University of Alberta

Intestinal Uptake of Barley Protein Nanoparticles as Delivery Vehicles for Bioactive Compounds

by

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Dedication

This thesis is dedicated to my parents, for without their early inspiration, coaching and support none of this would have happened.

This thesis is also dedicated to the University of Alberta, where my dream came true and a new journey of my life had started from.

Abstract

The use of nanoparticles as nutrient delivery vehicles enables the enhancement of the oral bioavailability and health promoting benefits of bioactive compounds. Barley protein nanoparticles were developed in previous study for hydrophobic compound delivery. The objective of the present study was to evaluate the cytotoxicity of the nanoparticles and to characterize their intestinal uptake properties using *in vitro* and *ex vivo* models. The nanoparticles showed low cytotoxicity in Caco-2 cells. Their cellular uptake was dependent on time, concentration and temperature, suggesting transcytosis pathway. Significantly greater β-carotene uptake (15%) was observed in Caco-2 cells when delivered by nanoparticles compared to control (2.6%). The nanoparticles also showed adhesion and permeation abilities in rat jejunum tissues. Findings from this study demonstrated the uptake improving effect of barley protein nanoparticles and suggested their potential as nutrient delivery vehicles for the development of novel functional foods.

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List of Abbreviations

NHPs	Natural Health Products
LDL	Low density lipoprotein
PUFAs	Polyunsaturated fatty acids
IFT	Institute of Food Technologists
GI tract	Gastrointestinal tract
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
PEG	Poly(ethylene glycol)
HPMCP	Hydroxypropyl methylcellulose phthalate
ADME	Absorption, deposition, metabolism and excretion
M cell	Microfold cell
GALT	Gut-associated lymphoid tissue
SLNs	Solid lipid nanoparticles
VIN	Vinpocetine
PIBCA	Poly(isobutyl cyanoacrylates)
WGA	Wheat germ agglutinin
BSA	Bovine serum albumin
HSA	Human serum albumin
nab-P	nanoparticle albumin-bound paclitaxel
FDA	US Food and Drug Administration
ENMs	Engineered nanomaterials
ROS	Reactive oxygen species

IBD	Inflammatory bowel disease
UC	Ulcerative colitis
CD	Chrohn’s disease
BNPs	Barley protein nanoparticles
GRAS	Generally Regarded as Safe
ATCC	American Type Culture Collection
DMEM	Dulbecco’s modified eagle medium
FBS	Fetal bovine serum
NEAA	Non-essential amino acids
HBSS	Hank’s balanced salt solution
DPBS	Dulbecco’s phosphate-buffered saline
MTT	Thiazolyl blue tetrazolium bromide
BMPs	Barley protein microparticles
NR-NPs	Nile red-labeled barley protein nanoparticles
TEM	Transmission electron microscopy
PTA	Phosphotungstate
CLSM	Confocal laser scanning microscopy
MFI	Mean fluorescence intensity
SNK test	Student-Newman-Keuls test
PDI	Polydispersity index
LDH	Lactate dehydrogenase
GSH	Glutathione
TBA	Thiobarbituric acid

TEER	Transepithelial electrical resistance
BC-NPs	β-Carotene encapsulated barley protein nanoparticles
BHT	Butylated hydroxytoluene
SDS	Sodium dodecyl sulfate
HPLC	High performance liquid chromatography

Chapter 1 Literature Review

1.1 Functional Foods Overview: Benefits and Challenges

Foods have been recognized for their role in resolving hunger and maintaining basic growth and development for the body. In the past two decades, however, the emphasis of foods has progressively switched to their potential to promote health beyond the conventional nutritional values (Niva, 2007). Such reshaping attitude towards food is due to many factors including an increasing aging population, health concerns raised by modern life style and, resulting from scientific advances and food regulations, the progressed public understanding that diet can alter physiological function and risks of chronic disease development. In response to consumers' needs for healthier foods, scientists and food manufacturers have launched intensive research and development of functional foods and nutraceuticals.

1.1.1 Concepts and benefits of functional foods

The term "functional foods", very frequently together with "nutraceuticals", has been used widely around the world, yet there is no consensus on their meanings. Health Canada defines a functional food as a conventional or formulated food that has physiological benefits or protection against a chronic disease and is consumed as part of a diet, while a nutraceutical is a product that has the same benefits but is isolated from foods and consumed in medicinal forms (Health Canada, 2002). Functional foods are usually discussed with, or categorized under, natural health products (NHPs), which need product license

for labeling and are controlled under Natural Health Products Regulation in Canada (Health Canada, 2012). Another commonly accepted concept for functional foods and nutraceuticals is that a functional food is a food that enhances physiological performance beyond its traditional functions; when a functional food aids in the prevention or treatment of diseases, it is called a nutraceutical (Brower, 1998; Kalra, 2003). Meanwhile, some scientists specify a nutraceutical in a narrower sense as a natural health-promoting ingredient in foods rather than a whole food product (Wildman, 2000). Kwak and Jukes (2001) have reviewed the regulatory concepts of functional foods and related biomedical terms in several countries. As the two concepts are closely related and very frequently mixed up when used in food, nutritional and biomedical studies, attention needs to be paid on their meanings in certain contexts. For the purpose of this thesis, a functional food is considered as a food that enhances physiological performances beyond its traditional nutritional values and may protect against or assist in treatment of a disease (general sense nutraceutical); a bioactive compound/ingredient is used to describe a natural beneficial component in food (narrow sense nutraceutical).

Functional foods offer many benefits in improving health condition and life quality, including the following aspects (Siró, Kápolna, Kápolna, & Lugasi, 2008). (i) "Add good to your life", for instance, yogurt containing probiotics can improve intestinal microflora and overall gastrointestinal functions. (ii) "Make your life easier", such as lactose-free and gluten-free products for certain consumer groups who are not tolerant to these components in natural foods (Siró

et al., 2008). (iii) Reduce risk or aid in treatment of chronic diseases. Many available functional foods are serving this purpose. For instance, breakfast cereals enriched with dietary fibers (e.g. β-glucan) can lower the level of low density lipoprotein (LDL) and protect against cardiovascular disease (Jenkins et al., 2002). Vitamin D fortified milk and orange juice are routinely consumed to maintain bone growth and prevent against vitamin D deficiency (Holick, 2007). Furthermore, eggs enriched with ω-3 fatty acids can be used as a source of polyunsaturated fatty acids (PUFAs) that may reduce cardiovascular disease risk and improve mental functions (Kris-Etherton, Harris, & Appel, 2002).

1.1.2 Concerns and challenges

Functional foods bring opportunities to improve health and well-being for consumers and marketing profit for food manufacturers. There are many challenges along the research and development chain, from the raw materials to processors and retailors, to bring a functional food to success. The expert panel of the Institute of Food Technologists (IFT) (2005) have identified a seven-step process that addresses majors aspects associated with the design, development and marketing of new functional foods: (1) identify a potential bioactive compound and its health benefit; (2) assess bioavailability and demonstrate the efficacy of the component; (3) evaluate the safety if the active component is new to food use; (4) select a suitable food matrix for the active component; (5) conduct independent expert review to ensure the health claim efficacy; (6) communicate with consumers about the health benefits; and (7) confirm the

efficacy of safety of the product in market. Though specific requirements and concerns for each step may vary depending on the particular bioactive compound and the functional food product of interest, getting through all the steps is a must for any new active compound and its application in foods.

The first five steps in the above process mainly emphasize the need for scientific proof of the functions of the product and the validation by regulatory bodies, while the last two highlight consumers' acceptance and verification of the claimed benefits. The application of an active compound in functional foods relies on the scientific evidence of its bioavailability and efficacy. Even though some products have been used for health-enhancing purposes for a long time, fundamental and clinical research is often insufficient (Brower, 1998). The health claims of functional foods need to be governed by policies and regulations, but the regulations vary greatly across countries because of the ambiguous definition and different levels of scientific evidence (Agriculture and Agri-Food Canada, 2009). The heterogeneous standard particularly challenges the global companies targeting different markets. A clear regulatory framework is in need for health claim evaluation, production, sales and advertising as well as for market growth and consumers' trust. The acceptance of a functional food by consumers is determined by their primary health concern, awareness of the association between health and foods, and also the labels and basic sensory quality of the product (Clydesdale, 2004). The first five steps above form the basis of the acceptance of consumers of the product, which is the key success factor for market orientation (Sirót et al., 2008).

The development and public education of functional foods relies on scientific evidences. Sustainable research is needed to identify new bioactive compounds, to provide insights into their health benefits, to seek for suitable food matrix and delivery vehicles if required, and to evaluate the health promoting efficacy of the product. Study on finding favorable vehicles for bioactive ingredients delivery is highlighted as one of the areas that are worthy of in-depth research (Clydesdale, 2004). In this context, the food matrix or delivery vehicles need to provide a stable environment for the ingredient and maximize its health benefits. As delivery vehicles play a critical role in the success of functional food development and in consumer compliance, research on modifying food matrix and finding effective nutrient delivery systems has witnessed rapid growth.

1.2 Nanoparticles as Potential Nutrient Delivery Systems

1.2.1 The need for delivery vehicles in functional foods

The effectiveness of a functional food in boosting health and preventing disease depends on preserving the bioavailability of the bioactive compounds in the products (Chen, Remondetto, & Subirade, 2006; Rein et al., 2013). Bioavailability is the amount of administrated dose that is available for utilization at the target organ or tissue; in food science, the bioavailability of orally administrated food compounds can be interpreted as the amount of an ingredient that enters the systematic circulation (Chen et al., 2006; Acosta, 2009). The bioavailability of food compounds can be affected by two major

factors: (i) formulation factors, such as the structure of the bioactive molecules and the food matrix in which they are located in; and (ii) physiological factors, including solubility, permeability and transport mechanisms in the intestinal lumen, interactions with digestive enzymes and other food ingredients, metabolism and elimination (Ponchel, Montisci, Dembri, Durrer, & Duchêne, 1997; Rein et al., 2013; Yu & Huang, 2013). Bioactive compounds need to be bioavailable in order to exert any beneficial effects (Rein et al., 2013). Developing compound delivery systems that can alter its physicochemical and biological properties is one of the main approaches to improve the bioavailability and health benefits (Ezhilarasi, Karthik, Chhanwal, & Anandharamakrishnan, 2013) .

Nanotechnology is a promising approach to enhance oral bioavailability of bioactive compounds. It has been extensively studied in pharmaceutical research for drug delivery and routed as the next revolution in agricultural and food industry (Ezhilarasi et al., 2013). After the year 2000, nanotechnology in food filed has experienced a rapid, almost exponential, growth. The worldwide market of nanotechnology-based food products, mostly in the application of food packaging and edible coatings, nanosensors and nanotracers for food safety sensing and nutrient encapsulation and delivery, has reached approximately one billion US$ in the last ten years and is estimated to surge to over 20 billion US$ in this decade (Chau, Wu, & Yen, 2007). As potential nutrient delivery systems, nanoparticles are of particular interest because not only they have high biological activities due to high surface area to volume ratio and unique physicochemical

properties but they are more stable than other colloidal vehicles, such as liposome and emulsion, in the gastrointestinal (GI) tract and during food storage (Des Rieux, Fievez, Garinot, Schneider, & Préat, 2006). Nanoparticle delivery can improve the bioavailability of bioactive compounds by many mechanisms. First, the use of various polymeric materials and fabricating methods enables the improvement of compound solubility and transport efficiency across the mucosa barrier by modulating their physicochemical characteristics such as particle size, surface charge and hydrophobicity (Win & Feng, 2005). Second, nanoparticles allow controlled release of the bioactive compounds, i.e. the compounds are available to be transported or utilized at desired site and time at a specific rate (Pothakamury & Barbosa-Cánovas, 1995). This can be achieved by tailoring the formulation of nanoparticles so as to carry out desired release mechanisms, such as delayed, prolonged and enzyme or pH triggered release (Galindo-Rodríguez, Allémann, Fessi, & Doelker, 2005). Finally, the particle surface can be modified with adhesive or targeting molecules that enhance the mucosal adhesion or cellular uptake, such as lectin and chitosan (Ezpeleta et al., 1999; Schipper et al., 1997). Consequently, it has been extensively demonstrated that nanoparticles protect bioactive compounds against the harsh environment in the GI tract and facilitate the transmucosal transport and overall bioavailability (Chen et al., 2006; Yu & Huang, 2013).

Although food scientists and industries have embraced nanotechnology, most of the research on nanoparticle vehicles has been focused on drug delivery in pharmaceutical field; the application in foods is still on the initial stage (Chau

et al., 2007). Nanotechnology-based delivery systems hold great promise in increasing the bioavailability of active compounds and fulfilling the effectiveness of functional foods in promoting the state of health.

1.2.2 Nanoencapsulation of nutrients and preparation techniques

Enhanced bioavailability of nutrients by nanocarriers is implemented by nanoencapsulation techniques. It is a process by which the compound of interest is packaged within a spherical structure in the scale under 1000 nm (Ezhilarasi et al., 2013; Pinto Reis, Neufeld, Ribeiro, & Veiga, 2006). Nanoparticles encapsulated with bioactive compounds can be categorized to nanospheres and nanocapsules (Figure 1.1); the term nanoparticle is commonly used for both. Nanospheres have a uniform matrix structure with the ingredient dispersed within the matrix or distributed near the surface. Nanocapsules have a core/shell structure with the nutrient confined within the inner core and enclosed by a solid polymeric wall (Couvreur, Dubernet, & Puisieux, 1995). Based on the shape and fine structure, nanocapsules can be further categorized into classes such as multiwall and multicore nanocapsules (Chaudhuri & Paria, 2012).

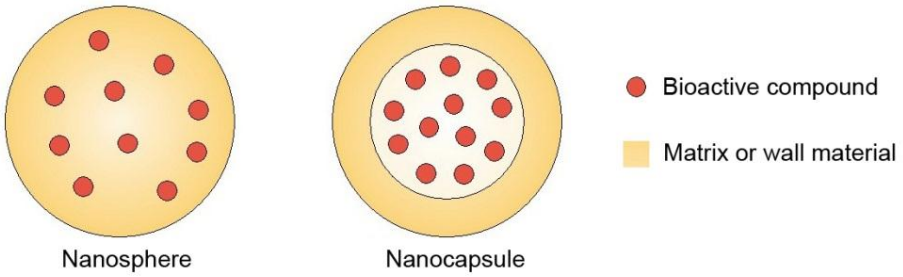

Figure 1-1 Schematic structures of nanoparticles: naonsphere and nanocapsule. Adapted from Orive, Anitua, Pedraz, & Emerich (2009).

There are two main strategies to prepare nanoparticle systems. The first one is the "top-down" approach, or mechanical approach, in which nanoparticles are produced by size reduction of materials through mechanical forces. The other one is the "bottom-up" approach, or chemical approach, where nanoparticles are generated by self-assembly of smaller molecules in the presence of certain chemical reagents (Chen et al., 2006; Sanguansri & Augustin, 2006).

In top-down approaches, compress, impact, shear and other physical forces are used to break bulk materials down to the nanometer size range by employing mechanical processes including milling, microfluidization and emulsification (Sanguansri & Augustin, 2006). Dry milling such as ball milling and jet milling is the most common method to prepare ultrafine powders by either using a grinding media or the produced particles to shear themselves. Milling techniques are intensively used in the preparation of ultrafine power of food ingredients, including soluble iron powder in aqueous solution (Lomayeva et al., 2000), wheat flour with high water-binding capacity and fine green tea powder that demonstrates higher antioxidant activity (Sanguansri & Augustin, 2006). Microfluidization and high-pressure homogenization uses high shear stress of liquid flow by subjecting the liquid material through narrow nozzles under high pressure (up to 20,000 psi), causing the formation of nanosized droplets or emulsion. Microfluidization has been used in food processing as a well-developed technology, especially for dairy products (Olson, White & Watson, 2003), and one of the main approach in the preparation of solid lipid

nanoparticles (SLNs) which are widely investigated as drug delivery systems for lipophilic ingredients (Müller, Mäder, & Gohla, 2000).

In bottom-up approaches, nanoparticles are formed by self-assembly or self-organization of polymers or polymer and surfactant. Various reactions such as polymerization, colloidal aggregation or coacervation, nanoprecipitation and supercritical fluid technique are used in this approach (Ezhilarasi et al., 2013). During preparation, bioactive compounds are molecularly dispersed in lipophilic, hydrophilic or amphiphilic solvents; biodegradable polymers are often used as particle matrix or wall materials (Horn & Rieger, 2001). Poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) are among the most commonly used polymers. These particles are frequently coated with poly(ethylene glycol) (PEG) to improve the stability and transport efficiency (Brannon-Peppas, 1995; Vila, Sánchez, Tobío, Calvo, & Alonso, 2002). Anand et al. (2010) encapsulated curcumin in PLGA nanoparticles with stabilizer PEG-5000 using nanoprecipitation technique; the particles enhanced the uptake of curcumin *in vitro* and *in vivo*. Other examples include capsaicin-encapsulated gelatin nanoparticles prepared by cross-linking with glutaraldehyde using coacervation process (Wang, Chen, & Xu, 2008), and lutein-loaded hydroxypropyl methylcellulose phthalate (HPMCP) nanoparticles prepared from supercritical antisolvent precipitation that protect lutein from thermal or light oxidation (Jin, Xia, Jiang, Zhao, & He, 2009). Polymeric nanoparticles, mostly prepared by polymerization and nanoprecipitation, have been received more attention as they

can efficiently form nanoparticles of around 100 nm size, which has been proved the most effective in achieving enhanced drug delivery (Pinto Reis et al., 2006).

Comparing the two main nanoparticle fabrication strategies, top-down approaches minimize the use of chemical additives and thereby mitigate potential safety concerns; however, high energy density input is required to provide the physical force (Yu & Huang, 2013). Bottom-up approaches are capable of produce smaller particles (average diameter 30-500 nm) compared to top-down methods (average diameter 200-1000 nm), whereas the process relies on understanding the accurate self-assembling properties of the molecules or atoms; it also involves the use of chemical additives and solvents (Sanguansri & Augustin, 2006). Currently most commercial nanomaterial products are prepared by top-down approaches; the use of bottom-up approaches and the combination of both strategies is progressively increasing as nanotechnology grows (Horn & Rieger, 2001).

1.3 Bioavailability Enhancement with Nanoparticle Delivery

1.3.1 Digestion and absorption of nutrients

It is essential to understand the biological processes that regulate bioavailability in order to design effective delivery systems for bioactive compounds. Although bioavailability is generally determined by absorption, deposition, metabolism and excretion (ADME), it is absorption and metabolism that principally influence orally administrated compounds becoming bioavailable

(Yu & Huang, 2013). In respect of absorption, it is directly determined by the food formulation and digestion process.

Smith and Morton (2001) elaborated on the digestion processes that foods encounter in the body. After the initial digestion in the oral cavity by mastication and salivary amylase, the food goes through esophagus and enters stomach. The food encounters dissolution in the stomach under the effect of the acidic gastric juice (pH 1.2-2), pepsin and other enzymes and the movement of the stomach wall. After a period of 0.5-3 h, depending on the food content (fats > proteins > carbohydrates), the partially digested food is mixed with gastric juice and becomes a semiliquid mass called chyme, which enters the small intestine and undergoes the majority of food digestion and nutrient absorption. In the first part of small intestine duodenum, the chyme is mixed with various digestive enzymes from pancreas including as amylase, trypsin, chymotrypsin and lipase. Carbohydrates and proteins are broken down to smaller units under the effect of these enzymes and become ready to be absorbed through the intestinal wall. The digested carbohydrates and proteins are predominately absorbed in the lower parts of small intestine: jejunum and ileum. The inner surface of small intestine is composed of polarized epithelial cells with microvilli oriented to the lumen to maximize the absorption area (approximately 250 m^2). Most nutrients are absorbed into the blood capillaries under the intestinal epithelium by transporting across the enterocytes. Some of the nutrients may be metabolized by the epithelial cells themselves before they are translocated across the cells. The metabolism may involve conjugation, reduction, oxidation and other processes

(Yu & Huang, 2013). The nutrients that survive intestinal metabolism can enter the blood stream upon absorption. The digestion of lipids does not start until they reach duodenum. Bicarbonate and bile are secreted to the duodenum and meet the lipids. The bile salts (e.g. taurocholate and lecithin) emulsify the fats into smaller droplets so that they can be digested by pancreatic lipase. Bile salts also form micelles with the lipids which make them soluble and ready to be absorbed by enterocytes (Iqbal & Hussain, 2009). After digestion, unlike carbohydrates, proteins and other nutrients that enter the blood capillaries upon absorption, lipid micelles are directly translocated to the lymphatic capillaries (lacteals) that underlie the intestinal epithelium. Finally, at the terminal section of small intestine, bile salts, vitamin B_{12} and any remaining nutrients are absorbed in the ileum. Ileum also contains specialized epithelial regions called Peyer's patches composing of specialized epithelial cells named microfold cell (M cell). Peyer's patches are part of gut-associated lymphoid tissue (GALT) and act as defensive system in the GI tract to prevent bacteria from entering the blood stream (Jepson et al., 1996).

It is important to note that before the nutrients can enter the systematic circulation upon absorption, they will take a detour directed by the blood that drains out of the digestive tract to the liver. The nutrient-rich blood moves through the liver via the hepatic portal system to provide essential nutrients needed for liver function and then direct the remaining nutrients to the general circulation. The metabolisms in the liver (hepatic metabolism) and in the GI tract (intestinal metabolism) directly affect the bioavailability of food ingredients.

Only the dose of nutrients and bioactive compounds that survive metabolism processes can eventually be available for body cell use. The exception is lipid absorption where lipids can enter the lymphatic system directly and bypass hepatic metabolism. Therefore, to avoid or to minimize metabolism is an effective way to improve bioavailability. However, scientists have not achieved significant success in manipulating metabolism (Yu & Huang, 2013). Other approaches used to enhance bioavailability include increasing the dissolution rate and absorption efficiency, which can be achieved by designing favorable nutrient delivery systems.

During micelle formation in the process of lipid digestion, the size of micelles that are formed with bile salts ranges from several nanometers to 100 nm; in a way they are human's natural nanoparticle delivery systems; the need for manufactured nanoparticle is sometimes under debate in respect of this issue (Acosta, 2009; Yu & Huang, 2013). However, intrinsic micelles can only make hydrophobic ingredients that are already dissolved in lipid available for absorption; they are not be able to facilitate the uptake if the compounds are not released from the food matrix or not consumed with sufficient dietary fat. In addition, different lifestyle and dietary habits may lead to some people having limited access to certain nutrients. In regards of meeting general nutrition needs and providing greater amount of available therapeutic compounds, nanoparticles offer the opportunity to incorporate desired dose of compounds to various food matrices.

1.3.2 Digestion and absorption of nanoparticles

Orally administrated nanoparticles undergo the similar digestive processes with other food contents through the GI tract. They may have unique biological behaviors and interactions in the digestive system due to their specific formulation and physicochemical properties. An insight to the digestion and absorption steps of nanoparticles that may determine the bioavailability of the bioactive compound is essential to design effective delivery systems.

Upon traveling through the esophagus after oral administration, dissolution of nanoparticles starts upon the contact with gastric juice in the stomach. Depending on the encapsulated ingredient, degradation of nanoparticle matrix in the stomach may or may not be favorable. Protection against gastric environment is often required in the delivery of proteins or peptides (e.g. insulin) so as to maintain their bioactivity before reaching the site of absorption. In this case, pH-sensitive materials such as polymers containing carboxyl groups which reduce the release and shield the encapsulated compound from gastric juice can be used to modify nanoparticles (Rekha & Sharma, 2009). Upon mixing with bile salts and pancreatic digestive enzymes in the small intestine, intact or partially digested nanoparticles (depending on the particle formulation and release profile) reach the surface of intestinal wall and ready for absorption. The epithelial lining is covered by a mucus gel layer mainly composed of anionic glycoprotein (mucin) (Ponchel & Irache, 1998). The epithelial cells and the mucus gel layer act not only as the absorption site of nutrients, but a barrier to potential pathogens (Crater & Carrier, 2010; Ensign, Cone, & Hanes, 2012). In

the GI tract, entrapped active compound are absorbed by either direct uptake of nanoparticles (Acosta, 2009) or cellular transport after being released from the particle matrix via various mechanisms (e.g. swelling, diffusion, biodegradation, etc.) (Pothakamury & Barbosa-Cánovas, 1995). The direct uptake of nanoparticles in GI tract has been intensively studied and proved effective in improving the absorption of bioactive compounds (Galindo-Rodríguez et al., 2005; Acosta, 2009; Ponchel & Irache, 1998; Ponchel et al., 1997).

The direct uptake of nanoparticles in the GI tract involves three basic steps: (i) mucoadhesion of particles to intestinal wall; (ii) cellular uptake of particles by epithelial cells; (iii) post-absorptive events. The first step is a dynamic event in the intestinal lumen (Figure 1.3). When nanoparticle suspension enters small intestine, a fraction of particles adheres to the mucus layer via specific ligand-receptor interaction (e.g. lectins with M cells, vitamin B_{12} with ileum mucosa) (Chalasani, Russell-Jones, Jain, Diwan, & Jain, 2007) or non-specific bioadhesive interaction (e.g. hydrogen bonding, van der Waals forces, etc.) (Ponchel & Irache, 1998). The particles progressively form a cover lining along the mucus as they travel through the lumen. Shear forces in the lumen result in the detachment of a portion of particles from their site of adhesion. The detached and non-adherent particles transit to the distal site and are eliminated to feces (Ponchel et al., 1997). The second step is the uptake of nanoparticles by intestinal epithelial cells, including enterocytes and M cells. If capable of travelling through the mucus gel layer (100-500 μm in the small intestine) (Atuma, Strugala, Allen, & Holm, 2001), the adherent nanoparticles are taken up

by enterocytes via various mechanisms (discussed in section 1.3.4) and may translocate across the cells and enter blood stream. Meanwhile, a fraction of nanoparticles enters lymph nodes via lymphatic uptake by M cells of the Peyer's patches in the small intestine (Ponchel & Irache, 1998). M cells are more accessible to nanoparticles due to the absence of mucus layer; M cells, however, represent less than 1% of the total intestine area, resulting in the limited extent of lymphatic uptake (Brannon-Peppas, 1995). Although it is reported that the interaction of nanoparticles with erythrocytes may result in increased particulate circulation time and the circulation half-life of the erythrocytes, the influence of nanoparticle flow in the blood and lymph remains unclear and needs more research (Kim, El-Shall, Dennis, & Morey, 2005). The third step is the postabsorptive phase. Limited knowledge is known about the distribution and destination of nanoparticles in the body after absorption. Florence (2005) outlined some events involved in nanoparticles following oral delivery at postabsorptive phase (Figure 1.4). Nanoparticles are firstly transferred to blood stream and mesenteric lymph after absorption by enterocytes and M cells respectively. Then the particles with encapsulated compound or released compound alone are directed by the blood flow to distant tissues and reach the sites of action by extravasating or diffusing through tissues. As bioavailability of an orally administrated dose is usually interpreted as the fraction that enters blood stream (Acosta, 2009), studies are insufficient on the biological behaviors after they enter the blood flow and the release kinetics in the systematic

circulation. Research on these issues is needed to obtain better understanding of the metabolic behaviors and the safety properties of nanomaterials in the body.

The suggested mechanisms by which mucoadhesion, cellular uptake and postabsorptive translocation occur are only crude description of the real complex conditions. The role of mucus layer in facilitating or delaying particle absorption is under debate (Florence, 2005); the effects of various influencing factors (stomach emptying, dilution of particles in GI fluids, mixing with other food contents, etc.) on particle mucoadhesion remain unknown (Ponchel & Irache, 1998). Moreover, the biological behaviors and potential risks of nanoparticles in the blood, lymph and distal sites after absorption are poorly understood.

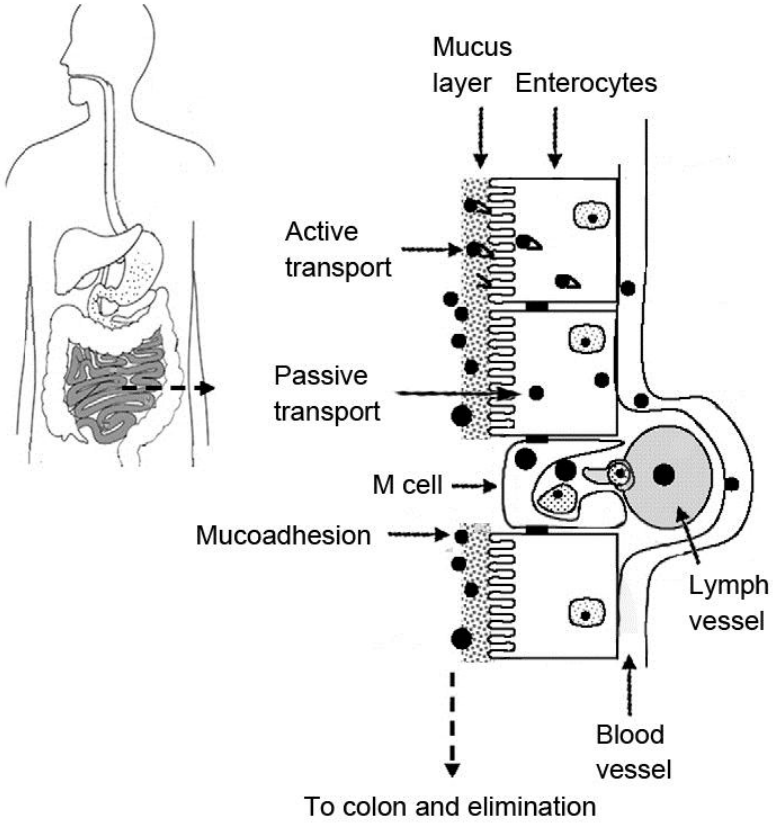

Figure 1-2 Direct uptake of orally administrated nanoparticles in the small intestine. Adapted from Acosta (2009).

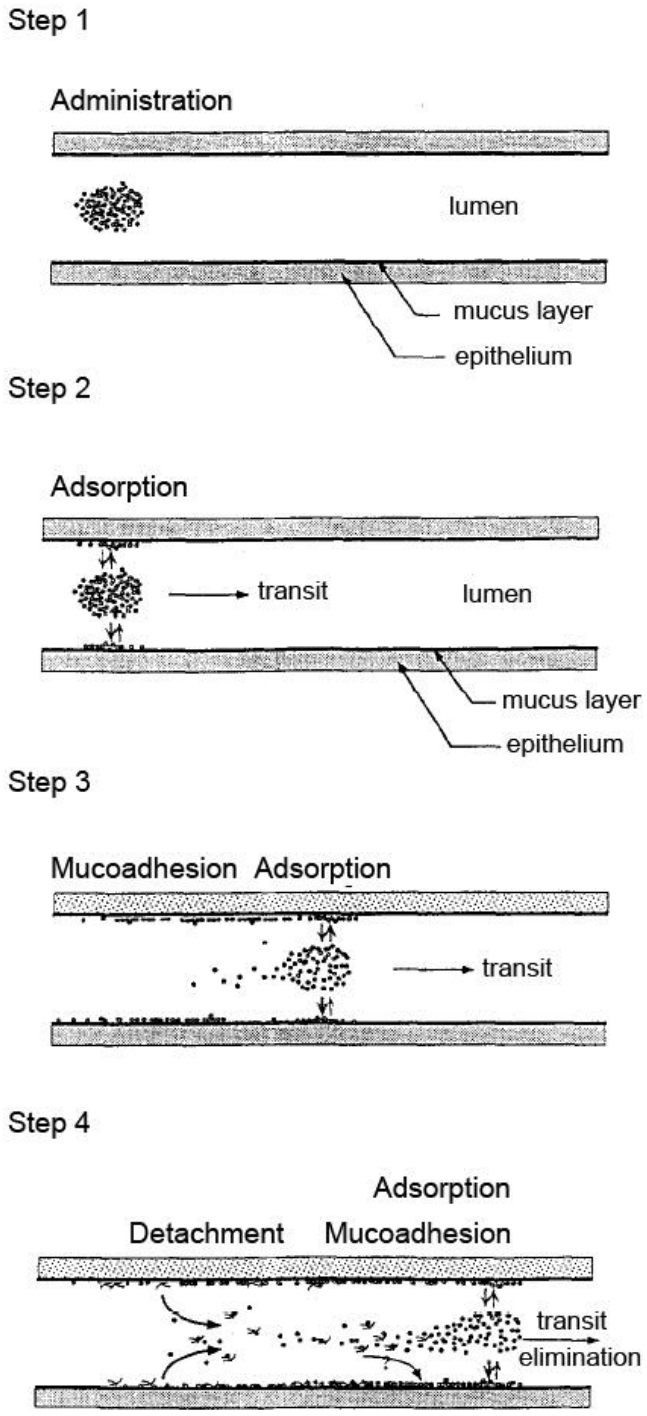

Figure 1-3 Mucoadhesive behaviors of orally administrated nanoparticles in the intestinal lumen. Step 1, nanoparticles enter the intestinal lumen after oral administration; Step 2, adsorption to the mucus gel layer on the surface and

particle transit in the lumen; Step 3, mucoadhesion while transit in the lumen; Step 4, detachment and faecal elimination of non-adherent nanoparticles. Adapted from Ponchel et al. (1997).

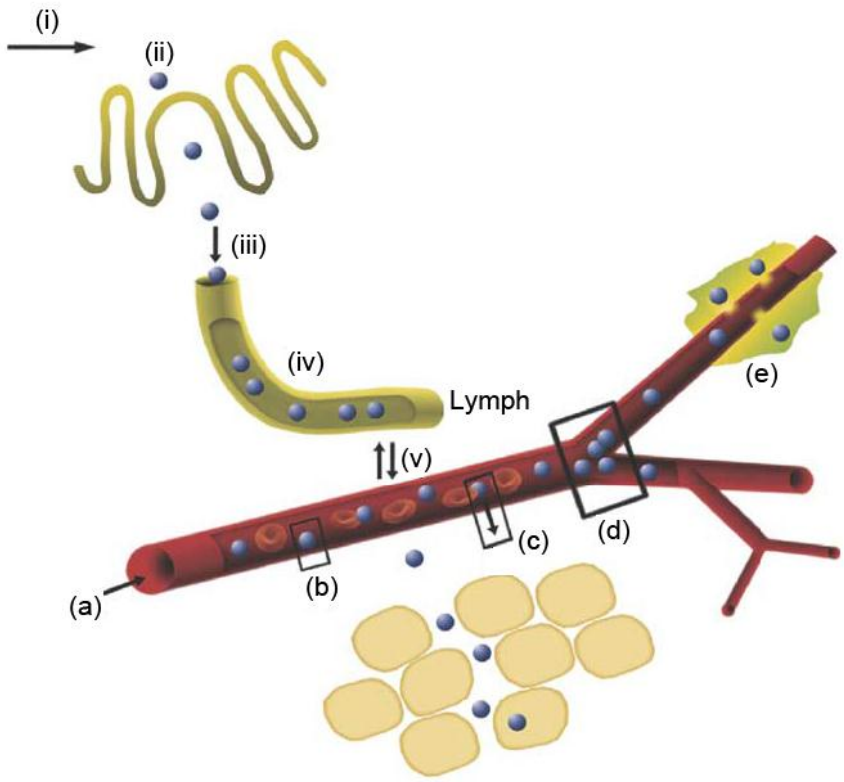

Figure 1-4 Postabsorptive events of orally administrated nanoparticles. (i) Particle flow in the intestinal lumen; (ii) translocation from the lumen to blood or lymphatic capillaries; (iii) passage into mesenteric lymph; (iv) flow in the lymph vessels and entrapment in the lymph nodes; (v) transport between lymph and blood; (a) passage into blood flow; (b) adhesion to capillary wall; (c) extravasation and flow in tissue; (d) flow at vessel bifurcations; (e) movement into target sites (e.g. tumors for drug delivery carriers). Adopted from Florence (2005).

1.3.3 Improvement of compound solubility

It is suggested that there are three steps for orally administrated nutrient to reach bioavailability: (i) solubilization: ingredients from food become soluble in the aqueous GI fluid; (ii) absorption: solubilized nutrients are absorbed across intestine wall; (iii) transport to systematic circulation: absorbed nutrients enter systematic circulation by either escaping from metabolism or directly entering lymphatic circulation (Yu & Huang, 2013). The design of nanoparticle delivery systems is accordingly focused on addressing challenges occurring in the three steps described above. The enhancement of nutrient bioavailability by nanoparticles may involve, but not limited to, mechanisms of increasing the solubility of bioactive compounds, improving the stability of compounds in GI tract, prolonging the residence time of compounds within GI tract, increasing the absorption efficiency and minimizing compound clearance due to metabolism (Bravo-Osuna, Vauthier, Farabollini, Palmieri, & Ponchel, 2007; Luo, Chen, Ren, Zhao, & Qin, 2006; Acosta, 2009; Roger, Lagarce, Garcion, & Benoit, 2009).

As being soluble in aqueous GI fluid is the first step of a bioactive compound to achieve bioavailability, various nanocarriers have been developed to improve the solubilization and dissolution of hydrophobic nutrients in GI tract. The nanomaterial formulations that can increase solubility include emulsions, micelles, dispersions and encapsulation (Yu & Huang, 2013). In emulsion (typically oil-in-water emulsion) and micelle systems, lipophilic compounds are predissolved in oil phase in the core of emulsion and micelle

complexes which are readily dissolved or homogenized in aqueous solution (Ahmed, Li, McClements, & Xiao, 2012). Alternatively, in nanoparticles, lipophilic ingredients are dispersed (nanoshpere) or encapsulated within polymeric matrices (nanocapsule); the nanoparticle carriers are dispersed in aqueous solution stabilized by the effect of surfactant or stabilizer. Luo et al. (2006) used solid lipid nanoparticles (SLNs) to encapsulate a lipophilic drug Vinpocetine (VIN) which has limited oral availability due to poor aqueous solubility and metabolism. Oral administration of VIN-loaded SLNs (70-200 nm) increased the absorption of VIN by 4.16 folds compared to that of VIN solution in rat models. Another strategy commonly used on poorly soluble compounds is NanoCrystal® Technology (Merisko-Liversidge, Liversidge, & Cooper, 2003), where large sized drug compounds are milled into nano-sized crystals and dispersed in a water-based stabilizer solution. The nanocrystalline particles (100-200 nm) are physically stable in an aqueous solution and can be processed into capsules, tablets and other forms suitable for oral delivery. Solubilization of a poorly soluble compound by this approach facilitates the dissolution of the compound and leads to higher absorption and bioavailability (Merisko-Liversidge et al., 2003).

The size of the nanoparticle carriers plays a key role in determining the solubility and dissolution rate of the bioactive compound. For poorly soluble compounds, small particle size leads to large relative surface area which drives dissolution (Merisko-Liversidge et al., 2003). Acosta (2009) investigated the relationship between nanoparticle size, relative solubility and relative

uptake/bioavailability from five different studies. It was concluded that in the range of 50-500 nm, the uptake/bioavailability and relative solubility of the active ingredient is a function of particle size; in other words, both bioavailability and solubility increase as particle size decreases. Considering the particulate systems and experimental methods from these studies are highly various, the similar correlation trend suggests that particle size is an essential factor in determining the solubilization of the active ingredient, which may also contribute to the fact drawn from a number of studies that particles uptake is size-dependent (Desai, Labhasetwar, Amidon, & Levy, 1996; Desai, Labhasetwar, Walter, Levy, & Amidon, 1997; Gaumet, Gurny, & Delie, 2009). It was also emphasized that both particle size and uptake of the particle carriers are important factors in improving the overall bioavailability of the active ingredient.

1.3.4 Improvement of permeability: mucoadhesion

Apart from solubility, the intestinal permeability of a compound is another essential determinant for the bioactive compound to reach its maximum absorption (Fade, 1998). In order to penetrate through the intestinal wall, the compound has to overcome the mucosal barrier, including proteolytic enzymes (pepsin, trypsin, chymotrypsin, etc.), mucus gel layer and the epithelial cell lining. Mucus gel layer is the first surface that nanoparticles encounter in GI lumen. It provides many opportunities for the design of adsorptive nanoparticles

to facilitate mucoadhesion, which can lead to higher compound concentration and prolonged retention time at the site of absorption (Jung et al., 2000).

Mucoadhesion can take place via building either non-specific (electrostatic interaction, hydrogen bond, hydrophobic interaction, etc.) or specific (ligand-receptor interaction) interactions between the particles and mucus layer. This can be enhanced by modifying the size, surface charge and other physicochemical properties of the nanoparticle systems. Chitosan is a commonly used cationic polysaccharide polymer for particle coating. The mucoadhesion capacity of chitosan-coated nanoparticles can be improved by establishing hydrogen bonds and electrostatic attraction/ionic bond between the cationic amino groups of chitosan and the anionic sialic acid residues of mucin in the mucus layer (Peppas & Huang, 2004). Bravo-Osuna et al. (2007) discovered that chitosan-coated poly(isobutyl cyanoacrylates) (PIBCA) nanoparticles showed up to 44-fold increased attachment to rat intestinal mucosa in comparison to the negatively charged uncoated PIBCA nanoparticles. Other adhesive chitosan-coated nanoparticles and the effects of chitosan molecular weight and crosslinking degree were reported in a number of studies (Behrens, Vila Pena, Alonso, & Kissel, 2002; Galindo-Rodríguez et al., 2005; Kawashima, Yamamoto, Takeuchi, & Kuno, 2000).

Despite of the promise of adhesive nanoparticle systems in improving non-specific adhesion of nanoparticles to the mucosa, a considerable amount of particles and active ingredients are subject to direct fecal elimination or mucus physiological turnover, i.e. the continuous process of loss and replacement of the

gel layer (Lehr, Poelma, Junginger, & Tukker, 1991). It is believed that interaction of decorated particles with surface receptors of enterocytes can lead to greater uptake (Hussain, 2000). In this context, adhesive nanoparticle systems employing specific interaction with the mucosa are developed. These particles are modified with a ligand which shows specific affinity to a receptor on certain areas of the mucus or underlying cells and can directly bind to the target surface via ligand-receptor interactions. In the previous example with chitosan-coated PIBCA nanoparticles, the researchers also investigated thiolated chitosan-coated particles which further improved their mucoadhesive properties via forming disulfide bond with the cysteine-rich domains of mucin (Bravo-Osuna et al., 2007). The most extensively studied binding ligands in mucoadhesive nanoparticle design are lectins, a type of natural proteins or glycoproteins derived from plants, animals or microorganisms that can specifically bind to carbohydrates. Lectin-conjugated nanoparticles can recognize the carbohydrate moieties located at the epithelial cell membrane and bind to the cells directly instead of the mucus layer (Galindo-Rodr ́guez et al., 2005). Florence, Hillery, Hussain and Jani (1995) studied the absorption of tomato lectin-conjugated polystyrene nanoparticles (500 nm) using growing rats. Compared to the control group, lectin-conjugated nanoparticles showed adhesive property to the enterocytes as well as M cells and increased the absorption by 10 times after 5 days daily dosing. Similar effects were observed on *Ulex europaeus* lectin-conjugated gliadin nanoparticles (Ezpeleta et al., 1999) and wheat germ agglutinin (WGA)-conjugated PLGA nanoparticles (Yin, Chen, Qiao, Wei, &

Hu, 2007). Irache et al. (1996) used different segments of rat intestinal mucosa samples to demonstrate different binding preference of polystyrene latex bead conjugated with different types of lectins including tomato lectin. It was also pointed out in the study that the use of lectins might increase the residence time of the particulate systems at their target sites of GI tract and thereby enhance compound bioavailability. It should be noted that such enhancing effect has shown to be less effective and inconsistent *in vivo*, mostly due to the delay in the stomach as well the unfavorable interactions between lectins and mucin, which may lead to the particle conjugates not capable of diffusing through the mucus layer and binding to underlying enterocytes (Bies, Lehr, & Woodley, 2004; Galindo-Rodr ǵuez et al., 2005). Therefore, in spite of the extensive research, the application of lectin in designing nanoparticle delivery systems in pharmaceutical and nutraceutical fields has achieved limited success. Hence, the overall role of mucosa in enhancing bioactive compound absorption is under debate. Some suggest that mucoadhesion can bring the particles and compound closer to the absorption site and prolong the retention time, while others argue that the entrapment of particles may delay or inhibit the uptake process (Florence, 2005).

1.3.5 Improvement of permeability: cellular transport

Once nanoparticles pass the preliminary events in GI tract (adhesion and transit through mucus layer) and reach the surface of intestinal epithelial cells, they can be taken up by the cells via three main mechanisms (Figure 1.5): (i)

paracelluar transport, by which particles transit through the space between cells; (ii) transcellular transport by enterocytes, where particles are internalized into cytoplasm, travel through the cell interior and externalized to the basolateral side, including passive diffusion, active transcytosis and carrier-mediated transport; (iii) transcellular transport by M cells, where lymphatic uptake takes place (Des Rieux et al., 2006). These mechanisms involve different physicochemical or biological interactions between nanoparticles. Efforts have been focused on designing nanoparticles with different properties to regulate their cellular uptake so as to improve the bioavailability of encapsulated compounds.

Nanoparticle uptake via paracellular route is a rare event due to the very small space between epithelial cells at the site of tight junctions (< 1 nm) (Figure 1.5). However, paracellular permeability can be enhanced by coating particles with polymers such as chitosan, poly(acrylate) and starch. These polymers are capable of opening tight junctions and facilitating paracellular transport of macromolecules or nanoparticles of small size (< 50 nm) (Jung et al., 2000). Chitosan-coated nanoparticles were used to deliver insulin to diabetic rats via the oral route. The blood glucose decreasing effect was sustained for at least 10 h, which was profoundly longer than effect of injected insulin (3 h) and significantly protected insulin from degradation in GI tract (Lin et al., 2007). It has been suggested that chitosans can interact with tight junction associated protein ZO-1as well as cytoskeletal F-actin via their positive charges, resulting in a transient opening of the tight junctions and thusly increased paracellular permeability (Schipper et al., 1997). As paracellular transport only applies to

very small particles and limited number of permeability enhancing polymers, the main focus of nanoparticle uptake study lies on improving their transcellular transport.

The transport effectiveness of nanoparticles via transcellular transport is mainly influenced the physicochemical properties of nanoparticles such as size, surface charge, hydrophobicity and presence of a ligand on the particle surface. It is also influenced by the *in vivo* condition of GI tract and the animal model used for transport study (Des Rieux et al., 2006). Efforts have been made on preparing nanoparticle systems with favorable physicochemical properties to enhance their direct cellular absorption; it is especially effective for active compounds that are soluble but with low permeability (Acosta, 2009). It is well established that the uptake of nanoparticles are size-dependent. Particles with diameter at approximately 100 nm demonstrate the highest uptake efficiency in Caco-2 cell model (Desai et al., 1997; Gaumet et al., 2009; Win & Feng, 2005) and in rat tissue models (Desai et al., 1996; Jani, Halbert, Langridge, & Florence, 1990a); meanwhile particles larger than 1 μm have very limited capability of penetrating through Caco-2 cell monolayer (Gaumet et al., 2009), mucus layer (Ponchel et al., 1997) or Peyer's patches (Jani, Halbert, Langridge, & Florence, 1990b). Apart from controlling particle size, coating or incorporating particles with polymers or surfactants has been used to modify their surface properties to regulate cellular transport. Eldridge et al. (1990) prepared microparticles with different hydrophobicity using different synthetic polymers and discovered the greatest particle uptake by Peyer's patches was correlated with the highest

hydrophobicity. While hydrophobic particles seemed to have more affinity for M cells than for enterocytes in this study, it was found in other studies that nanoparticles with hydrophilic coatings, such as PEG (Vila et al., 2002) and chitosan (Gaumet, Gurny, & Delie, 2010) demonstrated greater transport across Caco-2 cells and mucus layer, suggesting particles with hydrophilic surface have enhanced affinity to enterocytes (Des Rieux et al., 2006). Hydrophobicity and surface charge of nanoparticles are closely associated and affect the biological behaviors collaboratively. Shakweh, Besnard, Nicolas and Fattal (2005) observed that PLGA nanoparticles with neutral or negative charge and hydrophobic surface exhibited higher uptake by Peyer's patches; while Acosta (2009) demonstrated that chitosan-coated particles with positive and hydrophilic surface are widely used for their efficient uptake by mucus-covered enterocytes. Jung et al. (2000) summarized that uncharged or positively charged hydrophobic particles generally favor uptake by both M cells and enterocytes, whereas negatively charged hydrophilic particles may facilitate uptake via enhanced mucoadhesion.

Lymphatic uptake by GALT is interesting for compound delivery application is due to the great transcytotic ability of M cells (Des Rieux et al., 2006). Apart from their main role in antigen sampling, M cells are capable of transport a broad range of materials including nanoparticles (Frey & Neutra, 1997). Although accounting for only 1% of the total intestinal surface, Peyer's patches are considered the major site of particulates uptake attributed to transcytosis by M cells (Jepson et al., 1996). Besides modifying particle size and

surface properties as stated above, the main strategy to improve uptake by M cells is to decorate particles with specific ligands (Des Rieux et al., 2006). One of the mostly used ligands for M cell targeting is lectins; nanoparticles coated with these glycoproteins can be specifically recognized and captured by receptors of M cells. Another approach is the application exopolymers produced by toxic bacteria, where particles are coated with microbial adhesins as pathogen mimics and thusly can bind and interact with M cells (Des Rieux et al., 2006). Researches in this context show highly variable or even contradictory results, mostly attributed to the complex interaction of particular systems with M cells and the variant GALT conditions among different animal species (Acosta, 2009; Des Rieux et al., 2006). Finding an animal model that simulates human physiological conditions with comparative accuracy and extrapolating methods of data on animals studies to human are in demand.

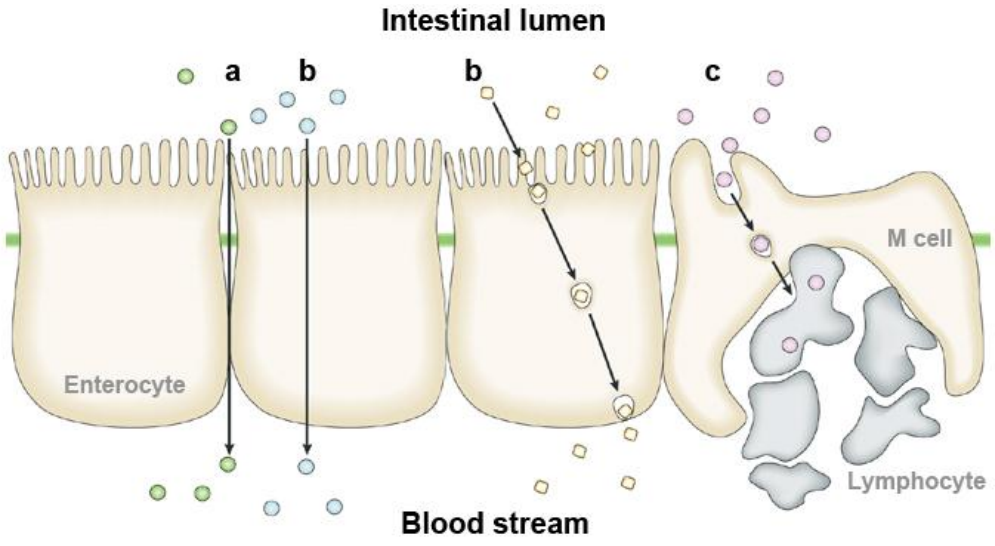

Figure 1-5 Transport routes of nanoparticles across intestinal epithelial cells: (a) paracellular transport, (b) transcellular transport by enterocytes and (c) transcellular transport by M cells. Adapted from Orive et al. (2009).

1.4 Barley Protein-based Nanoparticles in Nutrient Delivery

1.4.1 Proteins used in nanoparticle delivery systems

Biopolymer micro- and nanoparticles fabricated from proteins or polysaccharides have been extensively studied as encapsulation and delivery systems in food industry. They are mainly used as alternatives to liposome carriers as due to greater stability during storage and after oral administration as well as the effectiveness in enhancing absorption and bioavailability (Jahanshahi & Babaei, 2008). In addition, there are very few food-grade materials available for functional food development; it is financially challenging and time-consuming to introduce new food grade polymers. Proteins and polysaccharides are of particularly interest of food manufacturers owing to their natural origin and versatile physicochemical properties (Jones & McClements, 2010). Apart from the essential nutritional values, proteins have unique functionalities such as emulsification, gelation, foaming and water binding capacity. In food and pharmaceutical fields, they are fabricated into hydrogels, emulsion and colloid particles as active ingredient carriers. Thanks to the functional properties, protein-based micro- and nanoparticles are not only relatively easy to prepare for manufacturers but allow modifications with polysaccharides, lipids and other biopolymers as adhesion enhancers or ligands for cell targeting and controlled release (Chen et al., 2006).

A variety of animal and plant source proteins are used to fabricate nanoparticles. Chen et al. (2006) summarized the proteins that have been

investigated for compound encapsulation in past two decades with highlight of food protein applications. Albumin and gelatin are one of the proteins first being used for nanoparticle preparation. Albumin makes up the main protein of human plasma and is of special interest in pharmaceutical fields for its own multifold functionalities in the circulatory system such as the transportation and metabolism of numerous molecules. Albumin-based nanoparticles are biocompatible, biodegradable, less immunogenic and easy to prepare and control size range (Sundar, Kundu, & Kundu, 2010). Most extensively used for drug delivery, albumin nanoparticles release drug content by degradation of the protein wall by protease. The release profile can be regulated by surface modification. Many reactive groups (e.g. thiol, amino, carboxylic groups, etc.) that albumin carries can be used for covalent ligand binding and polymer crosslinking (Jahanshahi & Babaei, 2008; Wu et al., 1997). Both bovine serum albumin (BSA) and human serum albumin (HSA) have been employed to prepare nanoparticles as drug or active compound carriers or therapeutic enhancers (Elzoghby, Samy, & Elgindy, 2012b). Albumin particulate drug delivery system achieved its greatest success in nanoparticle albumin-bound paclitaxel (*nab*-P), which has been approved by the US Food and Drug Administration (FDA) in 2005 for breast cancer treatment (Petrelli, Borgonovo, & Barni, 2010). Gelatin nanoparticles possess many common advantages as albumin such as biodegradability, low toxicity and chemically modification capability. Moreover, they are mostly used for sustained drug release due to the outstanding adjuvant activity (Franz, Pokorová, Hampl, & Dittrich, 1998).

Attention needs to be drawn that cross-linkers such as glutaraldehyde are often introduced in the fabrication of these nanoparticles as drug carriers so as to improve their stability (Jahanshahi & Babaei, 2008; Wu et al., 1997). These cross-linkers, however, do not meet food grade standard and cannot be used in food application.

In addition to animal proteins, many vegetal proteins have also been exploited as nanoparticle matrices in the past twenty years. Gliadin, an alcohol-soluble protein fraction of wheat gluten, is one of the most used plant proteins in this context. Gliadin has low solubility in water due to its high content of neutral and lipophilic amino acid residues, which promote hydrogen bounding with intestinal mucus gel layer and hydrophobic interaction with other tissues (Arangoa et al., 2000). Gliadin nanoparticles have been used to delivery lipophilic compounds, such as retinoic acid (Duclairoir et al., 1999; Ezpeleta et al., 1996) and carbazole (Arangoa, Campanero, Renedo, Ponchel, & Irache, 2001) and shown increased bioavailability by providing sustained plasma concentrations due to their mucoadhesion property (Arangoa et al., 2000; Arangoa et al., 2001). Other vegetal proteins as potential particulate delivery systems include corn zein, soy proteins, barley proteins, etc. The particle matrices can be modified with polysaccharides, adhesive enhancers or other synthetic polymers to facilitate their physiochemical and biological properties (Chen et al., 2006).

While the recent advances have shown great potential of protein nanoparticle systems, most of them are currently extensively used in drug

delivery. Organic solvents are required in the fabrication process, which may limit their application for nutrient delivery in food area. For example, glutaraldehyde is commonly used as crosslinking reagent to stabilize the particle structure in the coacervation-based albumin nanoparticle fabrication; butadiene and formaldehyde are often needed in emulsification techniques (Elzoghby, Samy, & Elgindy, 2012a). These compounds are cytotoxic and may have undesirable reaction with the active compounds and biological tissues (Singh, Wang, Uludag, & Unsworth, 2010). In order to develop safe protein-based nanoparticles and extend their application in foods, alternative fabrication approaches and formulations are in demand. Attentions need to be focused on not only the preparation techniques but their biological properties and efficacy in nutrient delivery.

1.4.2 Barley proteins as potential nanoencapsulating materials

Barley is one of the most ancient cereal grains. It ranks the fourth place in world cereal production following wheat, rice and corn and accounts for 12% of the total cereal production (Jadhav, Lutz, Ghorpade, & Salunkhe, 1998). Historically barley has been an important food resource in parts of the world including the Middle East-North Africa and northeastern Europe. Nowadays, about two thirds of barley production is used as animal feed and a great amount is used in malting, brewing and distilling industries (L ászti ty, 1996). The proportion of barley grains in human foods occupies only about 2% and is underutilized in pharmaceutical and industrial products for human consumption.

Today pearled barley are most commonly used in breakfast cereals, porridge and bakery blends; barley flour has been incorporated into bread products (Gupta, Abu-Ghannam, & Gallaghar, 2010). Since FDA approved the health claim for soluble dietary fiber β-glucan of lowering cholesterol and reducing cardiovascular disease risk (Jenkins et al., 2002), barley consumption has been heightened because of its high β-glucan content (major fiber constitutes of barley) as well as other nutritious compounds including tocols, vitamins and minerals (Gupta et al., 2010).

Proteins are the second major composition of barely grains and accounts for 8-15% of total dry weight, following starch which contributes about 60% of grain content (Jadhav et al., 1998). Based on solubility, four protein fractions are distinguished in barley: albumins (water-soluble), globulins (salt-soluble), hordeins (alcohol-soluble) and glutelins (alkali-soluble). The amount of albumin and globulin is relatively low which takes 3-5% and 10-20% of the total protein respectively. The main proteins in barley are hordeins (one type of prolamins) and glutelins which account for 35-45% of the total protein each and have a great influence on the nutritional and technological proteins of barely (L ásztity, 1996). Hordeins, like other prolamins (wheat gliadins and corn zeins), have high proportions of proline, glutamine and glutamic acid and low lysine content. Based on their molecular weight, hordeins can be divided to A, B, C, D and γ hordein fractions. A hordeins are the smallest polypeptides (average molecular weight 15 kD) and D hordeins are high molecular weight prolamins (> 100 kD). B and γ hordeins (70-80% and < 5% of total hordeins respectively) are rich in

sulfur; while C hordeins (10-20% of total hordeins) are poor in sulfur content. Most B and D hordeins are linked by interchain disulfide bonds and γ hordeins are monomers folded with interchain disulfide bonds as well (Celus, Brijs, & Delcour, 2006). Glutelins are defined as the alkaline-soluble fraction obtained after hordein extraction. The components of glutelins have not been characterized as elaborately as hordeins. About ten bands can be observed in gel electrophorosis with molecular weight of 20-94 kD (Lásztity, 1996). Glutelins are traditionally extracted with acid or alkali, detergents (e.g. sodium dodecyl sulfate, SDS) and chaotropic agents (e.g. urea) with the presence of a reducing agent is more commonly used now (Celus et al., 2006). Both hordeins and glutelins are rich in neutral and hydrophobic amino acids and insoluble in aqueous solution at neutral pH. The amino acid composition of barley whole proteins is shown in Table 1.1. Barley proteins are one of the main components of by-products of barley starch processing industries (e.g. brewing industry); the application of the functional properties of barley proteins and development of value-added products need to be explored.

Wang et al. (2010) have developed an optimized extraction method for hordeins and glutelins by alcohol and alkali extraction from barley grain flour. The study also showed that glutelins have excellent oil-binding capacity and emulsifying stability, while hordeins exhibited great foaming ability. Based on these unique functional properties, barley proteins demonstrated promising potential to be used as wall materials for compound encapsulation. The hydrophobic nature of barley proteins may maintain greater encapsulation

integrity in aqueous environment compared to those fabricated with water-soluble proteins. Additionally, hordeins showed oxygen barrier properties, suggesting they may provide protective to food ingredients again oxidation during storage (Wang, 2011). In recent studies, novel microparticles were developed using barley hordeins and/or glutelins for encapsulation and delivery of lipophilic bioactive compounds (Wang, Tian, & Chen, 2011a). The barley protein microparticles, whether in the form of aqueous suspension or dried powder, could be administrated following the oral route and deliver active ingredient to the digestive system. The particles could steadily release core ingredients through proteolytic degradation of the protein matrix by digestive enzymes and showed effective in protecting fish oil from oxidation. A follow-up study (Wang, Tian, & Chen, 2011b) showed that nanoparticles could be generated by applying the barley protein microparticles in a simulated gastric tract. The nanoparticles could deliver lipophilic compound model β-carotene to GI tract in the study. The capability of forming nanoparticles suggests great potential of barley proteins in oral delivery of nutrients and drugs.

Table 1-1 Amino acid (AA) compositions of barley protein fractions. Adapted from Wang et al. (2010).

AA (%)	Hordeins	Glutelins	Pearled Grain Flour Protein[a]	Pearling Flour Protein[a]
Asx [b]	2.90	5.37	4.34	9.05
Ser	4.69	5.66	5.23	5.02
Glx [c]	32.17	20.21	26.74	15.15
Gly	2.54	8.41	3.54	8.31
His	1.22	2.64	2.32	1.81
Arg	2.67	4.78	3.91	4.14
Thr	2.76	4.71	3.50	4.52
Ala	2.90	6.33	4.52	9.29
Pro	21.24	11.18	16.57	8.09
Cys	1.83	0.72	1.33	0.31
Tyr	3.77	3.28	3.46	2.56
Val	5.25	6.12	5.61	8.03
Met	1.87	1.46	1.56	1.70
Lys	n.d.	3.78	1.81	5.03
Ile	3.51	3.44	3.46	3.05
Leu	5.75	8.04	7.56	7.22
Phe	4.93	3.89	4.54	3.16
Trp	n.d.	n.d.	n.d.	3.56

[a] The outer layer of barley grains (mainly bran and germ) are removed during pearling process. Therefore pearled grain flour is rich in hordeins and glutelins which are located in the endosperm; while pearling flour is rich in albumins and globulins which are mainly found in cytoplasm.
[b] Asx: Asn or Asp.
[c] Glx: Gln or Glu.
n.d.: not detected.

1.5 Safety Concerns of Nanoparticles in Foods

The unique physicochemical properties of nanoparticles drive increasing interest of them in food and biomedical application; these properties also raise safety concerns (Stern & McNeil, 2008). Nanoparticles have greater reactivity with biological tissues due to their large surface area and reactive surface, which

may cause adverse effects at the cellular level or toxic accumulation and damage to the lungs, liver or kidney (Rekha & Sharma, 2011). While the main focus of nanotechnology in food fields is addressed on the particle fabrication and biomedical application, the safety data of nanomaterials are relatively limited.

To date, research data have indicated that the physicochemical properties of nanoparticles have effects on the absorption, distribution, metabolism and excretion in the body (Bouwmeester et al., 2009; Martirosyan, Polet, Bazes, Sergent, & Schneider, 2012). Engineered nanomaterials (ENMs), defined by the American Chemistry Council as intentionally produced materials has a size about typically 1-100 nm (Jaffe, 2009), have been used as food additives, packing agents, nano-delivery systems and biosensors, such as nanoparticles of Ag, TiO_2, SiO_2, ZnO, Au and Pt (Martirosyan et al., 2012). These nanomaterials have been extensively studied for their exposure and deposit hazard risks following inhalation and dermal routes. *In vitro* and *in vivo* studies have shown that nanoparticles may generate reactive oxygen species (ROS) resulting in cytokine production and increased inflammation, which was elaborately reviewed by Nel, Xia, Mädler and Li (2006). It is also suggested that DNA damage, cell membrane disruption and cell death caused by nanoparticles may be triggered by increased oxidative stress and lipid peroxidation (Reeves, Davies, Dodd, & Jha, 2008). It is believed that these toxic responses are driven by the small size, large surface area and chemical composition of the nanoparticles; the toxicity of nanomaterials is more a result of their specific formulation rather than a general property of a type of nanomaterials (Martirosyan et al., 2012). Nel et al.

(2006) summarized the potential toxic effects of nanomaterials in various studies and their possible phathophysiological outcomes (Table 1.2).

In comparison to numerous pulmonary and cutaneous toxicity studies (Stern & McNeil, 2008), data on the toxic effects of nanoparticles on GI tract following the oral route are insufficient. It is believed that although nanoparticles in food can be absorbed by enterocytes and lymphatic tissues, most proportion is rapidly eliminated via feces; hence their potential adverse effects are usually disregarded (Nel et al., 2006). Bouwmeester et al. (2009) summarized available data of *in vitro* toxicity, acute and long term toxicity of oral nanoparticles to date. Acute and subchronic toxicity has been found on ENMs in animal models depending on the size and chemical composition of the particles (Martirosyan et al., 2012). Data on long term effects were scarce; some suggested that nanoparticles may activate pro-inflammatory cytokines in the lungs, liver, heart and brain as well as other adverse effects such as pro-thrombotic effects on the cardiovascular systems (Bouwmeester et al., 2009). It should be noticed that nanoparticles may have greater extent of interaction, absorption and toxicity in GI tract in the case of altered intestinal conditions such as diabetes and other digestive diseases (Hoet, Brüske-Hohlfeld, & Salata, 2004). For example, increased paracellular permeability was reported in the cases of inflammatory bowel disease (IBD) and ulcerative colitis (UC), which may alter nanoparticles absorption and result in disease progression (Martirosyan et al., 2012). It is also reported that insoluble nanoparticles, such as TiO_2, ZnO and SiO_2, may be responsible for Crohn's disease (CD) and UC as well as adjuvant triggers for exacerbation of the

inflammation in these diseases (Lomer et al., 2005). Although the association between ENMs and CD (and other diseases) was confirmed, the results were inconsistent and the role of nanoparticles in the progress of these diseases was under debate (Hoet et al., 2004). It is believed that those with digestive disorders have greater chance of being affected by any potential effects of dietary nanoparticles. It also needs to be noticed that nanoparticles may hold therapeutic potential for the diseases as drug delivery vehicles (Martirosyan et al., 2012).

Currently there is no conclusive evidence indicating toxicity of nanomaterials is a major problem or threat to public health (Nel et al., 2006). However, given the rapid growth of nanotechnology in food and pharmaceutical fields, the safety issues can no longer be neglected. The risk assessment and monitoring of nanomaterials are faced with many challenges. Physicochemical characterization, *in vitro* and *in vivo* studies are the key elements in toxicity evaluation, yet the data obtained apply only to the nanoparticles of interest with specific size, surface characteristics and chemical compositions. The knowledge gathered from one particular type of nanoparticles cannot be generalized to a wider class, which limits the usefulness of the data for risk assessment and prediction (Bouwmeester et al., 2009; Hoet et al., 2004). A set of effective, high throughput and low cost approach for nanotoxicity evaluation and corresponding regulations by legislators are in demand, especially for nanoparticles designed for delivery of drug and food components (Hoet et al., 2004).

Table 1-2 Nanomaterial effects as the basis for pathophysiology and toxicity. Adapted from Nel et al. (2006).

Experimental effects	Possible pathophysiological outcomes
ROS generation [a]	Protein, DNA and membrane injury [a], oxidative stress [b]
Oxidative stress [a]	Phase II enzyme induction, inflammation [b], mitochondrial perturbation [a]
Mitochondrial perturbation [a]	Inner membrane damage [a], permeability transition pore opening [a], energy failure [a], apoptosis [a], apo-necrosis, cytotoxicity
Inflammation [a]	Tissue infiltration with inflammatory cells [b], fibrosis [b], granulomas [b], atherogenesis [b], acute phase protein expression (e.g., C-reactive protein)
Uptake by reticulo-endothelial system [a]	Asymptomatic sequestration and storage in liver [a], spleen, lymph nodes [b], possible organ enlargement and dysfunction
Protein denaturation, degradation [a]	Loss of enzyme activity [a], auto-antigenicity
Nuclear uptake [a]	DNA damage, nucleoprotein clumping [a], autoantigens
Uptake in neuronal tissue [a]	Brain and peripheral nervous system injury
Perturbation of phagocytic function [a], "particle overload", mediator release [a]	Chronic inflammation [b], fibrosis [b], granulomas [b], interference in clearance of infectious agents [b]
Endothelial dysfunction, effects on blood clotting [a]	Atherogenesis [a], thrombosis [a], stroke, myocardial infarction
Generation of neoantigens, breakdown in immune tolerance	Autoimmunity, adjuvant effects
Altered cell cycle regulation	Proliferation, cell cycle arrest, senescence
DNA damage	Mutagenesis, metaplasia, carcinogenesis

[a] Effects supported by limited experimental evidence;
[b] Effects supported by limited clinical evidence.

1.6 Hypothesis and Objectives

Previous studies showed that barley proteins could be used as wall materials to prepare microparticles encapsulated with fish oil, providing protective mechanism against oxidation and prolonging storage time (Wang et al., 2011a). In addition, nanoparticles could be generated from enzymatic degradation of the barley protein microparticles; the active compound encapsulated within the particles was steadily released (Wang et al., 2011b). Data from these studies lead to the hypothesis of the current research: barley protein nanoparticles (BNPs) as potential vehicles for the oral delivery of bioactive compounds are compatible and generally safe to biological tissues, have effective intestinal uptake and may enhance the uptake of bioactive compounds.

The overall objective of the present study was to investigate the cytotoxicity and the intestinal uptake of BNPs using *in vitro* and *ex vivo* models, aiming to address the research hypothesis above. More specifically, the objectives of this research included:

(i) To test cytotoxicity of BNPs in Caco-2 cells based on the activity of cellular enzymes;

(ii) To confirm BNPs are capable of transporting across cell membrane and being taken up by Caco-2 cells, to characterize the uptake properties by testing the influence of time, particle concentration and temperature on their cellular uptake efficiency;

(iii) To evaluate the uptake of BNPs in Caco-2 cells using β-carotene as a model compound;

(iv) To assess the permeation ability of BNPs using rat jejunum tissue.

The present study was designed to provide knowledge on the biological properties of BNPs in cell and tissue models. Insights into the cellular uptake profile will allow a better understanding of the potential application of BNPs in nutrient delivery. It is an important initial step in bioavailability study that may facilitate the design of alternative particle formulation that has greater uptake and bioavailability and the subsequent *in vivo* study. Micro- and nanoparticles fabricated from barley proteins provide a new approach to develop novel functional foods as well as value-added products of the abundant barley grain resources.

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Chapter 2 Cellular Uptake of Barley Protein Nanoparticles as Delivery Vehicles for Bioactive Compounds

2. 1 Introduction

The field of nanoparticle delivery systems for bioactive compounds has been particularly expanding in the last decade (Desai & Park, 2005). Bioactive compounds from foods (e.g. vitamins, polyunsaturated fatty acids, bioactive peptides) are known for their physiological benefits and potential in the prevention of chronic diseases, yet many of them show limited bioavailability after oral administration due to their poor solubility or permeability and low absorption efficiency in the body. Encapsulation of these active ingredients in nanoparticle carriers is one of the most promising strategies to improve their bioavailability due to the unique physicochemical properties of nanomaterials. The mechanisms by which this occurs involve increasing solubility of hydrophobic ingredients (Horn & Rieger, 2001), prolonging retention time against intestinal clearance mechanism (Brannon-Peppas, 1995), overcoming the mucosal barrier (bioadhesive properties) (Ponchel & Irache, 1998) and direct uptake of nanoparticles (Acosta, 2009). Although numerous synthetic polymeric nanoparticles have been thoroughly developed and successfully used in drug delivery, their application in food industry is restricted to the generally regarded as safe (GRAS) standard (Acosta, 2009). To meet this challenge, food

biopolymers such as lipids, proteins and polysaccharides have been used to prepare nanocarrier delivery systems in recent years.

Among these GRAS materials, food proteins are of particular interest due to their high nutritional values and unique functionalities. They have excellent gelation, foaming and emulsification properties, which provide opportunities to use proteins as wall materials to encapsulate both hydrophilic and hydrophobic bioactive compounds (Chen, Remondetto, & Subirade, 2006). Additionally, the defined primary structure of proteins suggests protein-based nanoparticles have various modification possibilities to form complexes with lipids, polysaccharides and nutrient compounds via covalent or non-specific interactions (Chen et al., 2006; Jahanshahi & Babaei, 2008). Moreover, technologies have been developed to modify particle size and surface properties to achieve time-specific, site-targeting delivery and enhanced absorption (Liu, Wu, Selomulya, & Chen, 2013; Shakweh, Besnard, Nicolas, & Fattal, 2005; Win & Feng, 2005). Various proteins have been used in nanoencapsulation. Gelatin and albumin are studied in pharmaceutical field as they are biodegradable, non-toxic and easy to be chemically modified for drug attachment (Jahanshahi & Babaei, 2008; Jahanshahi, Sanati, Hajizadeh, & Babaei, 2008). Plant prolamines, gliadin and zein from wheat and corn respectively, are also under elaborate research due to their sustained release profile and bioadhesive ability to mucus layer (Duclairoir et al., 1999; Liu, Sun, Wang, Zhang, & Wang, 2005). In these studies, however, organic solvents (e.g. glutaraldehyde, acetone, dichloromethane) were used for particle fabrication as cross-linking reagents or surfactants, which may arouse

safety risks in food applications. Investigations on the underlying mechanisms involved in nanoparticle uptake and translocations are also limited.

Novel microparticles based on barley proteins have been recently developed in our group by an emulsification-homogenization process (Wang, Tian, & Chen, 2011a). The nanoparticle fabrication is organic solvent and surfactant free, utilizing only barley proteins, oil and active compound of interest. The particles showed excellent oil loading efficiency and sustained content release in simulated gastric and intestinal juice. They can form nanoparticles from degradation of the bulk protein matrix during digestion, the nanoparticles can deliver bioactive compounds to small intestine where they can be released steadily (Wang, Tian, & Chen, 2011b). However, the uptake properties and associated mechanisms of the nanoparticles remain unknown. In this study, the cytotoxicity of BNPs was tested by measuring the activity of cellular enzymes with the presence of the nanoparticles. The cellular uptake capability was confirmed and the effects of temperature, time and particle concentration on the uptake process were investigated. Finally, potential mechanisms involved in cellular uptake were discussed based on the uptake profile.

2. 2 Materials and Methods

2.2.1 Materials

Barley grain (Falcon) was provided by Dr. James Helm, Alberta Agricultural and Rural Development, Lacombe, Alberta. Human colorectal adenocarcinoma cell line Caco-2 was purchased from American Type Culture Collection (ATCC,

Manassas, VA). Cell culture reagents including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), non-essential amino acids (NEAA), HEPES solution, trypsin-EDTA, Hank's balanced salt solution (HBSS), Dulbecco's phosphate-buffered saline (DPBS), Alexa Fluor dyes, DAPI and mounting medium were from Life Technologies (Burlington, ON). Cell culture flasks and multi-well plates were purchased from Corning (Tewksbury, MA). Thiazolyl blue tetrazolium bromide (MTT), pepsin, Nile red and β-carotene were from Sigma-Aldrich (St. Louis, MO). All other reagents and chemicals were purchased from Fisher Scientific (Waltham, MA).

2.2.2 Preparation and characterization of nanoparticles

Barley protein microparticles (BMPs) were prepared with barley hordeins and glutelins as the coating material and canola oil as the lipid phase (Wang et al., 2011a). The oil phase was added to aqueous protein suspension using a homogenizer at 30,000 rpm (PowerGen 1000, Fisher Scientific Inc., CA). The pre-mixed emulsion was then passed through a high pressure homogenizer NanoDeBee (BEE International, Inc., MA). Concentration of BMPs was determined by their dry weight in the solution and was adjusted to 40 mg/mL with deionized water for further test. BMPs coulkd stay stable at 4 ℃ for at least 6 months. Nanoparticles (BNPs) were freshly prepared from BMPs before experiments. Briefly, BMP dispersion was diluted to 10 mg/mL with acidic phosphate buffer (pH 2.0) and incubated with 1 mg/mL pepsin solution (40 mg/mL pepsin from porcine gastric mucosa dissolved in 0.1 M HCl) at 37 ℃ for

15 min. The reaction was terminated by boiling the mixture for 5 min in water-bath. The BNP dispersion could be stored at 4 ℃ for 24 h. For cellular uptake study, Nile red-labeled BNPs (NR-NPs) were prepared by using canola oil dissolved with 0.025% (w/v) Nile red following the same procedure above. The average size and zeta potential of BNPs were measured by dynamic light scattering using a zetasizer (Nano-ZS, Malvern Instruments Ltd., UK) at room temperature. The refractive index of the particle and dispersion medium was set at 1.45 and 1.33 respectively. The morphology of nanoparticles was examined by transmission electron microscopy (TEM). Briefly, one drop of BNP dispersion was placed onto a 300 mesh copper grid coated with carbon and let stand for 5 min. Then the excess water was removed by gently tapping filter paper at the edge of the grid without disturbing the surface. The grid was then stained with phosphotungstate (PTA) for 30 sec and examined under Philips-FEI transmission electron microscope (Morgagni 268, FEI Co., Hillsboro, OR) operated at 80 kV.

2.2.3 Cell culture

Human colon carcinoma cell line Caco-2 was cultured in T-75 flasks at 37 ℃ in a humidified atmosphere of 95% air and 5% CO_2. High glucose (4500 mg/L) DMEM with sodium pyruvate (110 mg/L) and L-glutamin was used with supplementation of 20% FBS (v/v), 1% NEAA and 25 mM HEPES. The medium was changed every other day and the cell morphology was monitored. Upon reaching 80% confluence, cells were removed with 0.25% trypsin in 1 mM

EDTA solution, gently centrifuged, resuspended and transferred to new flasks. Cells between passage 30 and 50 were used for experiments.

2.2.4 Cytotoxicity assay

The cytotoxicity of BNPs was examined by MTT assay, a colorimetric assay measured the activity of dehydrogenase in the cells and presented cytotoxicity as cell viability with the presence of the nanoparticles. Caco-2 cells were harvested and seeded onto 96-well plates at a density of 8000 cells per well in 100 μl culture medium. After allowing the cells to grow for 24 h for attachment, BNP dispersion was added to each well at final concentrations of 0.002, 0.02, 0.2 mg/mL and incubated with cell for 2-24 h at 37 ℃. Upon removal of BNP dispersion, 10 μl of MTT solvent (5 mg/mL in PBS) was added to each well and incubated with cells for a further 4 h at 37 ℃. The medium was then removed from each well without disturbing the cells and 100 μl of DMSO was added. The plates were examined at 570 nm with a reference wavelength at 620 nm using a microplate reader (SpectraMax, Molecular Devices, USA). Cell viability was expressed by the percentage of living cells in the test wells as a ratio to living cells in the control wells.

2.2.5 Confocal laser scanning microscopy

The uptake of the nanoparticles was studied by examining the fluorescence-labeled nanoparticles NR-NPs in Caco-2 cells using confocal laser scanning microscopy (CLSM). Cells were harvested and seeded onto glass bottom

microwell dishes (P35G-1.5-14-C, MatTek Corp., USA) at the density of 1×10^5 cells per dish and cultured for 5-7 days until reached a confluent monolayer. On the day of experiment, the medium was replaced with HBSS (supplemented with 25 mM HEPES, without phenol red) and allowed to equilibrate at 37 ℃ for 30 min. Then HBSS was replaced with NR-NP dispersion (0.02 and 0.2 mg/mL in HBSS) and incubated with the cells for 1-6 h at 37 ℃ and 4 ℃. At each endpoint, the cells were gently rinsed 3 times and fixed with 4% paraformaldehyde for 15 min at 37℃. Upon washing the cells, WGA-Alexa Flour 488 conjugate labeling solution (5 μg/mL in HBSS) was applied to cover the cells for membrane staining. After incubating for 15 min in dark at room temperature, the labeling solution was removed and cells were washed. DAPI solution (0.1 μg/mL in PBS) was then added and incubated with cells for 10 min in dark at room temperature for nucleus staining, followed by washing and mounting with Prolong Gold Antifade Reagent and sealed with coverslips. The cells were examined using CLSM 510 Meta (Carl Zeiss, Jena, Germany) equipped with a diode, an argon laser and a helium/neon laser providing excitation at 405 nm, 488 nm and 543 nm respectively. An oil immersion objective (40×) was used. Images were processed with ZEN 2009LE software (Carl Zeiss MicroImgaing GmbH, Germany).

2.2.6 Flow cytometry

The nanoparticle uptake by Caco-2 cells was quantified by flow cytometry, which estimated the relative amount of fluorescence labelled BNPs in the cells

by measuring the fluorescence intensity associated with the cells. Cells were seeded onto 6-well plates at the density of 1×10^5 cells per well and cultured for 5-7 days until reached a confluent monolayer. The medium was then replace with NR-NP dispersion (0.02 and 0.2 mg/mL in HBSS) for 1-6 h at 37 ℃ and 4 ℃. At each endpoint, cells were trypsinized, washed and resuspended in DPBS. The fluorescence associated with cells was measured with a flow cytometer (B.D. Biosciences FACSort, CA) using FL2 detector at the wavelength of 580 nm. Nile red fluorescence was plotted versus the number of cells and mean fluorescence intensity (MFI) was evaluated. 10000 cells were analyzed for each sample and repeated in triplicates.

2.2.7 Statistical analysis

BNP and NR-NP dispersions were prepared in three batches from corresponding microparticles. Size and zeta-potential measurements for each type of nanoparticles were performed in duplicates for each batch; flow cytometry analysis of NR-NPs was conducted in triplicates for each group. Data were presented as mean ± SEM. Student's t-test was used for comparisons between two samples and one-way ANOVA for more than two samples. When a significant effect ($P < 0.05$) was detected, comparisons among multiple means were conducted by Student-Newman-Keuls (SNK) test. Statistical analyses were performed using SAS 9.0 for Windows (SAS Institute Inc., Cary, NC).

2. 3 Results and Discussions

2.3.1 Preparation and characterization of nanoparticles

Barley protein nanoparticles were prepared by a two-step process based on previous study with slight modifications (Wang et al., 2011b). First, BMPs were obtained by pre-emulsification and high pressure homogenization. Then BNPs were generated by digesting BMPs with pepsin for 15 min. BNPs had spherical shape with a thick protein wall and oil encapsulated in the core (Wang et al., 2011b) with size of 50-500 nm in diameter (Figure 2.1). Measurements by Zetasizer showed BNPs had negatively charged surface with the zeta potential of -30.7 ± 0.7 mV and an average size of 351.2 ± 3.6 nm. The average polydispersity index (PDI, an index that describes the heterogeneity of a distribution) of BNP dispersion was 0.329, which was slightly higher than the acceptable homogeneity level of 0.3 suggested by (Dragicevic-Curic, Gräfe, Gitter, Winter, & Fahr, 2010). Yet this heterogeneous size distribution was in correspondence with the particle size variety observed in TEM micrographs (Figure 2.1). Particle size is one of the most important determinants of particles stability, cellular uptake, tissue permeability and controlled release properties of encapsulated ingredients (Desai, Labhasetwar, Walter, Levy, & Amidon, 1997; Lamprecht, Schäfer, & Lehr, 2001; Win & Feng, 2005). Particle size can be easily controlled when particles are prepared with synthetic polymers (e.g. PLGA) as it is mainly determined by solubility of the organic solvent used in preparation and the cosolvent constituents (Peltonen, Koistinen, Karjalainen, Häkkinen, & Hirvonen, 2002; Roger, Lagarce, Garcion, & Benoit, 2009; Song et al., 2006). BNP formation, on the other hand, is resulting from unrestricted

proteolytic reaction of the protein matrix of BMPs; the size of liberated nanoparticles could not be precisely controlled. Yet consistent results of average size and size distribution were obtained in repeated experiments. It was observed that BNPs were generated from BMPs very quickly (10-15 min) and remained stable after 2 h incubation in simulated gastric juice (Wang et al., 2011b).

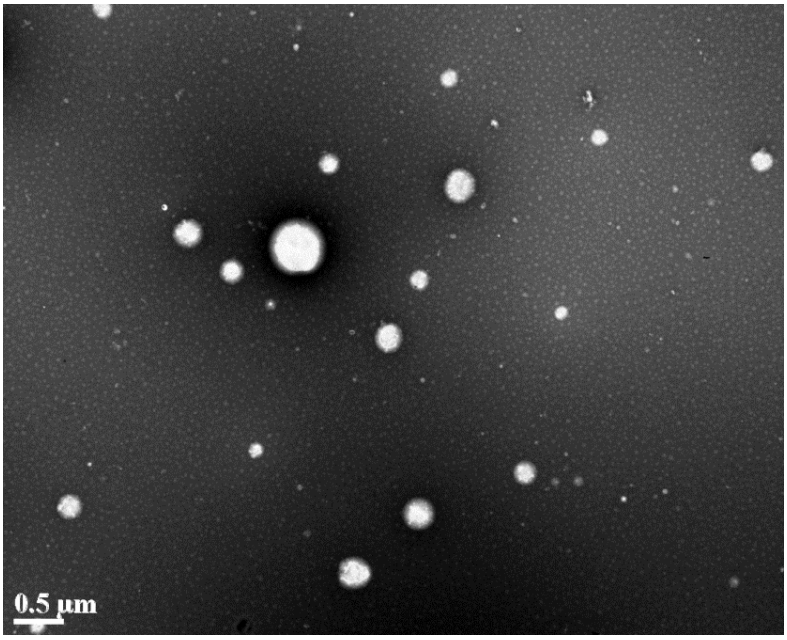

Figure 2-1 TEM micrographs of barley protein nanoparticles.

2.3.2 Cytotoxicity of nanoparticles

Although numerous nanoparticles have been widely studied for pharmaceutical and food application and some nano-food related products are already available in the market, very little is known about the safety and potential risks of these novel materials (Bouwmeester et al., 2009). Studies have shown that nanoparticles may induce oxidative stress and inflammation depending on the size and surface properties of particles (Nel, Xia, Mädler, & Li,

2006). In the present study, *in vitro* cytotoxicity of BNPs was examined by MTT assay on Caco-2 cells and presented as changes of cell viability when exposed to BNPs (Figure 2.2). Compared to cells without BNPs exposure (control group), more than 80% cells were viable throughout 24 h exposure at a BNP concentration range of 0.002-0.2 mg/mL. The fluctuation of cell viability might be the result of dynamic adjustment process of cells in response to the changes of environmental stimuli. The toxic effects of BNPs on cells were dependent on particle concentrations. As there is lack of a reference nanoparticle system to use as a standard for *in vitro* cytotoxicity testing, gold nanoparticles are suggested as a reference system for low cytotoxicity. Gold nanoparticles demonstrated around 15% reduction in cell viability at 0.2 mg/mL and elicit toxic effects at high concentrations (Lewinski, Colvin, & Drezek, 2008). As a preliminary estimation of the safety of BNPs, results from the present study are similar to those of gold nanoparticle tests. It is reasonable to conclude that BNPs have low cytotoxicity on Caco-2 cells at a concentration lower than 0.2 mg/mL.

The MTT assay used in this study measures the viability of cells based on the changes in metabolic activity of cells, more specifically mitochondrial dehydrogenase activity (Lewinski et al., 2008). MTT assay is particularly suitable for nanoparticles cytotoxicity investigation because one of the main toxic effects of nanoparticles is the perturbation of mitochondria due to ROS generation, such as mitochondrial membrane damage (Nel et al., 2006). The potential interactions between nanoparticles and mitochondria may explain the fluctuation of Caco-2 cell viability during the 24 h incubation in this study

(Figure 2.2). Alternative cytotoxicity study methods can be performed in future work to explore different effects BNPs may have on biological tissues. For example, cell membrane integrity can be tested using Trypan blue-light microscopy examination and lactate dehydrogenase (LDH) assay; oxidative stress in cells can be detected by glutathione (GSH) assay and thiobarbituric acid (TBA) assay (Lewinski et al., 2008). More comprehensive understanding on the safety issue is needed through further studies on acute or long-term toxic effects of BNPs using animal models.

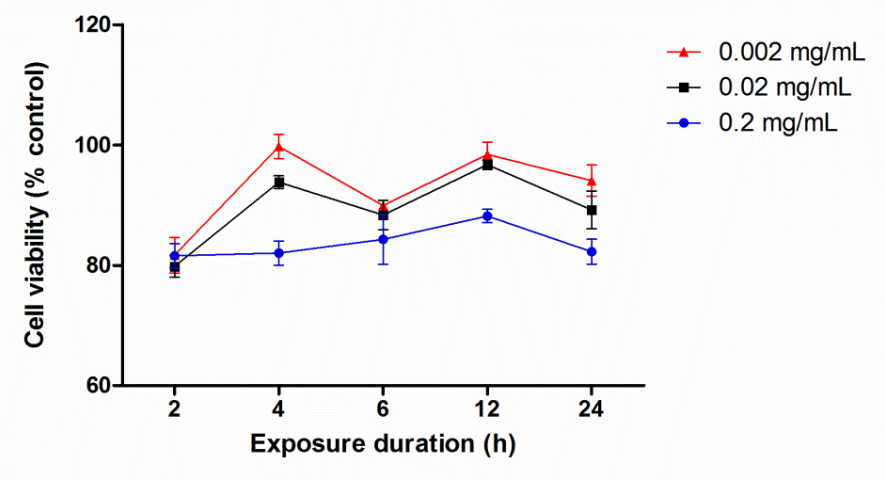

Figure 2-2 Cell viability of Caco-2 cells after incubation with barley protein nanoparticles. Viability was presented by a percentage of living cells as a ratio of the living cells in the control group (without nanoparticles). Data are presented as mean ± SEM, n = 6.

2.3.3 Cellular internalization and localization of nanoparticles

Efficient cellular uptake and sufficient retention time within cells is essential to the development of effective delivery systems (Win & Feng, 2005). Caco-2

cells, a well-recognized model for predicting oral absorption and metabolism of compounds (Biganzoli, Cavenaghi, Rossi, Brunati, & Nolli, 1999), was used to investigate the cellular uptake profile of BNPs and associated mechanisms. In order to visualize the uptake of BNP into cells, canola oil dissolved with fluorescent probe Nile red was used to prepare fluorescent nanoparticles NR-NPs. The cellular uptake of NR-NPs was examined by confocal microscopy and the corresponding quantitative data were obtained by flow cytometry.

Confocal microscopic image with three dimensional projections of Caco-2 cells after 6 h incubation with NR-NPs with a final concentration of 0.2 mg/mL at 37 ℃ was shown in Figure 2.3. Most NR-NPs were present in cytoplasm and accumulated around the nuclei, demonstrating their direct internalization by cells. Interestingly, some NR-NPs were also found within nuclei. Localization of nanoparticles within nuclei is seldom observed. It is well established that direct uptake of BNPs may facilitate the absorption of encapsulated active ingredients (Galindo-Rodríguez, Allémann, Fessi, & Doelker, 2005). Meanwhile the capability of nucleus internalization suggested their potential in the delivery of gene or oligonucleotides. Potential effects of BNPs on genetic functions of cells also need further investigation.

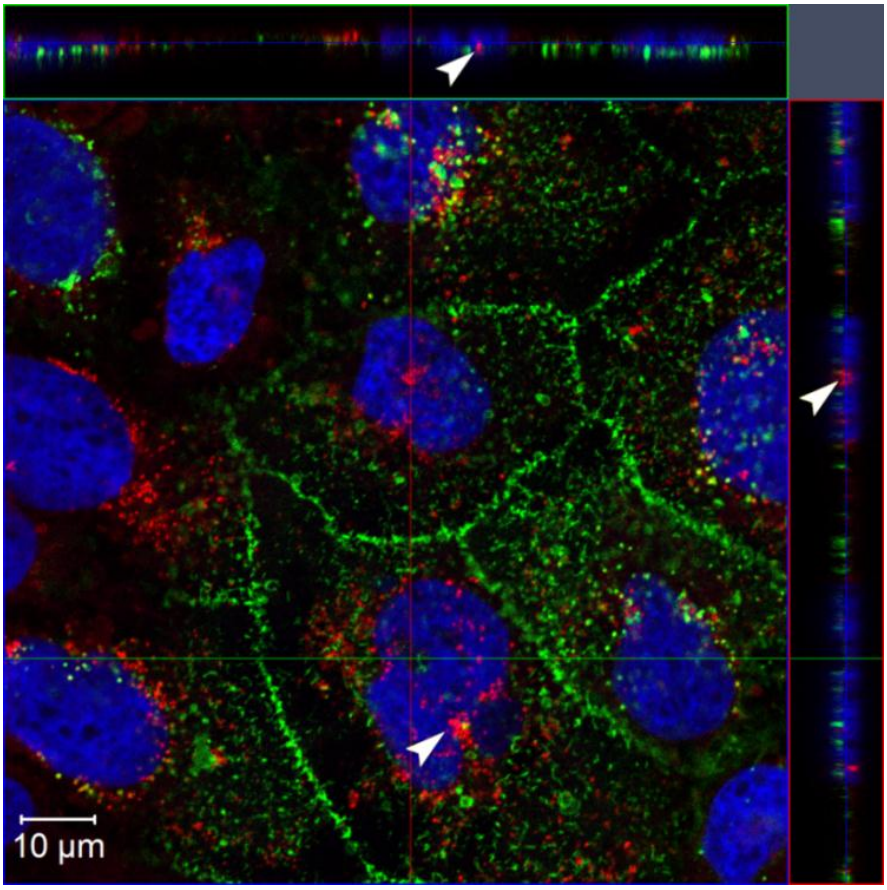

Figure 2-3 Confocal microscopic image of Caco-2 cells after 6 h incubation with Nile red labeled barley protein nanoparticles (0.2 mg/mL) at 37 ℃, presenting three-dimensional analysis of the optical xy-section (center square) with xz- and yz- projections (side panels). Image shows distinct uptake of nanoparticles by the cells; arrows heads indicate the presence of the nanoparticles within nuclei. Red: Nile red-labeled barley protein nanoparticles; green: cell membrane; blue: nuclei.

2.3.4 Uptake profile and mechanisms

Caco-2 cells were incubated with NR-NPs at different particle concentrations, temperatures for various incubation times to investigate effects

of these factors on the particle uptake. It is reported that free Nile red cannot be taken up by cells (Roger et al., 2009), thus the intracellular fluorescence detected by CLSM and flow cytometry was only due to the internalized NR-NPs rather than potential Nile red released from particles. Figures 2.4a-c showed that NR-NPs uptake degree increased with the incubation time within 6 h when concentration and temperature were fixed (0.2 mg/mL, 37 ℃). Apart from time, NR-NP uptake was also affected by particle concentration and temperature. Cells incubated with NR-NPs at 0.2 mg/mL distinctly exhibited more intracellular fluorescence signal (Figures 2.4c, d) and increased the uptake level by 18% and 70% compared to those at 0.1 mg/mL and 0.02 mg/mL respectively, under the same time and temperature condition (Figure 2.5b). When incubation was performed at 4 ℃, particle uptake decreased by 51.7% compared to that at 37 ℃ with equivalent particle concentration and time (Figure 2.5c). Hence, lesser amount of particles was observed in cells (Figures 2.4c, e). These findings indicated that the cellular uptake of BNPs was dependent upon particle concentration, incubation time and temperature.

Understanding the cellular uptake profile of nanoparticles helps interpret the associated mechanisms and design delivery carriers with the optimal uptake properties. Nanoparticles administrated by oral route can transport across intestinal epithelial cells by (i) paracellular pathway via tight junctions, (ii) transcellular pathway by enterocytes including passive diffusion and active transcytosis and (iii) lymphatic transcytosis by microfold cells (M cells) (Des Rieux et al., 2006). Paracellular transport requires particles to have very small

size (< 50 nm) due to the tiny space at tight junctions between epithelial cells (pore diameter < 1 nm). Surface modification with some polymers (e.g. chitosan, polyacrylate or starch) can enhance paracellular transport of nanoparticles by reversely opening tight junctions (Jung et al., 2000). This enhancement mechanism, however, is limited to cationic polymers while negatively charged particles are not capable of disrupting tight junctions (Lin et al., 2007; Roger et al., 2009). Therefore, it was hypothesized that the uptake BNPs was not via paracellular route. This was confirmed by monitoring the transepithelial electrical resistance (TEER) of Caco-2 cell monolayer. No significant TEER change was observed during the transport study (data not shown), given that the opening of tight junctions could lead to decrease in TEER (Yamashita et al., 2000).

In transcellular transport, particulate materials can be internalized across the cell membrane barrier by passive diffusion or active transcytosis followed by travelling through the interior via membranous vesicles and excreted to exterior (Tuma & Hubbard, 2003). Considering the particle size, BNPs are most probably to be internalized by cells via transcytosis, by which cells take up macromolecules and particles (Conner & Schmid, 2003). Moreover, transcytosis is a temperature- and time-dependent process (Jung et al., 2000), which is compatible with the pronounced increase observed in NR-NP uptake at higher temperature and longer incubation time. Therefore, it was hypothesized that the uptake of BNPs was a transcytosis process. More explicitly, the nanoparticles firstly entered cells from the apical side by endocytosis, i.e. a process where the

particles were carried into the cell by membrane-bound vesicles derived from the plasma membrane, then crossed the cytoplasm with the vesicles and finally exited cells by exocytosis (a reverse process of endocytosis).

There are different mechanisms may be involved in endocytosis: phagocytosis (uptake of large particulates), macropinocytosis (uptake of large fluid and solutes), clatherin-mediated endocytosis, caveolea-mediated endocytosis and mediator-independent (or adsorptive) endocytosis (Conner & Schmid, 2003). Phagocytosis is primarily used for clearing large pathogens such as bacteria, yeast or large debris such as dead cells (Aderem & Underhill, 1999). Fluid-containing particles can be internalized by pinocytosis. While caveolea-mediated endocytosis is generally used for particulates smaller than 60 nm, clathrin-mediated endocytosis and mediator-independent endocytosis are used for particles around 90-150 nm; macropinocytosis is typically exploited for the uptake of larger particulate materials but samller than 5 μm (Des Rieux et al., 2006). In order to understand by which mechanism BNPs were taken up, transport efficiency experiments can be performed in the future with the presence of different inhibitors (e.g. Filipin for caveolae disruption and chlorpromazine for clathrin disassembly) (Roger et al., 2009). It is reported that nanoparticles about 100-200 nm can be internalized by receptor-mediated endocytosis, while larger particles are taken up by adsorptive endocytosis (Couvreur & Puisieux, 1993). Considering that BNPs are generated in heterogeneous size, it is deduced that multiple endocytosis mechanisms may be

employed during cellular uptake. Lymphatic uptake was not studied in current study; it might be used by BNP uptake and will be investigated in future work.

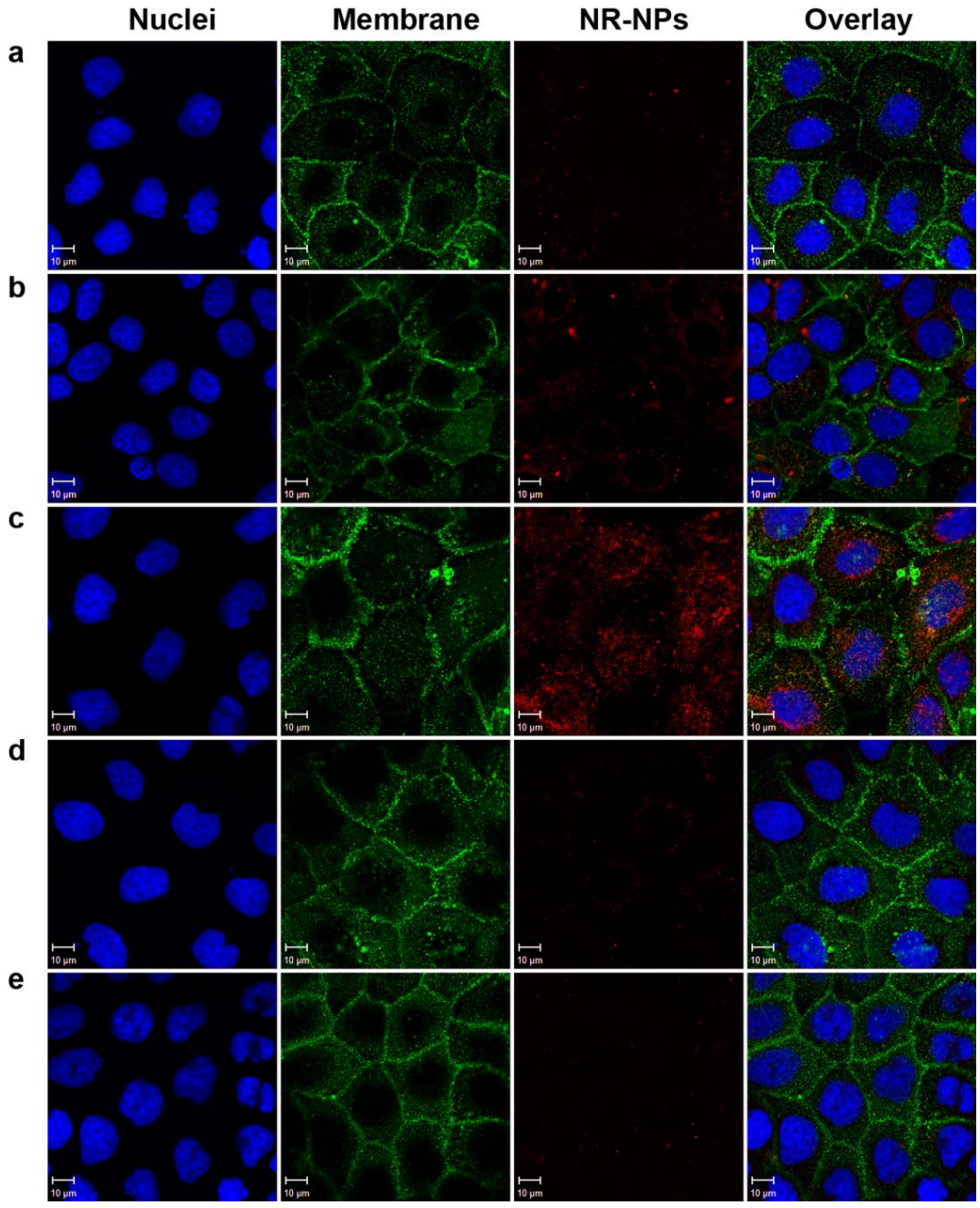

Figure 2-4 Confocal microscopic images of Caco-2 cells incubated with Nile red labeled barley protein nanoparticles for different incubation times, at different concentrations and temperatures. (a), 0.5 h, 0.2 mg/mL, 37 ℃; (b), 2 h, 0.2

mg/mL, 37 ℃; (c), 6 h, 0.2 mg/mL, 37 ℃; (d), 6 h, 0.02 mg/mL, 37 ℃; (e), 6 h, 0.2 mg/mL, 4 ℃. Red: NR-NPs, green: cell membrane, blue: nucleus.

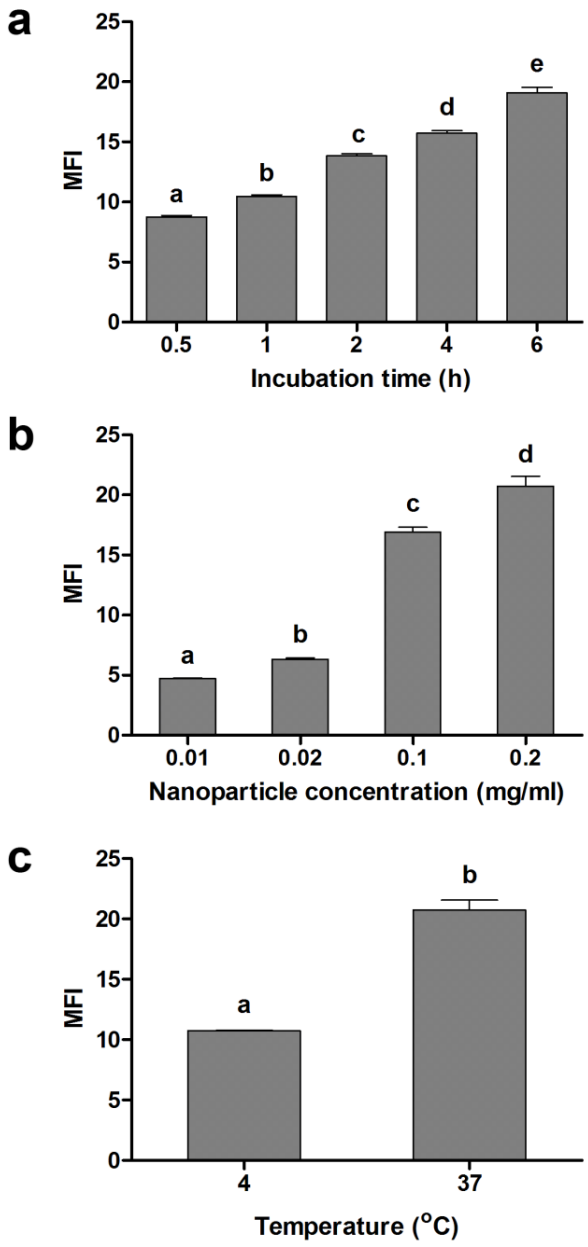

Figure 2-5 Flow cytometry analysis of cellular uptake of Nile red labeled barley protein nanoparticles by Caco-2 cells, showing the effect of incubation time (a) (0.2 mg/mL, 37 ℃), nanoparticle concentration (b) (6 h, 37 ℃) and temperature

(c) (0.2 mg/mL, 6 h). Different letters indicate significant difference ($P < 0.05$). MFI: mean fluorescence intensity. Data were presented as mean ± SEM, n = 3.

2. 4 Conclusions

Biodegradable nanoparticles were prepared with barley proteins and canola oil using high pressure homogenization and enzymatic degradation techniques. Lipophilic bioactive compounds were dissolved in the oil during preparation and encapsulated within the nanoparticle matrix. BNPs had an average size of 350 nm and negatively charged surface (-30 mV). The particles showed low cytotoxicity on Caco-2 cells; more than 80% cells remained viable with exposure to BNPs for up to 24 h when particle concentration was up to 0.2 mg/mL. By confocal microscopy, it was established that BNPs were capable of travelling across cell membrane and entering Caco-2 cells. Quantitative study by flow cytometry demonstrated that the uptake process was particle concentration-, incubation time- and temperature-dependent. Paracellular transport was not observed in BNP uptake; transcytosis was speculated to be the pathway by which BNPs were taken up by cells. Multiple endocytosis mechanisms may be involved in the internalization of BNPs, such as adsorption endocytosis and receptor-mediated endocytosis. The characterization of cellular uptake properties of BNPs implied their capability of interactions with intestinal cells. Knowledge of the BNPs cellular uptake mechanisms may facilitate in achieving ideal intestinal absorption. Data from the present study suggest the potential of using BNPs as delivery vehicles for oral delivery of lipophilic bioactive compounds. Novel functional foods may be developed by incorporating barley protein micro-

or nanoparticles into the food matrices, aiming to enhance the oral bioavailability and health promoting benefits of bioactive compounds.

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Chapter 3 Transport of Barley Protein Nanoparticles as Nutrient Delivery Vehicles in Human Cell Line and Rat Tissue

3. 1 Introduction

Functional foods containing bioactive ingredients are experiencing a growing consumption due to their health promoting and potential disease preventing benefits. The effectiveness of functional foods relies on bioavailability, the amount that is available for utilization at target sites, of the active ingredients in human body (Chen, Remondetto, & Subirade, 2006). However, some bioactive compounds have shown limited bioavailability following oral administration due to their instability during food processing and/or digestion, low solubility and/or permeability in the GI tract and fast elimination prior to absorption (Ponchel et al., 1997). Biodegradable nanoparticles have been developed as delivery systems for drugs and nutrients in pharmaceutical and food areas (Acosta, 2009). Owing to their unique physicochemical and biological properties, nanoparticles have great potential in improving oral bioavailability of bioactive compounds by improving their solubility and permeability (Luo, Chen, Ren, Zhao, & Qin, 2006; Peppas & Huang, 2004; Yu & Huang, 2013), prolonging the retention time and enhancing uptake efficiency (Jung et al., 2000; Win & Feng, 2005). It is a simple and effective approach to develop novel functional foods by incorporating bioactive compound-encapsulated nanoparticles into food matrices (Chen et al., 2006).

Once administrated following the oral route, nanoparticles need to overcome the physical and chemical barriers in GI tract to be absorbed in the small intestine. The thick mucus gel layer and underlying epithelial cells in the intestinal wall function as a barrier for the penetration and translocation of nanoparticles to blood stream (Crater & Carrier, 2010; Cone, 2009). Mucus can prevent the adsorption of nanoparticles to the intestinal wall, or stick to particles and trap them inside the porous network and prevent them from penetrating to epithelial cells. Multiple low-affinity hydrophobic interactions may be involved in the adhesion and entrapment of particles to mucus layer (Cone, 2009). In addition, because of its protective mechanism against pathogens and foreign matters, mucus is secreted continuously which may result in rapid clearance of particles prior to absorption (Ensign, Cone, & Hanes, 2012; Cone, 2009). Different strategies can be used to improve nanoparticle adhesion and translocation across the mucus-epithelium barrier, such as modifying particle size and surface charge (Shakweh, Besnard, Nicolas, & Fattal, 2005), coating particles with mucoadhesive polymers (Bravo-Osuna, Vauthier, Farabollini, Palmieri, & Ponchel, 2007; Kawashima, Yamamoto, Takeuchi, & Kuno, 2000), and enhancing the interaction with immune system and cell targeting (Ensign et al., 2012).

Nanoparticles investigated in previous studies are mostly aimed at drug delivery in pharmaceutical field. Synthetic polymers and organic solvents are usually required in fabrication as cross-linkers or stabilizers (Sanguansri & Augustin, 2006), making the nanoparticles not suitable for nutrient delivery in

food area because of safety concerns. In our previous study, barley protein nanoparticles (BNPs), prepared with food grade materials only, were developed and investigated for their potential as delivery systems for hydrophobic bioactive compounds. The safety and uptake properties of BNPs in human intestinal cell line Caco-2 were characterized in the last chapter; their biological properties in the whole intestinal epithelium remained unknown. Therefore, this study was designed to investigate the uptake properties of BNPs in biological tissues. Nanoparticles were firstly digested with simulated gastric and intestinal juice to mimic the digestion process they encounter in the GI tract. Digested nanoparticles encapsulated with β-carotene (model hydrophobic bioactive compound) were investigated on Caco-2 cell monolayers for their ability of enhancing the uptake of β-carotene. Finally, mucus adhesion and tissue permeation properties were studied using fluorescence-labeled BNPs on rat jejunum tissues.

3. 2 Materials and Methods

3.2.1 Materials

Barley grain (Falcon) was provided by Dr. James Helm, Alberta Agricultural and Rural Development, Lacombe, Alberta. Human colorectal adenocarcinoma cell line Caco-2 was purchased from ATCC (Manassas, VA). Cell culture reagents including DMEM, FBS, NEAA, HEPES solution, penicillin-streptomycin solution, trypsin-EDTA, HBSS and Prolong® Gold Anitfade reagent were from Life Technologies (Burlington, ON). Cell culture flasks and multiwall plates

with Transwell® inserts were purchased from Corning (Tewksbury, MA). Pepsin, pancreatin, bile extract and β-carotene were from Sigma-Aldrich (St. Louis, MO). All other reagents and chemicals were purchased from Fisher Scientific (Waltham, MA).

3.2.2 Preparation and *in vitro* digestion of nanoparticles

Three types of nanoparticles were prepared in this study. Original barley protein nanoparticles (BNPs) without bioactive compound were used for TEM imaging on Caco-2 cells; β-carotene encapsulated BNPs (BC-NPs) were used to assess transport efficiency; Nile red labeled nanoparticles (NR-NPs) were used for fluorescence imaging on tissues. The same preparation procedure was used as in section 2.2.2. β-Carotene and Nile red encapsulation was done by dissolving 0.14% (w/v) β-carotene and 0.025% (w/v) Nile red in canola oil and following the same steps as described in Chapter 2.

Nanoparticles were digested for transport study using an *in vitro* digestion model (Garrett, Failla, & Sarama, 1999). Briefly, BC-NP or NR-NP dispersion was equilibrated at room temperature for 1 h before adjusting the pH to 2.0 by drop-wise adding 1 M HCl. Pepsin solution described above was added at a final concentration of 1 mg/mL and the mixture was incubated on a shaker (95 rpm) at 37 ℃ for 1 h. After gastric digestion, 0.9 M sodium bicarbonate solution was added to the sample to raise the pH to 5.3. Next, a mixture of pancreatin and bile extract (pancreatin from porcine pancreas and bile extract porcine dissolved in 0.1 M sodium bicarbonate solution) was added to final concentrations of 0.4 and

2.4 mg/mL respectively. The pH was then increased to 7.4 by the addition of 1 M sodium hydroxide followed by incubating the mixture on a shaker (95 rpm) at 37 ℃ for a further 2.5 h. The reaction was terminated by boiling for 5 min in water-bath. Sample vials were blanketed with nitrogen at all incubation stages to avoid oxidation.

3.2.3 Preparation of Caco-2 cell monolayers

Caco-2 cells were harvested upon 80% confluence and seeded onto 12-well polyester clear Transwell® inserts (pore size 3 μm) at 2.6×10^5 cells/cm^2 (Hubatsch, Ragnarsson, & Artursson, 2007). High glucose (4500 mg/L) DMEM with sodium pyruvate (110 mg/L) and L-glutamin was used with supplementation of 10% FBS (v/v), 50 IU/mL penicillin and 50 μg/mL streptomycin, 1% NEAA and 25 mM HEPES. The medium in apical and basolateral compartments was changed every other day for at least 21 days to allow the cells to differentiate and form a confluent monolayer. The integrity of the cell monolayer was measured by routinely monitoring the TEER using an epithelial tissue voltohmmeter (EVOM^2, World Precision Instruments, Saratosa, FL). Monolayers with TEER values higher than 500 Ωcm^2 were used for transport study. TEER was also measured before and after the transport experiments. Cells between passage 30 and 50 were used in this study.

3.2.4 TEM imaging of Caco-2 cell monolayers

Caco-2 cell monolayers were washed 3 times with HBSS and pre-incubated at 37℃ for 1 h to reach equilibrium. BNP dispersion in HBSS at 0.2 mg/mL

were applied to the apical compartment and incubated at 37 ℃ for 4 h. The cells were then rinsed with HBSS 3 times to remove non-adherent particles. At this point, the Transwell inserts were removed from the wells. The polyester membrane grown with cells was then carefully cut off from the insert support and placed in a new 12-well plate. The monolayers were prefixed with 2.5% glutaraldehyde and 2% paraformaldehyde overnight at room temperature, followed by washing 4 times with 0.1 M phosphate buffer (pH 7.4) for 10 min per change. The cells were then post-fixed with 1% osmium tetroxide (OxO4 in 0.12 M cacodylate buffer, pH 7.2) for 1 h and washed 3 times with phosphate buffer for 10 min per change. Samples were dehydrated with 50%, 70%, 90% and 100% ethanol solutions for 15 min in each solution and 3 times in 100% ethanol. The polyester membranes were then carefully cut into small pieces and infiltrated with Spurr resin by replacing the 100% ethanol with ethanol:Spurr mix (1:1, v/v) and pure Spurr resin for 2 h in each step. The small pieces of samples pieces were carefully transferred in molds filled with Spurr resin in favorable orientation. The molds were incubated at 70 ℃ for 20 h for resin polymerization.

Following trimming the blocks, thin section of the samples were performed using a mircrotome (Reichert-Jung Ultracut E Ultramicrotome). Sections with thickness of 80 nm were collected on copper grids. Samples were stained with 4% uranyl acetate for 30 min and lead citrate for 7 min in a CO_2 free chamber. Then grids were thoroughly rinsed, dried and examined with Philips-FEI

transmission electron microscope (Morgagni 268, FEI Co., Hillsboro, OR) operated at 80 kV.

3.2.5 *In vitro* transport of nanoparticles across Caco-2 monolayers

β-Carotene encapsulated nanoparticles digested with simulated gastric and intestinal fluids, referred as BC-NP digesta, were used to assess the transport efficiency in comparison with β-carotene without delivery carriers. A previously reported Tween 40 method (During, Albaugh, & Smith Jr., 1998) was used to prepare free β-carotene suspension. Briefly, required amount (same dosage as the corresponding BC-NPs) of β-carotene in hexane and 1 mL acetone containing 20% Tween 40 (w/v) was introduced into a glass vial and mixed thoroughly. The solvent was evaporated under nitrogen stream and the residue was dissolved in phosphate buffer. The β-carotene residue and BC-NPs were transferred to the *in vitro* digestion system described in section 3.2.3 prior to experiment.

Caco-2 cell monolayers were washed 3 times with HBSS and pre-incubated at 37 ℃ for 1 h to reach equilibrium. BC-NP digesta (0.5 mg/mL in HBSS) and free β-carotene digesta (4 μg/mL in HBSS) were applied to apical compartments. HBSS in basolateral compartments was replaced with HBSS containing 0.5 mM taurochlorate, 1.6 mM oleic acid and 45 mM glycerol (Netzel et al., 2011), as it is believed that with the supplementation of taurocholate and oleic acid Caco-2 cell monolayers can produce chylomicrons, which can incorporate β-carotene and excrete the compounds to the basolateral side (During & Harrison, 2005).

Following a 16 h (overnight) incubation at 37 ℃, solutions in apical and basolateral compartments were collected and the cells were removed from the inserts for β-carotene extraction.

3.2.6 β-Carotene extraction and HPLC analysis

Solutions collected from apical and basolateral compartments of four wells from a Transwell plate were pooled together for β-carotene extraction using the method of Barba et al. (2006) optimized by Aheme et al. (2010). Briefly, samples were extracted twice with hexane/ethanol/acetone (v/v/v= 50/25/25), mixed vigorously for 2 min and centrifuged at 2,000 g for 15 min. The hexane supernatant was pooled and evaporated under nitrogen stream with β-carotene residue left in the vial. β-Carotene extraction from the cells was performed using the method described by Peng et al. (1995). Cells were pipetted off the Transwell inserts and pelleted by spinning at 800 g for 10 min at 4 ℃. 1 mL of PBS containing 0.5 g/L butylated hydroxytoluene (BHT) and 2% (w/v) pronase E (protease from *Streptomyces griseus*) was added to the cell pellet. Cells were vortexed and incubated at 37 ℃ for 45 min. Then 1 mL of ethanol containing 0.5 g/L BHT and 10 g/L sodium dodecyl sulfate (SDS) was added, the mixture was vortexed for 1 min. After lysing the cells, β-carotene was extracted from the lysate using the same hexane/ethanol/acetone extraction method described above. The extracted residues from all samples were dissolved in 300 μL acetonitrile/ethanol (v/v = 65/35) and stored in -20 °C until analysis. β-Carotene concentration was analyzed using a high performance liquid chromatography

(HPLC) system (1200 series, Agilent Techonologies, Inc., Santa Clara, CA) based on the method of Wang Wang, Liu, Mei, Nakajima, & Yin (2012) with slight modifications. Quantitative measurement was carried out on a Kenitex 150 × 4.6 mm, 2.6 μm C18 column (Phenomenex, Inc., Torrance, CA) with a mobile phase of acetonitrile/ethanol (65:35, v/v) at the flow rate of 1 mL/min. β-Carotene was eluted at 9 min and at the wavelength of 450 nm. The concentration of β-carotene in each sample was calculated based on a standard curve (concentration versus area under the curve) constructed by linear regression (R^2 = 0.9966) of a series of standards. The limit of detection was 0.01 mg/L which is comparable with previous studies (Aherne et al., 2010; Wang et al., 2012). The transport efficiency was expressed as the amount of β-carotene in the basolateral media (ng) for unit area (1 cm^2)of Caco-2 cell monolayer; it was also presented with the percentage of β-carotene in the basolateral media compared to the total β-carotene in the apical media before incubation.

3.2.7 *Ex vivo* adhesion and permeation of nanoparticles in rat jejunum

The *ex vivo* study on the adhesive ability and permeability of BNPs was performed on a modified Ussing chamber model (Vine, Charman, Gibson, Sinclair, & Porter, 2002). The animal care and experimental procedures were conducted in accordance with Canadian Council on Animal Care and approved by the University of Alberta Animal Care and Use Committee (Livestock). Male 10-week old Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and fed standard chow diet (Laboratory chow

5001). Animals were given free access to food and water. On the day of experiment, animals were anesthetized with isoflurance-oxygen mix (3.5%) and sacrificed by exsanguination. The jejunum was removed distal to the ligament of the Treitz and immediately placed in ice-cold Krebs buffer supplemented with sodium L-glutamate (4.9 mM), disodium fumarate (5.4 mM), sodium pyruvate (4.9 mM) and D-glucose (11.5 mM) and continuously bubbled with O_2/CO_2 (95%/5%). Individual segments were cut from the jejunum and mounted in modified Ussing chambers (Harvard Apparatus Inc, Holliston, MA). Each mounted area available for permeation was 1.15 cm^2. Tissues were allowed to equilibrate in oxygenated Krebs buffer at 37℃ for 30 min. Upon equilibration, NR-NP digesta was added to the mucosal chamber to a final concentration of 0.5 mg/mL. After incubating for 60 and 90 min, solutions from mucosal and serosal chambers were collected and tissues were carefully removed. A small piece was cut from each segment and immediately mounted in OCT embedding media (Lamb-OCT, Thermo Scientific) on dry ice. Upon frozen, tissue blocks were stored at -20℃ until cryosection. Tissues were mounted on glass slides using Prolong® Gold Anitfade reagent without staining and examined with CLSM.

Solutions from each chamber were used to extract total lipid for Nile red quantification. Hexane/ethanol/acetone (50:25:25, v/v/v) was added to all solutions followed by centrifuge at 10,000 g for 15 min. The hexane supernatant was pooled and dried under nitrogen stream. The residue was redissolved in 300 µl methanol and measured by SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA) with the excitation and emission wavelength at 552

and 636 nm for Nile red fluorescence intensity. A standard curve indicating the quantitative relationship between Nile red intensity and NR-NP concentration was created by linear regression (R^2 = 0.9994). The standard was used to estimate the original NR-NP concentration in each chamber. The permeation of NR-NPs was expressed as the amount of the digesta (μg) in the serosal side for unit area (cm^2) of jejunum tissue.

3.2.8 Statistical analysis

Size and zeta-potential measurements for BC-NP digesta were obtained from three individual digestion batches and performed in duplicates for each batch. The quantification of β-carotene and Nile red was carried out in duplicates. Data were expressed as mean ± SEM. Student's t-test was used for comparisons between two samples. The significant level was set as $P < 0.05$. Statistical analyses were performed using SAS 9.0 for Windows (SAS Institute Inc., Cary, NC).

3. 3 Results and Discussions

3.3.1 Nanoparticle transcytosis: TEM

It is crucial for bioactive compounds to become bioavailable to exert physiological benefits (Rein et al., 2013). In the context of food science, this means the compounds need to be absorbed by the intestinal wall and translocated to the blood stream. As delivery vehicles for bioactive ingredients, direct uptake of nanoparticles can facilitate the bioactive compounds to overcome the mucosal

barrier and enhance bioavailability (Des Rieux et al., 2006). The bioavailability improving effect of BNPs in terms of increasing uptake efficiency was highlighted in the present study. It was suggested that uptake of BNPs by Caco-2 cells was via transcytosis pathway in the previous chapter; the transcytotic process was further investigated in this study.

Transport of BNPs across Caco-2 cell monolayers was explored by examining Caco-2 cells and the solutions in apical and basolateral compartments after incubation with BNPs using TEM imaging. Upon growing for 21-25 days, Caco-2 cells progressively differentiated to polarized monolayers with well-defined microvilli on the apical side and tight junctions between cells (Figure 3.1a). After 4 h incubation with BNPs at 37 ℃, non-adsorptive BNPs were washed off prior to TEM sample preparation. From the cross-section of cell monolayer, spherical particulates with diameter of 200-300 nm were observed adhered to the microvilli and within the cells (Figure 3.1b). No presence of similar intracellular vesicles with such size was found within or adhering to the control cells without BNPs (Figure 3.1a) despite of the fact that transcytosis is utilized by epithelial cells for various cargos (Tuma & Hubbard, 2003). Additionally, considering that no cell organelles with globular shape were of this size range in intestinal cells (Alberts et al., 1994), the particulates within membrane-bound vesicles were most likely exogenous BNPs rather than intrinsic cellular structures. In order to identify directly that the particulates are indeed BNPs in the process of transcytosis across Caco-2 cells, particle labeling

such as immunogold labeling can be conducted in future study. A standard cargo can also be introduced to cells as an internal control.

In the micrographs of the transport solutions (Figure 3.2), nanoparticles were observed in the apical compartment after 4 h incubation; particulates with diameter around 100 nm were found in the basolateral compartment. It could not be clarified whether these nano-scaled particulates were the fraction of BNPs with smaller size translocated to the basolateral side or new objects reassembled in enterocytes and secreted to the medium. Yet they were exclusively found with the presence of BNPs, implying that interactions with biological tissues may affect the structure of nanoparticles during transport. Meanwhile in the control group, where cells were incubated with HBSS in the absence of BNPs, only fragments and a porous-looking background was observed, which also could be found in the experiment group as well as the TEM image of pure BNP dispersion (Fig 2.1). These findings suggested that BNPs could be taken up by Caco-2 cells as well as transport across the cell layer via transcytosis to the basolateral side. Following the transport, they could be translocated from the intestinal lumen to bloodstream and directed to body cells.

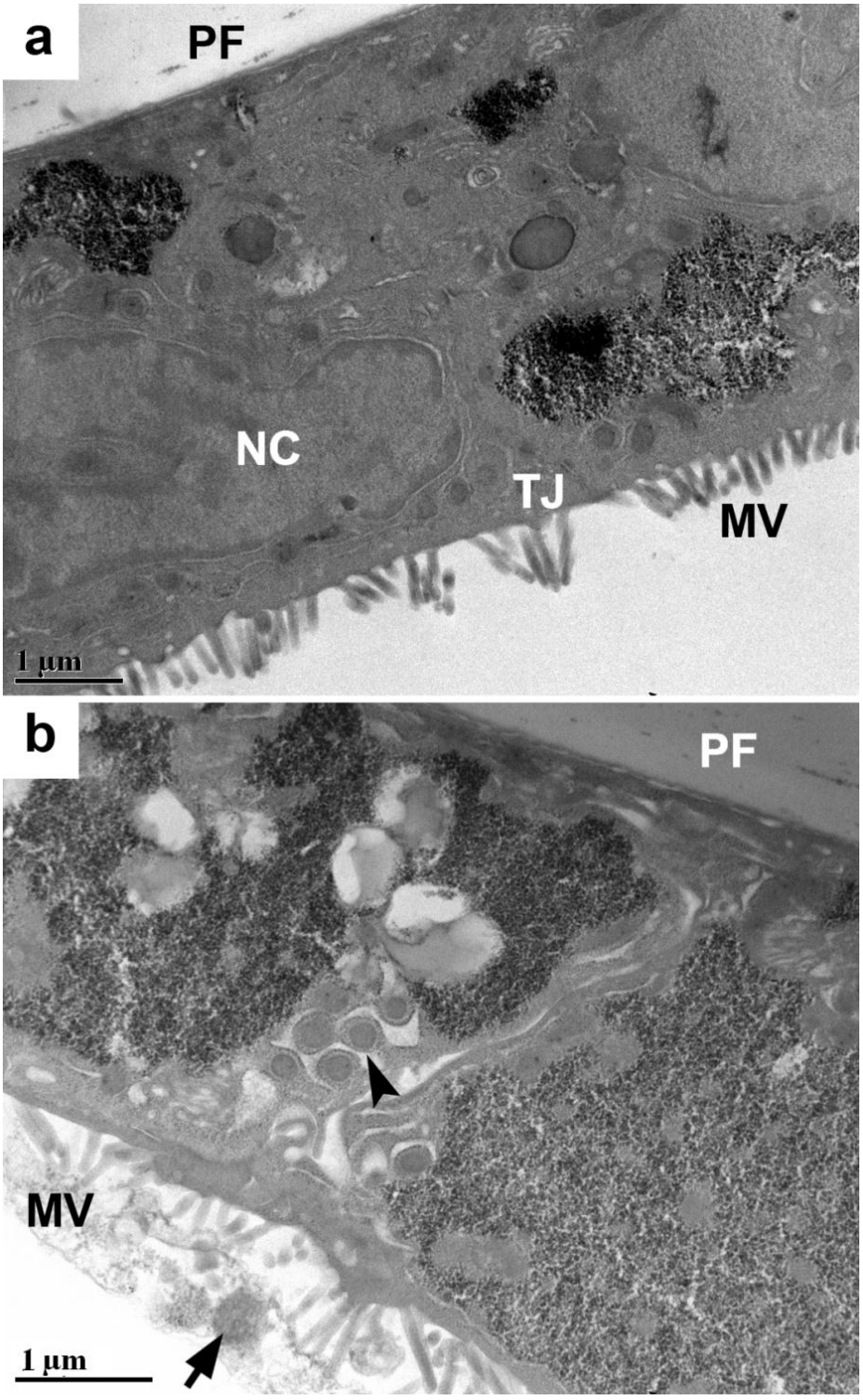

Figure 3-1 TEM micrographs of Caco-2 cell monolayers on Transwell inserts incubated without (a) and with barley protein nanoparticles (b) for 4 h. Arrow

heads show nanoparticle-conveyed vesicles within cells; arrows show nanoparticles adhered to the microvilli. PF: polyester membrane, DS: desmosome, NC: nucleus, TJ: tight junction, MV: microvilli.

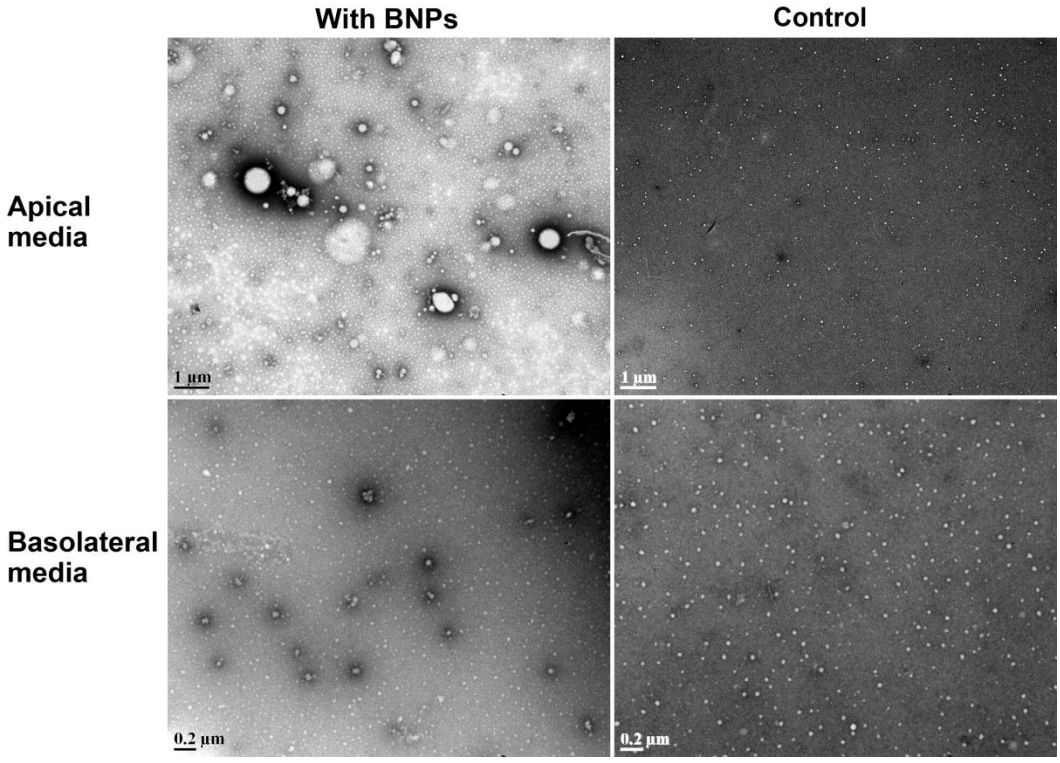

Figure 3-2 TEM micrographs of apical and basolateral media of Caco-2 cell monolayers on Transwell inserts incubated with or without barley protein nanoparticles (control) for 4 h. BNPs: barley protein nanoparticles.

3.3.2 Uptake of β-carotene encapsulated nanoparticles

The uptake enhancing effect of nanoparticles was assessed using BC-NPs on an *in vitro* digestion/Caco-2 cell monolayer transport model. After digested with pepsin and pancreatin, BC-NP digesta was added to the apical compartment of

Transwells and incubated with cells overnight (16 h). The uptake of β-carotene into Caco-2 cells and across the cell monolayer was represented in Figure 3.3. When delivered by nanoparticles, 9.38 ± 0.46 ng β-carotene was taken up into the cells for 1 cm^2 of cell monolayer, accounting for 11.45 ± 0.57 % of the total β-carotene applied to the apical chambers. The result was significantly higher than the control group, where only 2.88 ± 1.14 ng/cm^2 β-carotene (1.97 ± 0.11 %) was found in the cells. Likewise, more β-carotene (1.84 ± 0.10 ng/cm^2, 3.52 ± 1.39 %) could be taken up across Caco-2 cell monolayers and secreted into the basolateral media when delivered by nanoparticles compared to that without delivery vehicles (0.64 ± 0.04 ng/cm^2, 0.68 ± 0.04 %). Statistical significance level was not reached in terms of the β-carotene in the basolateral media, which might be due to the small sample size used in the experiment (n = 2) and thereby reduced statistical power. Experiments with a greater number of replicates will be needed in future work.

β-Carotene has numerous biological functions including antioxidant activity, potential role in the prevention of cancer, heart disease and ocular disorders and provitamin A activity (Wang et al., 2012). However, β-carotene from raw vegetables exhibits very limited bioaccessibility (about 3%) due to its low solubility in aqueous environment (Hedrén, Diaz, & Svanberg, 2002). Its absorption relies on the disruption of food matrix and the formation of micelles with the presence of lipid and bile salts (Hedrén et al., 2002). When encapsulated within nanoparticles, β-carotene molecules exist as well-dispersed suspension in the GI tract and are easier to be directly absorbed. As the particles are

progressively degraded while traveling down the small intestine, it is presumed that disrupted protein wall and released β-carotene may form an emulsion co-existed with intact nanoparticles; both forms can be absorbed by Caco-2 cells as shown in our study. Apart from improving the solubility, BNPs can protect the active ingredients against the harsh gastric environment until release them at the site of absorption. Furthermore, the large surface area of nanoparticles results in higher adsorption to the mucosa and/or more efficient cell-particle interaction, which leads to prolongation of the residence time in GI tract and higher absorption efficiency. In summary, under the present experimental conditions, BC-NPs improved the absorption and transport of β-carotene. The total cellular transport efficiency (approximately 15%) of β-carotene via BC-NPs is comparable and slightly higher than the corresponding uptake of carotenes released from cooked carrots in Caco-2 cell Transwell models (approximately 11%) reported in previous study (Netzel et al., 2011). Considering the highly variable diet preference for each individual as well as the cooking process depending on each person's lifestyle, the access to β-carotene source and its bioavailability in the body cannot be guaranteed. Nanoparticles encapsulated with the compound can be incorporated into other food matrices as a simple method to develop functional foods. This provides a convenient and reliable source of regular β-carotene intake with enhanced bioavailability.

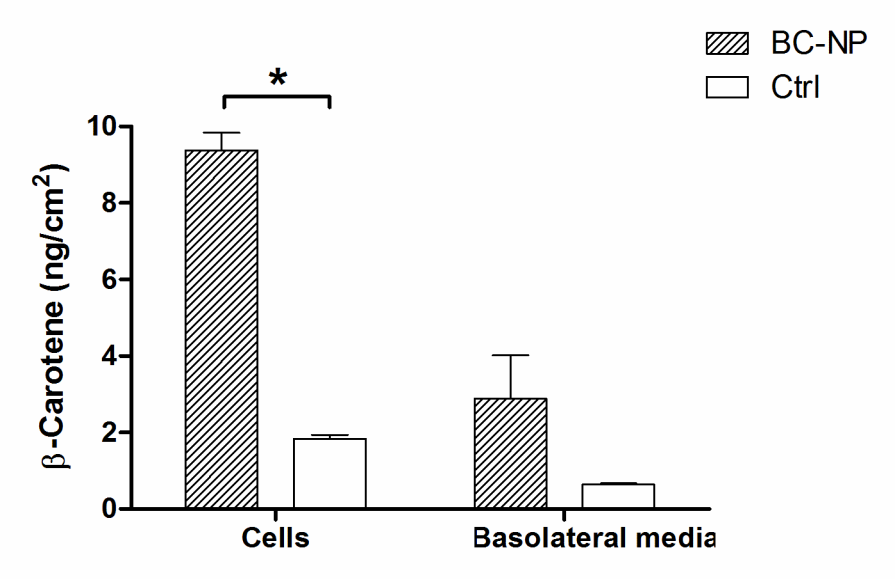

Figure 3-3 Uptake of β-carotene delivered with and without barley protein nanoparticles in Caco-2 cell monolayers after 16 h incubation. Star shows significant difference compared to control ($P < 0.05$). Data are presented as mean ± SEM, n = 2. BC-NP: β-carotene encapsulated barley protein nanoparticles; Ctrl: control, β-carotene delivery without nanoparticles.

3.3.3 Bioadhesion and permeation of nanoparticles in rat jejunum

The surfaces throughout GI tract are covered by mucus which is composed of high molecular weight anionic glycoproteins (mucins) (Acosta, 2009). The mucus forms a continuous adherent gel layer covering the underlying epithelial cells and functions as a barrier to prevent the absorption of potential harmful substances or pathogens (Atuma, Strugala, Allen, & Holm, 2001). When traveling in GI tract, nanoparticles firstly diffuse to the liquid medium in the lumen and then pass through the mucus gel layer prior to the direct interaction

with epithelial cells. The bioadhesion ability to intestinal mucosa, or "mucoadhesion", of nanoparticles is a prerequisite for them to pass through the mucus gel layer and interact with epithelium (Bravo-Osuna et al., 2007). The *ex vivo* bioadhesion and permeation properties of BNPs were studied using NR-NPs on an *in vitro* digestion/rat jejunum model using Ussing chamber technique. Original BNPs without fluorescence labeling were used as negative control to correct the autofluorescence associated with the particles and tissue debris in the media. Results showed, interestingly, that although the nanoparticles demonstrated distinct uptake in Caco-2 transwell model, their adhesion and permeability on the tissue model was not high under current experiment condition. After incubated with digesta of NR-NPs for 60 and 90 min, relatively dull fluorescence was observed in the tissues (Figure 3.4). Quantitative assessment of NR-NP digesta in the mucosal chambers was calculated using on a standard curve of NR-NP amount vs. fluorescence intensity (R^2 = 0.9994); autofuorescence in the media was corrected by deducting the fluorescence signal in the serosal media when tissue was incubated with negative control BNPs). Results showed that 2.24 ± 1.10 and 6.04 ± 0.14 μg NR-NP digesta was permeated across for 1 cm^2 tissue and secreted into the serosal media after 60 and 90 min incubation respectively (Figure 3.5). The results confirmed the ability of BNPs of adhering to and permeating through intestinal tissues; yet higher adhesion and permeation efficiency was needed for more effective delivery systems. Uptake study with greater sample size including the use a model compound is required in the future.

Studies have shown that most polymeric nanoparticles adhere to mucus gel layer by non-specific bioadhesion mechanisms, such as hydrogen bonding, van der Waals force, and more specifically for hydrophobic particles, the inherent tendency of developing contacts with mucosal surfaces (Ponchel & Irache, 1998). The interaction between particles and mucus gel can be dramatically affected by particle size, surface characterization, crosslinking degrees and the mucus absorbent (Galindo-Rodríguez, Allémann, Fessi, & Doelker, 2005). We suggest that the surface charge of BNPs, demonstrated by a zeta-potential of lower than -30 mV, may be the primary cause to their mucoadhesion properties showed in this study. The energy barrier of electrical repulsion between negative nanoparticles and the negatively charged mucus layer could result in inefficient adherence. Studies employing cationic chitosan coating as an adhesion enhancer also suggested negatively charged particles were less adhesive than non-ionized and positive carriers (Galindo-Rodríguez et al., 2005; Ponchel & Irache, 1998). Findings from the present *ex vivo* study suggested the need of surface modification with cationic polymers or adhesive conjugate for BNPs to achieve ideal bioadhensive property and *in vivo* absorption. It also implied the complexity of *in vivo* absorption process and the limitation of *in vitro* cell models, such as the absence of mucus gel layer and variability of permeability due to culture conditions (Artursson, Palm, & Luthman, 2001). It is necessary to investigate the biological behaviors of nanoparticles using tissue and animal models so as to obtain accurate results and comprehensive understanding.

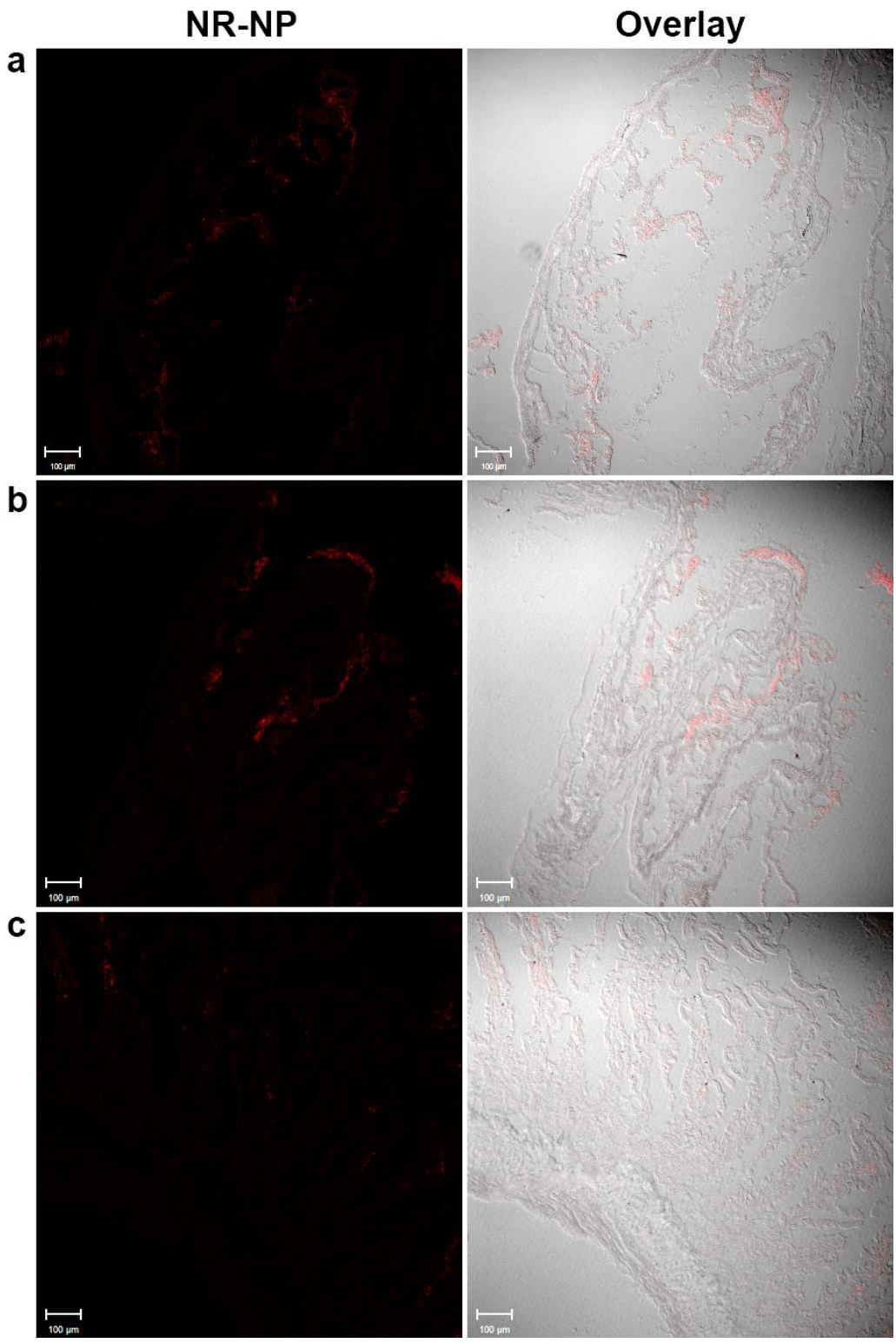

Figure 3-4 Confocal images of rat jejunum tissues incubated with digested Nile red-labeled barley protein nanoparticles for 60 min (a) and 90 min (b) and with

non-fluorescent nanoparticles (negative control) (c) using Ussing chamber model. NR-NP: Nile red-labeled barley protein nanoparticle.

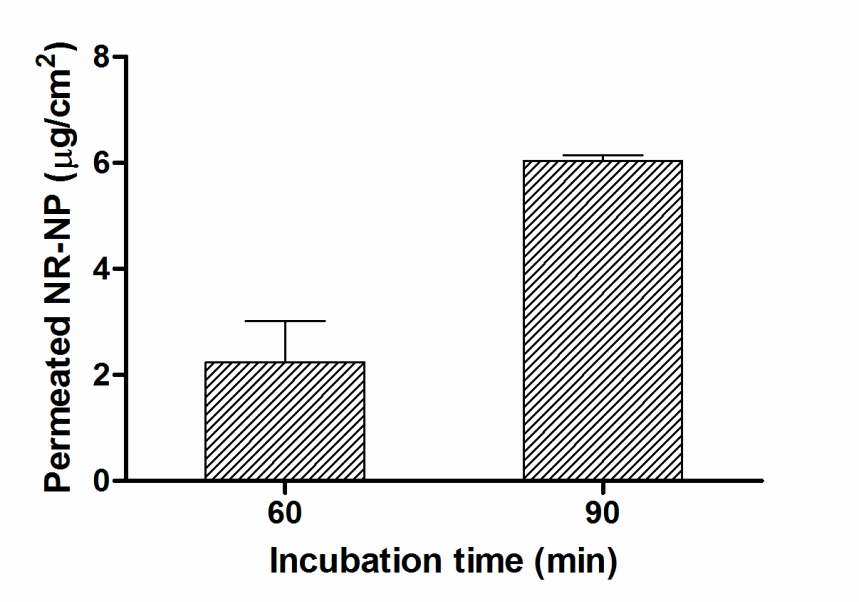

Figure 3-5 Permeation of digested Nile red-labeled barley protein nanoparticles across rat jejunum in Ussing chambers. Data are presented as mean ± SEM, n = 2. NR-NP: Nile red labeled barley protein nanoparticles.

3. 4 Conclusions

Barley protein nanoparticles can encapsulate hydrophobic bioactive compounds and deliver them to human body following the oral route. Owing to the unique properties of barley proteins, BNPs are resistant to gastric digestion but can be degraded in the small intestine. As a result, the encapsulated compounds can be protected in the stomach and released in the small intestine. This protective mechanism and controlled release property make up the major

advantage of BNPs as delivery vehicles in preserving the stability and bioactivity of compounds prior to their absorption. In this study, BNPs were directly and efficiently internalized and transported across Caco-2 cell monolayers via transcytosis mechanisms. When β-carotene encapsulated nanoparticles were delivered to Caco-2 cell monolayers, around 15% of total β-carotene was taken up into or transported across the cells, which was significantly higher than free β-carotene without nanoparticle carriers. This demonstrated the promise of BNPs in enhancing the overall bioavailability in β-carotene delivery. Results of *ex vivo* study using rat jejunums showed limited mucoadhesion capability of BNPs, which might be explained by the electric repulsion between nanoparticles and negatively charge mucus gel layer lining the intestinal surfaces. Based on Ussing chamber experiment, approximately 2.24 and 6.04 μg digested particle were able to permeate through each cm^2 intestinal tissue and translocated to the serosal side after 60 and 90 min respectively. Delivery dosage can be assessed by considering the total absorption area of small intestine and the daily needs of the compound of interest. Results from current study showed that BNPs could improve β-carotene in *in vitro* models and indicated the great potential in enhancing bioavailability of lipophilic bioactive compounds. Particle surface modification is needed to overcome the mucosal barrier. Studies on lymphatic uptake properties of BNPs and their biological properties on animal models are also needed in future research.

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Chapter 4 Final Remarks

4. 1 Summary of Present Research

Solid microparticles were prepared from barley proteins (hordein : glutelin = 1:1, w/w) with canola oil (protein : oil = 1:1, w/w) using pre-emulsification and high pressure homogenization techniques based on our previous studies (Wang, Tian, & Chen, 2011a). Lipophilic bioactive compounds can be encapsulated into the particles by dissolving the compound in canola oil prior to microparticle preparation. It is suggested that the particles can be used as delivery systems for the oral delivery of hydrophobic bioactive compounds due to their encapsulation and controlled release properties (Wang, Tian, & Chen, 2011b). In the present study, nanoparticles (BNPs) were generated as a result of partial degradation of the protein matrix by digesting microparticles with pepsin solution for 15 min. BNPs have negatively charged surface and a size range of 50-500 nm. The cytotoxicity, cellular uptake profile and mechanisms, transport efficiency of BNPs were investigated using human cell and animal tissue models.

BNPs showed low cytotoxicity on human intestinal epithelial cell line Caco-2 cells when particle concentration is lower than 0.2 mg/mL. BNPs can be internalized into the cytoplasm and nucleus by Caco-2 cells. The cellular uptake process of BNPs is particle concentration-, incubation time- and temperature-dependent. Transcytosis pathway was suggested being used by BNPs as it is the main route that cells transport macromolecules and particulate matters; the uptake profile of BNPs discovered in this study is in correspondence with transcytosis. Furthermore, paracellular pathway was excluded as the integrity of

Caco-2 cell monolayer was not compromised during nanoparticle transport. Taking the size range of BNPs, multiple transcytosis mechanisms (e.g. adsorptive and receptor-mediated endocytosis) may be involved. β-Carotene was used as a model compound to be encapsulated in BNPs and delivered to Caco-2 cell monolayer. After overnight incubation, approximately 15% of the total β-carotene was taken up into or across the cells. This proportion was significantly higher than that of free β-carotene without nanoparticle carriers, suggesting uptake enhancing ability of BNPs. In *ex vivo* study using rat jejunum tissues and Ussing chamber techniques, an observable portion of nanoparticles was adhering to the tissue. About 6.04 μg particles were permeated through to the serosal side for each cm^2 of tissue after 90 min incubation. These results implied that surface modification of nanoparticles may be needed to achieve ideal bioadhesion and permeation efficiency.

4. 2 Potential Applications and Future Research

Protein nanoparticles are of increasing interest in the application of drug and nutrient delivery. Advantages of proteins being wall materials for nanoencapsulation include their gelling and emulsifying ability, high biocompatibility, easy to control their physicochemical properties, as well as their high nutritional values and abundant resources (Chen, Remondetto, & Subirade, 2006; Sundar, Kundu, & Kundu, 2010). Compared with synthetic polymers used in drug delivery, proteins are GRAS grade or food grade and thus are safe for food application. In addition to the advantages above, barley protein

nanoparticles demonstrate unique characteristics to form excellent nanoencapsulation for nutrient delivery. Firstly, due to the hydrophobic nature, barley proteins can form solid micro- and nanoparticles without the addition of cross-linking reagents or surfactants in the preparation process, eliminating the safety concerns that are usually encountered in other nanoparticle systems (Jahanshahi & Babaei, 2008; Wu et al., 1997). Secondly, BNPs can protect the encapsulated compound in the stomach and steadily release it in the small intestine. This controlled release property can preserve the activity of the compound, prolong its residence time in GI tract and improve absorption efficiency. Thirdly, functional foods can be developed by incorporating BNPs into liquid or semi-liquid food matrix to maximize the health-boosting benefits of bioactive compounds. BNPs based functional foods are also promising value-added products of barley grains, which are abundant and inexpensive resources but of limited use for human consumption (Gupta, Abu-Ghannam, & Gallaghar, 2010).

Further study is needed to address issues listed, but not limited to, as follows:

(i) Modification on BNPs fabrication and surface propertie to achieve higher adhesion and permeation efficiency in intestinal tissue;

(ii) Comprehensive investigation on lymphatic uptake of BNPs;

(iii) *In vivo* study to evaluate the safety and bioavailability enhancing effectiveness on animal models;

(iv) Influence of BNPs on the sensory properties of foods, stability of BNPs and encapsulated compound during food storage;

(v) Understanding the formation mechanisms of BNPs and principles to manipulate their physicochemical properties so as to design the ideal delivery systems.

4. 3 Bibliography

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