitosan grafting β -cyclodextrin beads for the separation of GMP from milk whey, and chitosan-carrageenan PEC for the pH-sensitive delivery of protein drugs. The detailed findings are:

BSA adsorption native/cross-linked CS:

A phase inversion technique was used to fabricate native CS hydrogel beads (diameter ~ 1.7 mm), which were subsequently cross-linked by GLU and EPI, respectively (diameter ~ 1.7 mm). The BSA adsorption was investigated on native/cross-linked CS beads in a batch adsorber under different pH value, ion strength, and cross-linking ratios. SEM characterized the morphologies of adsorbent beads before/after adsorption. It was found that the adsorption kinetics follows the pseudo 2nd order rate equation. In single component aqueous solution, pH affects the adsorption performance significantly and the electrostatic interactions between BSA and amine groups of CS play a major role in

the adsorption capacity while the adsorption kinetic rate is an important factor for the cross-linked CS as well. At a high pH > 6.0, cross-linking with either agent will decrease the adsorption capacity. But at a low pH of ~5.5, cross-linking will promote the protein adsorption capacity due to the better acidic stability and more protonated amine groups. EPI cross-linked CS beads (mass ratio: 0.2) are better adsorbent with a maximum adsorption capacity of 193.6 mg/g-adsorbents at pH 5.5. Fixed bed breakthrough experiments of BSA was conducted on native/EPI cross-linked CS beads for potential industrial application, it was found that Bohart & Adams and Clark model are good candidate for the simulation of the fixed bed kinetics.

Separation of GMP from milk whey:

The cationic property of CS was used to adsorb GMP selectively from milk whey. β -cyclodextrin (BCD) was successfully grafted to native CS beads with 1,6-hexamethylene diisocyanate as spacer in a two-step method. CS was used as supporting materials to solve the solubility of BCD in aqueous solution. The nano scale cavity structure of BCD enhanced the adsorption and selectivity towards GMP. The selectivity towards GMP of CS beads, EPI cross-linked CS beads and CS-HMDI-BCD beads were evaluated in multiple proteins aqueous solution without any precipitation process. Overall, the selective adsorption mechanisms on the adsorbent are complicated, generally the electrostatic interactions and hydrophobic interactions are involved, but the formation of inclusion complex between cavities of BCD and glycosylated part of GMP is the major mechanism for the selective adsorption towards GMP. As a result, in the absence of any precipitation using TCA/ethanol, the CS-HMDI-BCD beads present superior adsorption affinity and adsorption capacity towards GMP in the whey proteins

solution comparing to native and EPI cross-linked CS beads. A simple acidic ninhydrin assay specific to sialic acid existing in GMP was developed for the rapid and sensitive analysis of GMP and it was proved to be reliable and feasible by RP-HPLC analysis. According to the quantitative analysis of RP-HPLC, the contents of α -lg var. A and β -lg var. B in whey protein powder prepared were $\sim 21.1\%$, $\sim 24.6\%$ and $\sim 46.6\%$, respectively. At pH 3.0, 88.63 % of total GMP was adsorbed with a maximum adsorption capacity corresponding to 12.87 mg of sialic acid/g-adsorbent, while minimum, 6.25 % of other whey proteins were adsorbed. Most GMP and other whey proteins adsorbed can be eluted using 1 M NaCl solution. Adsorption-desorption cycle experiments showed that the CS-HMDI-BCD beads could be regenerated and re-used in many cycles without significant decreases in the capacity and selectivity towards GMP.

PEC delivery of protein drugs:

.CS- κ -carrageenan PEC was prepared by salt induced impeding of polyplex formation method and encapsulated BSA. The FTIR spectra showed the successful formation of the PEC under the experimental conditions. The surface area and average pore diameter of PEC particulate were found to be $4.09\text{ m}^2/\text{g}$ and 17.66 nm, respectively. The prepared BSA encapsulated CS- κ -carrageenan PEC are stable under stronger acidic conditions and show good pH responsibility. In the simulated gastric fluid (pH 1.2), only 11 μg of BSA is released from 3 mg of PEC due to desorption of loosely bound BSA from the surface of particle with little swelling, while in the simulated intestinal fluid (pH 7.5), 180 μg of BSA is released from 3 mg of PEC in four hours due to significant swelling and disintegration of PEC. In simulated gastrointestinal media (SGF followed by SIF), 185 μg of BSA is released from 3 mg of PEC, which is similar to the one released in

single SIF.

The presence of digestive enzymes in the gastrointestinal tract is detrimental to the activity of protein drugs because the enzymes could digest the released protein or peptide. Most of BSA is degraded in SGF with pepsin and up to 20% degradation of BSA is observed in SIF with pancreatin after 1 hour.

These results show that the PEC matrix based CS is suitable for oral delivery of protein and peptide drugs. It is the first time to show that the CS- κ -carrageenan PEC particles prepared in the presence of NaCl can be tailored to the pH responsive oral delivery of protein drugs. The mucoadhesive and mucopermeation properties of CS would offer the particles to adhere in intestinal mucosa and release the drug in sustained manner to increase bioavailability of protein or peptide drugs.

7.2 Recommendation for future studies

This project constitutes an important part of work in the research on chitosan for bio-separation and controlled delivery of proteins. More efforts are still needed to develop more advanced chitosan-based materials and evaluate their potential performance for the bio-separation. The following directions are suggested for the future researches:

- (1) Grafting poly (acrylamide)/poly (acrylic acid) onto CS for adsorption of single protein in fixed bed column.
- (2) Investigating the separation of GMP from milk whey in fixed bed column packed

with CS-HMDI-BCD materials.

- (3) Conducting the analysis of amino acids to evaluate the residual level of aromatic amino acids in GMP product via isolation and purification process.
- (4) Preparing CS beads loaded with metal ions as ligands for separating GMP from milk whey in affinity chromatography.
- (5) Studying the protein release behavior using CS-carrageenan PEC *in vivo* and evaluating the bioactivity of proteins *in vivo*.

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