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Past, Present, and Future Technologies for Oral Delivery of Therapeutic Proteins

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Abstract

Biological drugs are usually complex proteins and cannot be orally delivered due to problems related to degradation in the acidic and protease-rich environment of the gastrointestinal (GI) tract. The high molecular weight of these drugs often results in poor absorption into the periphery when administered orally. The most common route of administration for these therapeutic proteins is injection. Most of these proteins have short serum half-lives and need to be administered frequently or in high doses to be effective. So, difficulties in the administration of protein-based drugs provides the motivation for developing drug delivery systems (DDSs) capable of maintaining therapeutic drug levels without side effects as well as traversing the deleterious mucosal environment. Employing a polymer as an entrapment matrix is a common feature among the different types of systems currently being pursued for protein delivery. Protein release from these matrices can occur through various mechanisms, such as diffusion through or erosion of the polymer matrix, and sometimes a combination of both. Encapsulation of proteins in liposomes has also been a widely investigated technology for protein delivery. All of these systems have merit and our worthy of pursuit.

Keywords

nanoparticles; nanospheres; microparticles; microspheres; poly(lactic/ glycolic) acid (PLGA or PLA); polymeric drug delivery systems; oral drug delivery; protein delivery; vaccine delivery; mucosal delivery

INTRODUCTION

Proteins perform many important physiological and biological processes of the host. Protein ligands bind their receptors that result in actions or changes due to sometime distant signals. Enzymes are involved in many biotransformational reactions or *de novo* generation or catalysis of a multitude of substrates. Antibodies can actively participate in neutralizing toxins or host factors (e.g., TNF- α). The knowledge and translation of the human genome, has greatly increased the desire to discover new proteins and understand their function,

usefulness as a therapy as well as to devise drug delivery systems (DDSs) for these molecules. While designing novel DDSs is not essential for successful and efficacious protein drug delivery, effective DDSs would enable these therapeutic proteins to be delivered via mucosal routes to increase efficacy and patient compliance as well as reduce medical errors in administration (e.g., intravenous delivery).

DDSs should be designed to reduce adverse reactions while achieving site-specific delivery, convenient administration, improved patient compliance, and increase product shelf-life. Over the past few decades, interest in developing effective DDSs for biologicals has grown considerably as the number of recombinant proteins being investigated for therapeutic applications has increased.¹ Success of these new therapeutics hinges on efficient DDSs that allow drug access to their target site(s) at the right time, duration and dose. Four factors must be considered to create these conditions: route of administration, drug release pattern, delivery method, and fabrication/ formulation.

Unfortunately, most protein drugs are therapeutically useful only when a regimen requiring multiple injections is followed without tissue targeting (Fig. 1). Such therapies are frequently administered under close medical supervision. This necessitates novel technologies to refine and control therapeutic protein delivery. In addition, the biochemical and structural complexity of proteins compared to conventional drug-based pharmaceuticals makes formulations design for biologicals a formidable task. In this regard, the development and evaluation of effective DDSs for therapeutic proteins must consider the biophysical, biochemical, and physiological characteristics of proteins, including their molecular size, biological half-life, immunogenicity, conformational stability, dose requirement, site and rate of administration, pharmacokinetics, and pharmacodynamics.²

Several technologies have been used to deliver complex molecules. Although the concepts of microencapsulation and sustained release are well established, the convergence of these concepts and their applications to control release from polymeric microspheres occurred <15 years ago. Somatostatin encapsulation in polylactic glycolic acid (PLGA) microspheres and thyroid releasing hormone microspheres were successfully prepared by spray drying techniques.^{3,4}

Microparticles comprised of biodegradable and non-biodegradable polymers have been investigated for sustained release. Non-biodegradable polymers pose problems of toxicity, ease of removal or degradation and achieving a constant rate of release.⁵ To overcome some of these problems investigations into biodegradable polymers for sustained release and the development of parenteral DDSs began in the early 1970s. Yolles et al.⁶ was one of the first to report the use of polypeptides in parenteral DDSs. These methods were developed for two reasons. Surgery was required to remove drug-depleted DDSs made with non-biodegradable polymers since non-removal posed toxicological problems. Second, diffusion-controlled systems, although an excellent means of achieving predetermined rates of drug delivery, were limited by polymer permeability and drug characteristics. With the basic mechanism of nonbiodegradable devices being diffusion, drugs having either a high molecular weight (>7500 Da) or poor polymer solubility are not amenable to classic diffusion-controlled release.

In the last decade, there have been major advancements using biodegradable polymers. The most notable is for prostate cancer treatment, where a single (once-a-month) injection has replaced 30 daily injections of luteinizing hormone-releasing hormone agonist. Additional promising treatments for cancer, viral and bacterial infections, birth control, and AIDS are being investigated.^{7–12} Indeed, the delivery of genetically engineered products as vaccines, for example, soluble recombinant human immunodeficiency virus (HIV) proteins, has led to increased efficacy by entrapment of vaccine antigens in PLGA microspheres.¹³ Therapeutic proteins, e.g., recombinant erythropoietin,¹⁴ have also been encapsulated in PLGA microspheres as well as plasmid DNA, antisense oligos, and synthetic double-stranded DNA.^{15–18}

A variety of synthetic and naturally occurring biodegradable polymers have been studied over the past 30 years, including polyesters, polyanhydrides, polyorthoesters, polyphosphazenes, and pseudo amino acids, out of which polyesters have found more widespread use. A number of other studies have used natural polymers for DDSs that have centered-around proteins (e.g., collagen, gelatin, albumin) and polysaccharides (e.g., starch, dextran, insulin, cellulose, hyaluronic acid). Despite many advantages of polyesters, like PLGA, these polymers have inherent shortcomings. By in large, polymers are more hydrophobic compared with most of the proteins to be encapsulated. Indeed, a lack of protein polymer compatibility leads to stability problems during storage or under *in vivo* release conditions. Hydration and degradation of polyesters are prerequisites for the release of protein during the bioerosion phase; however, this can result in an acidic microenvironment (due to formation of lactic and glycolic acids), which might denature encapsulated proteins. One approach to improve protein polymer compatibility is by co-encapsulating buffer salts and stabilizers for proteins, which are thought to modify the internal pH of microspheres. Another way might be realized by modifying the polymer structure itself.

CHALLENGES IN THERAPEUTIC PROTEIN DELIVERY

Physiological Obstacles

A major obstacle for the oral absorption of macromolecules is their vulnerability to proteolytic degradation in the GI tract. Many macromolecules, especially protein and peptide drugs, are susceptible to rapid degradation by digestive enzymes. The proteolytic activity is highest in the stomach and duodenum, and is significantly reduced in the mouth, pharynx, esophagus, ileum, and colon. Degradation of proteins during their transit via the mouth, pharynx and esophagus is minimal. Saliva contains mucus, amylase and lysozyme and digestion is limited to polysaccharides hydrolysis by amylase. Indeed, no absorption of food material occurs in the mouth. The secretions present in the esophagus are entirely mucoid in character and maintain a well-lubricated esophageal lumen. Movement of food through the pharynx and esophagus takes between 6 and 10 s. After traveling through the esophagus, food reaches the stomach where it is stored and digested. Based on anatomical and histological characteristics, the stomach is divided into the fundus body and antrum. These regions coordinate and control the motility function of the stomach.

The digestive juices of the stomach are secreted by gastric (or oxyntic) glands. These glands are responsible for secretion of hydrochloric acid, pepsinogen and mucus along with other components. The pyloric (exocrine) glands secrete mucus and some pepsinogen. In this regard, pepsinogen is converted into pepsin by hydrogen chloride secreted by the oxyntic glands. Pepsin is active at low pH, but is rapidly inactivated above pH 5.0. Pepsin is most efficient at cleaving bonds between aromatic amino acids: phenylalanine, tryptophan, and tyrosine. No absorption of food takes place through the stomach. Additional digestion and the majority of absorption occur in the small intestine.

The duodenum, jejunum and ileum of the small intestine have disparate secretion and uptake physiologies. While small intestinal cells secrete enzymes, e.g., aminopeptidase, this part of the GI tract does not significantly contribute to the digestive process. The exocrine glands in the small intestine largely secrete mucus that lines the inside of the intestinal wall. In fact, protein digestion in the small intestine mainly occurs due to pancreatic secretions of amylase and lipase. Pancreatic secretion also contains sodium bicarbonate that neutralizes the acidity of the contents emptied by the stomach. The pancreatic proteolytic enzyme secretions contain trypsinogen, chymotrypsinogen and procarboxypeptidase. Trypsinogen is converted by an autocatalytic reaction to its active form, trypsin, by an enzyme called enterokinase present in the wall of the duodenum. Trypsin converts chymotrypsin and procarboxypeptidases into their active analogues. These enzymes act on specific amino acid linkages and convert peptide fragments into small peptides and amino acids.

Size and Charge of Particles

The low oral bioavailability of macromolecules is due primarily to their large molecular weight and variable solubility. Bioavailability is essentially independent of molecular mass for drugs <700 Daltons (Da); however, bioavailability decreases sharply when molecular mass increases beyond this threshold. A minimum level of hydrophobicity is also needed for macromolecules to permeate the epithelium and to be transcellularly absorbed through passive diffusion. Without this minimum degree of lipophilicity, no passive absorption can take place unless it is through the paracellular pathway, which is restricted to relatively small compounds (<200 Da). Unfortunately, most macromolecules being evaluated as biotherapeutics are typically >700 Da and hydrophilic. This poses a major obstacle for DDS formulation methods.

Challenges in Formulation Methods

Out of the host of microencapsulation techniques, the most commonly used methods of microencapsulation of proteins are spray drying, multiple emulsion, and phase separation methods. The difficulties associated with developing effective formulations for proteins have been discussed in various articles.^{19–23} Despite many attractive features, proteins as therapeutic agents have some serious limitations. Proteins are relatively large molecules with often-complex structures. Unlike low-molecular weight drugs, they possess secondary, tertiary, and in some cases, quaternary structures with labile bonds and side chains of chemically reactive groups. Disruption of these structures or modification of side chains can lead to loss of immunogenicity or activity.

The fragile nature of protein therapeutics requires the processes involved in the fabrication of DDSs must not damage the protein, reduce its biological activity, or render the protein immunogenic. For example, aggregated human growth hormone (hGH) has less biological activity than its native monomeric form.²⁴ Recombinant therapeutic proteins hGH can undergo non-chemical changes such as folding and unfolding (denaturation), which leads to loss of native structure and enables the protein to interact with its surroundings, which might lead to surface absorption or aggregation.²⁵ Aggregation of insulin has been well characterized and depends on the unfolding of insulin.²⁶ Chemical degradation may also occur at many points during formulation and delivery processes. The most common adverse chemical modification associated with DDSs is oxidation.²⁴ Deamidation contributes to a reduction in the catalytic activity of lysozyme and ribonuclease at high temperatures.²⁷ This reaction is also widely observed in therapeutic proteins. Peptide bond hydrolysis results in the loss of activity when proteins are heated.²⁵ Aggregation of lyophilized formulations of bovine serum albumin, β -lacto-globulin, and glucose oxidase are attributed to disulphide bond interchange.²⁸

It is important to devise formulation strategies to preserve protein stability. These approaches include adding stabilizing agents and developing fabrication processes for delivery systems that are benign to proteins. Stabilizing additives used in the formulation of proteins are diverse and include proteins, sugars, polyols, amino acids, chelating agents, and inorganic salts. These additives can stabilize proteins in solution and also in frozen and dried states, although not all additives confer stability under all three conditions. For example, carbohydrates in particular have the ability to stabilize dried proteins.²⁹ Sugars such as trehalose, sucrose, maltose, and glucose are used as collagen, ribonuclease, and ovalbumin stabilizers.³⁰ Cyclodextrins have also been used as stabilizing excipients in protein formulations.^{31,32} In particular, this dextrin protects growth hormones from thermal and interfacial denaturation.³³ Heparin stabilizes acidic fibroblast growth factor by increasing its unfolding temperature by $>15^{\circ}\text{C}$.³⁴

Surfactants have also been used as protein stabilizers. Nutropin[®] (recombinant hGH) contains surfactant polysorbates as stabilizers.²⁴ Polysorbate 20 was found to be useful in stabilizing hGH incorporated in a PLG polymer matrix. It is presumed that these surfactants protect proteins against denaturation during several stages from formulation to release at the site of delivery. Certain transition metals have also been shown to confer protein stability. Zinc stabilizes the hGH against urea-induced denaturation.³⁵ Zinc-hGH complex was more stable in PLG microspheres compared with hGH alone.

Lyophilization or spray drying also increases the storage stability of proteins.³⁶ Freeze drying itself exposes the protein to destabilizing stresses, therefore suitable excipients are included in freeze drying formulations.³⁷ Freeze-drying protectants such as dextran, glycols, glycerol, and cyclodextrin have been found to minimize instability in freeze-dried formulations of luteinizing hormone release hormone (LHRH),³⁸ monoclonal antibodies,³⁹ and tumor necrosis factor.³⁷

The incorporation of therapeutic proteins into solid delivery matrices exposes them to a high surface-to-volume environment creating ample opportunity for absorption to the delivery

device, which limits the amount of free unabsorbed protein available for release. Incorporating surface-active agents to compete for protein binding sites might reduce protein retention by sustained delivery systems. For example, adding albumin to an insulin solution was found to reduce the absorption of the latter to solid surfaces. In many sustained delivery matrices, protein drugs are exposed to changing environments as the delivery matrix degrades over time. This degradation might lead to the generation of acidic oligomers (lactic/glycolic acids), resulting in increased acidity making the protein prone to degradation.⁴⁰ To overcome the potential of acidic microenvironment in the DDSs, basic salts such as sodium bicarbonate or magnesium hydroxide may be incorporated as buffering agents into the matrix.

APPROACHES FOR MUCOSAL DELIVERY OF THERAPUTIC PROTEINS

Besides parenteral delivery, which is the most widely followed route for delivery of proteins, considerable emphasis has gone into exploring non-injectable methods of protein delivery including oral,⁴¹ rectal,⁴² buccal,⁴³ transdermal,⁴⁴ nasal,⁴⁵ and ocular⁴⁶ routes. One obstacle associated with oral delivery of protein-based drugs, as discussed earlier, is the poor permeation across biological barriers, such as the intestinal lumen (Fig. 2). Since the lumen is lined with proteases and peptidases, this can lead to protein degradation. The tight junctions, or zonula occludens,⁴⁷ across the intestinal epithelium is another physiologic barrier against paracellular diffusion of large molecules, aberrant charge or hydrophilic nature. These characteristics generally lead to low oral bioavailabilities (<1%) and short *in vivo* half lives (<30 min).^{48,49}

Rectal Delivery of Protein Drugs and Vaccines

In contrast to the oral route of administration, rectal delivery of proteins provides the advantage of greater systemic bioavailability.² Various absorption enhancers like surfactants, bile acids, and sodium salicylate have been used to enhance the uptake of insulin, gastrin, lysozyme, and heparin following rectal administration.⁵⁰ However, the absorption rate of rectal administered macromolecules is relatively poor when compared to parenteral routes of administration. Moreover, rectal delivery of biologicals has poor cultural acceptance in several countries.

Ocular Administration

The ocular route may be used for systemic delivery of therapeutic proteins. Absorption occurs mainly through the nasolacrimal system. In addition to insulin, thyrotropin-releasing hormone, LHRH, enkephalin, calcitonin, and glucagon have been administered via the ocular route. However, the availability of ocular administered proteins is still expected to be significantly lower than that of conventional small drug molecules because of their unfavorable molecular size, hydrophilicity, and susceptibility to degradation by peptidases in various compartments of the eye. Fortunately, the systemic absorption by this route is relatively fast. Absorbed proteins also bypass portal circulation to the liver thus avoiding first pass metabolism. Even though using the ocular route for systemic delivery is acceptable it may not be possible. Ophthalmic administration of particles can result in irritation and can induce lachrymation with possible consequences of reducing drug bioavailability. However,

the topical use of growth factors to heal eye injury is promising, as these injuries heal very slowly from a lack of blood supply.

Buccal Delivery

The intestinal mucosa has greater permeability and perfusion than the skin, while the oral cavity provides an environment almost free from the acidity and protease activity encountered elsewhere in the mucosa.⁵¹ However, recent studies revealed the presence of aminopeptidase activity along the buccal mucosa, which could be inhibited by enzyme inhibitors.⁵² In addition, blood vessels of the oral mucosa drain directly into the jugular vein avoiding “first pass extraction” by the liver. There is also regional variation in drug permeability in the buccal mucosa.⁵³ The factors affecting buccal permeability also include DDS formulation factors that effect penetration enhancement. Many buccal delivery systems for numerous peptide/protein drugs have been described since the first attempt at a bioadhesive system,⁵⁴ yet none have reached the market.

In a series studies using cationic, anionic, and neutral polymers, anionic poly (acrylic acid) (PAA) was found to have the highest binding affinity for epithelial cells.⁵⁵ PAA was also shown to be a potent inhibitor of proteolytic enzymes. There is increasing evidence that the interaction between various types of bio(muco)adhesive polymers and epithelial cells can directly influence mucosal epithelia permeability. Indeed, DDS adhesion to the intestinal epithelial cell surface leads to enhanced uptake by nonspecific receptor-mediated endocytosis. An area for improvement in this technology is to increase the localization or retention of DDSs at a desired region.⁵⁶ Currently, mucoadhesive systems do not remain for longer periods of time at the site of attachment. DDSs are also susceptible to the high turnover and sloughing rates of the mucosa⁵⁷ and displacement by mucus excretion.⁵⁸

Nonetheless, delivery of calcitonin, LHRH, and glucagon-like-peptide I using an adhesive tablet showed 37%,⁵⁹ 100%,⁶⁰ and 41%⁶¹ bioavailabilities, respectively. There are still many issues to resolve before this effective and convenient route of drug delivery can be thoroughly and safely utilized. Challenges still remain for reproducible and maximal bioavailability of protein drug delivery across the buccal mucosa.

In contrast, others studies of protein absorption and permeability through the buccal mucosa were shown to present a number of problems. Although a higher dose of protein could be administered via the buccal route than parenteral, the resulting plasma levels of the therapeutic were much lower than when systemically delivered.⁶² It is believed that these limitations were due to reduce permeability of proteins delivered by this route. For example, calcitonin with a molecular weight of 3500 Da is not able to permeate through the buccal mucosa. Similarly, protein delivery via the transdermal route is also limited. Without chemicals to alter skin permeability the mere size and charge of proteins prevents passive absorption through the skin.⁶³

Thus, poor absorption and low bioavailability of therapeutic proteins delivered by these non-injectable routes, has forced biologicals to be parenterally administered by subcutaneous or intramuscular injection. However, the half-lives of parenterally injected proteins are only a few hours in most cases, necessitating multiple injections per week for therapeutic

effectiveness. As a result, patient compliance is a concern with systemically administered drugs. This problem could be resolved by sustained release of proteins to obtain well-defined pharmacokinetic profiles. Due to rapid clearance by the mononuclear phagocytic system (MPS) and because site-specific targeting of intravenously administered particulate drug carriers is not yet possible, it is extremely difficult to maintain DDSs in the bloodstream. Future DDSs should avoid the MPS and possess optimal surface characteristics to minimize interactions with opsonins that lead to phagocytosis. The main parameters governing these interactions are surface charge⁶⁴ and hydrophobicity.⁶⁵

POLYMERS AND GELS USED IN PROTEIN DDSS

Polyethylene Glycol

Strategies to circumvent the MPS include grafting polyethylene glycol (PEG) to the surface of the DDS or protein. PEG is considered to be a nontoxic hydrophilic polymer with FDA approval. PEG-grafting results in a steric barrier at the surface of DDSs that reduces absorption of various proteins and diminishes complement activation.⁶⁶ The chain length and density of PEG domains are major parameters that determine the extent of uptake or lack thereof by the MPS. The presence of hydrophilic chains reduces the influence of serum proteins on particle internalization by monocytes.

While PEG-conjugated proteins may not be a micro- or nanoparticle *per se*, PEGylation of protein drugs was developed and commercialized by Dr. Abraham Abuchowski. Three FDA-approved protein drugs: ADAGEN[®], ONCAS-PAR[®], and PEG-INTRON[®] were among the first to use this technology. Currently, the PEGylated biological market is approximately \$1 billion (USD) per year. Industry analysts expect this market will grow to over \$6 billion by 2008.

PEGylation technology was also exploited to extend the product (i.e., patent) lifecycle of approved protein therapeutics. For example, Schering Plough's PEG-Intron[®] is a PEGylated version of Intron and Amgen's NEULASTA[®] is PEGylated Neupogen. PEGASYS[®] (PEGylated interferon) is another PEGylated interferon marketed by Roche. Various other PEG-grafted proteins include: soluble TNF receptor-type I (PEG-sTNF-RI), synthetic thrombopoietin, arginine deaminase, anti-growth factor receptor antibody fragment, anti-IL-1 β antibody fragment and anti-PDGF β -receptor antibody fragment.

Polyesters

In addition to their biocompatibility, thermo plasticity, high tensile strength, stability, controlled degradation rates, adjustable hydrophilicity/hydrophobicity, tailored release rates, and proven non-toxic biodegradable polymers are uniquely suited for incorporation in DDSs. A variety of synthetic and naturally occurring polymers have been intensively studied over the last 30 years. Of these, polyesters have found the most widespread use.⁶⁷

Features that attracted investigations to using polyesters in protein formulation include pre-existing toxicological and chemical data as well as their biocompatibility, predictable biodegradation kinetics, ease of fabrication, versatility, commercial availability and perhaps most importantly—regulatory track record.⁶⁸ A broad spectrum of performance

characteristics with these polymers can be obtained by careful manipulation of four key variables: monomer stereochemistry, co-monomer ratio, polymer chain linearity, and polymer molecular weight. Hence, different polymers can modulate the structure, range of hydrophilic behavior, and solubility of DDSs. Together, these factors ultimately affect the biodegradation and release profile of the resulting DDS. For example, crystalline domains and stereo irregularity inhibit the degradation of the polymer; hence, stereo irregularity in lactides can determine degradation time.

Poly L – lactide	> PolyDL – lactide	> <i>Polyglycolide</i>
(Crystalline; stereoirregular)	> (Amorphous; stereoirregular)	> (Crystalline; stereoregular)

Varying co-polymer ratios results in different crystallinities, transition temperature and hydrophilicities (or hydrophobicities), which affect biodegradation profiles. Polymer chain linearity affects the hydrophilicity of the polymer, which in turn affects its degradation rate. The extent of block or random structure in the copolymer also affects hydration rate and the degradation profile. Polyesters are commercially available in a wide range of molecular weights. Higher molecular weight polyesters have higher viscosities that affect entrapment efficiency as well as sphere size and shape. For example, the size of calcitonin-encapsulated microspheres increased when polymers with higher viscosities were used in their formulation.⁶⁹

Degradation of aliphatic polyesters occurs by random, non-enzymatic, and/or hydrolytic cleavage of ester linkages. The nature of degradation can be heterogeneous or homogeneous. Heterogeneous degradation is confined to the surface of the polymeric carrier where it is interfaced with the physiological microenvironment. The external degradation rate is constant, while the non-degraded carrier core retains its chemical integrity. As expected, carriers possessing higher surface to volume ratios undergo faster degradation.

Homogeneous degradation occurs in bulk, where erosion takes place throughout the DDS and the rate of water penetration is greater than its conversion to water-soluble fragments. Initially during this process, there is random removal of hydrogen bonds, due to hydration, followed by cleavage of covalent bonds. PLA and PLGA spheres degrade by this process where their chains are cleaved to monomeric acids, for example, lactic and glycolic acids, that can be metabolized *via* the Krebs cycle. The mass of these polymers decreases due to continuous cleavage and solubilization of low molecular weight fragments and their absorption. However, the role of enzymes in the biodegradation of these polymers is not clear.

Natural Polymers

The use of natural biodegradable polymers to deliver drugs continues to be an area of active research despite the advent of synthetic biodegradable polymers. In light of the benefits of polyesters, these polymers remain attractive primarily because they are “natural” products of living organisms that are readily available, inexpensive, and capable of a multitude of chemical modifications. Most investigations using natural polymers as matrices in DDSs

have centered on proteins (e.g., collagen, gelatin and albumin) and polysaccharides (e.g., starch, dextran, cellulose, etc.).

Collagen has been extensively tested in DDSs because of its unique structural properties. It has been fabricated into a wide variety of forms including: cross-linked films, meshes, fibers, and sponges. Collagen, as a biomaterial, offers several advantages. It is biocompatible and non-toxic in most tissues. It can be easily isolated and purified in large quantities and has well-documented structural chemical and immunological properties.⁷⁰ However, certain properties of collagen have adversely influenced its use as a DDS. These properties include: poor dimensional stability due to swelling; low mechanical strength and elasticity; anti-collagen immune responses; tissue irritation due to residual aldehyde crosslinking agents; poor patient tolerance (e.g., ocular inserts); and variability in drug release kinetics.

Albumin, gelatin, casein, and fibrinogen in the form of microspheres and nanoparticles continue to be exploited as DDSs. Albumin microspheres have been extensively used in diagnostic nuclear medicine for the evaluation of organ function and circulatory studies following administration by a variety of routes. The exploitable features of albumin include its biodegradation into natural byproducts, lack of toxicity and non-antigenicity, and availability. Although the literature contains many examples of albumin microsphere use, there are few reports describing gelatin systems. Gelatin offers several advantages for use in DDSs. For example, this protein weakly interacts with other proteins or drugs, which reduces the chance of encapsulated molecules being altered or aggregated. Moreover, this protein is considered to have low antigenicity.

Hence, natural polymers particularly in the form of microspheres have an important role in DDSs and targeting to selective sites. Yet many concerns must be addressed before they will have widespread use. Among these issues are a better understanding of the kinetics of drug release, more effective ways to control natural polymer DDS, greater understanding of drug-polymer interactions and their effect on shelf life stability. The field would also benefit from additional animal studies to determine host responses to natural polymers, tissue adsorption, biodegradation, and metabolic rates. Perhaps most importantly, well-designed clinical studies are necessary to assess efficacy in relation to current therapies.

Hydrogels

Much attention has focused on developing stimuli sensitive hydrogels that exhibit dramatic changes in network structure or swelling behavior in response to change in pH, temperature, electric field or ionic strength.⁷¹ Most of these systems rely on the sensitive nature of specific interpolymeric interactions within the hydrogel. By exploiting the sensitive nature of these gels, external (magnetic field, light etc.) or internal triggers (pH, enzymes, etc.) can be used for temporal and/ or spatial delivery of biomolecules in the host. Delivery systems can also be designed to release macromolecules in response to increased concentration of a specific compound or changes in the surrounding environment.^{72,73}

These polymer complexes are prepared by free radical solution⁷⁴ or dispersion⁷⁵ polymerization methods. For example, methacrylic acid (MAA) and methoxy-terminated

PEG mono methacrylate added with tetra-ethylene glycol (EG) dimetha-crylate crosslinks the gel matrix. These materials exhibit pH-dependent swelling behavior due to the formation and dissociation of interpolymer complexes.^{76,77} Hydrogels comprising of MAA and EG in equimolar amounts exhibit maximum change in the mesh size or the correlation length of the network due to the pH shift. With an increase in the amount of MAA in the network, the average mesh size in the acidic media increases due to the steady decrease in the number of MAA–EG interactions. Depending on the pH of the surrounding medium, the average mesh size in a network with MAA:EG ratio of 1:1 changes by a factor of 3-fold between swelling states, which corresponds to a 10-fold change in the effective area for diffusion of the encapsulated drug. This results in changes in the diffusion coefficient of the drug.⁷⁸

Thus, hydrogels are ideal for the oral delivery of peptides and proteins due to their large change in network structure over a small pH range. Hence, in the acidic environment of the stomach, drugs would be entrapped in the collapsed gel and protected from degradation. However, in the near neutral environment of the intestine, where protein drugs can be better absorbed, the peptides and proteins could be released albeit susceptible to digestive enzymes. In addition, the polymer structure and composition can be altered by changing parameters such as the crosslinker, crosslinker density and relative amounts of monomers added to achieve controlled delivery of proteins and peptides of therapeutic interest.

PREPARATION OF MICROSPHERES

Biodegradable polymers can be used to prepare microspheres by several methods, each with advantages and disadvantages. It is essential to select an encapsulation process fulfilling the requirements of the desired DDS. The requirements to consider are optimal protein loading, high yield of microspheres, stability of the encapsulated protein, batch uniformity and inter-batch reproducibility, adjustable release profiles, low burst effect, and free-flowing or non-aggregating microspheres.

The encapsulation efficiency of the formulation process should be high so that the contents are not wasted. The protein:polymer ratio should be as large as possible to reduce the mass of the material to be administered. The process of encapsulation should generate high yield of particles of the desired size, depending on the tissue target and route of administration. Importantly, the biological activity of the encapsulated protein should be maintained throughout the formulation process. It is desirable to use a process where exposure to potentially denaturing solvents or heat is low. The process should be simple, reproducible, and scaleable so that different batches of the DDS have the same properties and release characteristics. The encapsulation method used should ideally produce free-flowing microspheres that do not aggregate. This will help to produce uniform, reproducible bioavailability and uptake.

As with all parenteral products, microspheres need to be sterile. This can be ensured by a terminal sterilization step or through aseptic processing. Further, in relation to safety requirements, the excipients and processing solvent used should either be nontoxic or removed from the final product. There are many procedures for preparing lactide–glycolide microspheres for protein delivery like phase separation–coacervation, double emulsion,

spray drying, interfacial deposition, phase inversion microencapsulation, *in situ* polymerization, chemical and thermal crosslinking, to name a few. The most widely used techniques for microsphere preparation of proteins are: spray drying, double emulsion, and phase separation–coacervation.

Spray Drying

In principle, the biodegradable polyester is dissolved in a volatile organic solvent, such as dichloromethane or acetone, the drug in solid form is dispersed in polymer solution by high-speed homogenization, and this dispersion is atomized in a stream of heated air. As the droplets form, the solvent evaporates instantaneously yielding microspheres typically 1–100 μm depending on conditions. The microspheres are collected from the airstream by a cyclone separator and residual solvents can be removed by vacuum drying. Spray drying in a nitrogen atmosphere is technically feasible. Important advantages of this technique over other encapsulation methods are reproducibility, well-defined control of particle size, control of drug release properties of resulting microspheres, and the process is quite tolerant to small changes of polymer specifications. The disadvantages include high capital investment, encapsulation requires lyophilization of protein before dispersion, and homogenization in the organic polymer solution. These process conditions are likely to induce aggregation and denaturation to sensitive proteins and antigens. Hence, stability of micro-encapsulated proteins during processing, release, and storage becomes a major concern.

Double Emulsion Method

In this process, protein in an aqueous solvent is emulsified with a non-miscible organic solution of polymer to form a water in oil emulsion. The organic solvent dichloromethane is frequently used while the homogenization step is carried out using either high-speed homogenizers or sonicators. This primary emulsion is rapidly transferred to an excess of an aqueous medium, containing a stabilizer, usually polyvinyl alcohol. Again homogenization or intensive stirring is necessary to initially form a double emulsion of water-oil-water. Subsequent removal of organic solvents by heat, vacuum or both results in phase separation of the polymer and core to produce microspheres. Instead of solvent evaporation, solvent extraction can also be undertaken yielding microspheres containing protein. The advantages of this method are that the proteins can be encapsulated from an aqueous solution, and high yields and encapsulation efficiencies are obtained. The disadvantages include a complex process, protein sensitivity to polymer and solvents, limited control of release profiles of drug from microspheres, limited shelf life and stability of this DDS.

Phase Separation

Protein is dispersed in solid form into solution containing dichloromethane and polymer. Oil (e.g., silicon) is added to this dispersion at a defined rate, reducing solubility of polymer in its solvent. The polymer-rich liquid phase (coacervation) encapsulates the dispersed drug particles and ‘embryonic’ microspheres are subjected to hardening and washing steps. This process is quite sensitive to polymer properties and residual solvents.

EFFECT OF PARTICLE SIZE, CHARGE AND HYDROPHOBICITY ON MUCOSAL UPTAKE

Particle Size

Particle size charge and size distribution are arguably the most important characteristics of mucosal DDSs. They determine the *in vivo* distribution, biological fate, toxicity, uptake and tissue targeting. In addition, they can also influence the drug loading, release kinetics and stability of particles. Many studies have demonstrated that particles of sub-micron size have a number of advantages over microparticles as a DDS.⁷⁹ Generally, nanoparticles have relatively higher intracellular uptake compared to microparticles and are available to a wider range of biological targets due to their small size and relative mobility. Desai et al. found that 100 nm nanoparticles had a 2.5-fold greater uptake than 1 μ m microparticles, and 6-fold greater uptake than 10 μ m microparticles by Caco-2 cell line.⁸⁰ Indeed, nanoparticles penetrate the submucosal layers of rat intestinal loops, while microparticles were predominantly localized to the epithelial lining.⁸¹

Drug release is also affected by particle size. Smaller particles have a larger surface area; therefore, most of the protein drug is associated at or near the particle surface leading to faster drug release. Whereas, larger particles have large cores, that allow more drug to be encapsulated, but might take longer to release.⁸² However, nanoparticles can have a greater risk of aggregation during storage and dispersion. DDS degradation can also be affected by size. For instance, the rate of PLGA polymer degradation was found to climb with increasing particle size.⁸³ It was thought that encapsulated contents of smaller PLGA particles, can diffuse more readily; large particles have degradation products that remain with the DDS matrix longer, to cause autocatalytic degradation of the polymer material. However, PLGA particles of different sizes were shown to have similar polymer degradation rates *in vitro*.⁸⁴

Many studies regarding size effects on nanoparticle absorption by intestinal epithelia have been performed using polystyrene standard particle suspensions of defined size distributions. Particles with mean diameters of 50 and 100 nm showed a higher uptake in the rat intestine than larger particles.^{85,86} The nanoparticle uptake was followed by its appearance in the systemic circulation and distribution to different tissues. After administration of equivalent doses 33% of the 50 nm and 26% of the 100 nm particles were detected in the intestinal mucosa and, in the case of 500 nm particles only 10% were localized in intestinal tissues. Particles >1 μ m in diameter were exclusively localized in Peyer's patches. Although particles >3 μ m were found occasionally in follicle-associated epithelia and showed no passage to associated lymphoid tissues.

Summarizing numerous absorption studies of polystyrene particles in intestinal tissues reveals important facts that should be considered when designing an oral DDS. Particles <100 nm show higher rates of uptake by absorptive enterocytes than particles >300 nm. The uptake of particles <100 nm by the follicle-associated epithelia is more efficient than uptake via absorptive enterocytes. Uptake of particles >500 nm by absorptive enterocytes is an unlikely event and only particles <500 nm reach the general circulation. The size-dependent

particle passage to mesenteric lymph nodes is still the subject of controversy. The uptake of 100 nm PLGA nanoparticles in the rat intestine was significantly increased compared to larger particles of 1–10 μm . Nearly identical uptake rates were observed in Peyer's patch regions and enterocytes for 100 nm size particles, while particles >100 nm were only detected in the Peyer's patches. In summary, size is an important parameter controlling the internalization of DDSs by the GI tract. As a rule of thumb, sizes <500 nm will be required for optimal uptake and bioavailability.

Hydrophobicity and Surface Charge

Apart from particle size, DDS surface properties also influence how they are taken up by intestinal epithelia. Uptake of particles prepared from hydrophobic polymers seems to be higher than those with more hydrophilic surfaces. Poloxamer coating of polystyrene nanoparticles caused a decrease in GI uptake *in vivo*. Moreover, hydrophobic polystyrene nanoparticles seem to have a higher affinity for M cells than for absorptive epithelia. Less hydrophobic PLGA particles show interactions with both cell types.⁸⁷ These results are in accordance with observations by Norris and Sinko⁸⁸ who investigated the *in vitro* mucus permeability of particles consisting of polymers with varying hydrophobic/hydrophilic characteristics. They found that in contrast to more hydrophilic particles, hydrophobic beads showed poor mucus penetration.

The affinity of charged carriers to intestinal tissues is a subject of great interest. Carboxylated polystyrene beads have significantly lower affinity for intestinal epithelia, especially to M cells, than compared to positively charged or uncharged polystyrene beads.⁸⁵ Coincidentally, nanoparticles consisting of negatively charged polyanhydride copolymers of fumaric and sebacic acid were highly adhesive to the cell surfaces.⁸⁹ After administration, these particles were detected in paracellular spaces, enterocytes and Peyer's patches demonstrating increased absorption rates of encapsulated dicoumarol, insulin and plasmid DNA. In summary, these results showed that uncharged or positively charged nanoparticles consisting of hydrophobic polystyrene have an affinity for follicle-associated epithelium as well as absorptive enterocytes, whereas negatively charged polystyrene nanoparticles show only low affinity for intestinal tissues. Negatively charged nanoparticles comprised of hydrophilic polymers show high bioadhesive properties and are readily absorbed by both M cells and absorptive enterocytes. Hence, surface charge in combination with hydrophilicity of the DDS matrix material affects GI uptake of particles.

When nanoparticles are administered intravenously, they are easily recognized by the host immune system and frequently cleared by phagocytes. Apart from the size of nanoparticles, their surface hydrophobicity determines the amount of adsorbed blood components, mainly opsonins that bind and influence DDS bioavailability.⁹⁰ The association of protein drug with conventional carriers leads to modification of the protein therapeutic biodistribution profile, as it is mainly delivered to the mononuclear-phagocyte system (MPS). Specifically, the MPS might direct DDSs to the liver, spleen, lungs and/or bone marrow. Indeed, once in the bloodstream, non-modified nanoparticles are rapidly opsonized and cleared by macrophages. Hence, DDS formulation should minimize the potential of particle opsonization to prolong their bioavailability. This can be achieved by surface coating with hydrophilic polymers/

surfactants. Alternatively, biodegradable copolymers with hydrophilic segments such as polyethylene glycol (PEG), polyethylene oxide, polyoxamer, poloxamine and polysorbate 80 (Tween-80) can be grafted to nanoparticles to reduce interactions with the MPS. PEG surfaces in brush-like and intermediate configurations reduce phagocytosis and complement activation, whereas PEG surfaces in branched conformations activate complement and favor phagocytosis.^{91,92} Hence, this versatile polymer can be used to modulate mucosal uptake, bioavailability and tissue targeting.

APPROACHES FOR OPTIMIZING UPTAKE AND BIOAVAILABILITY

Enzyme Inhibition

The strategy of employing enzyme inhibitor(s) and absorption enhancers to protect the DDS contents from various enzymatic actions is a popular approach for oral protein drug delivery.²² Due to the nature of enzyme distribution and quantities, the use of digestive protease inhibitor(s) for oral delivery of therapeutic protein would be difficult. However, some successful results have been reported for insulin administration with sodium glycocholate, camostat mesilate, and bacitracin to rats⁹³ and FK-448 with other protease inhibitors.^{20,94} Indeed, CYP3A4 showed a marked increase in the oral bioavailability of cyclosporine.⁹⁵ The absorption of large peptides, cholecystokinin/enkephalin analogs and protein drugs was improved by using protease inhibitor cocktails with attention to specific absorption sites.^{96–98}

A new and interesting method for oral delivery of protein drugs makes use of a polymer-enzyme inhibitor conjugates that protect the therapeutic protein from enzymatic degradation.⁹⁹ Chitosan and its derivatives showed multiple effects on enhancement of insulin, calcitonin, and buserelin absorption, following oral administration by exploiting enzyme inhibitors and their mucoadhesion properties.¹⁰⁰ In similar work, pepstatin analogs were covalently joined to mucoadhesive polymers to inhibit proteolysis of model protein drugs.¹⁰¹ This system provided some advantages by increasing contact-time with the mucosa and maintaining a controlled as well as sustained drug release. It also reduced toxic effects of the inhibitor because of its attachment to the non-absorbable polymer backbone. However, from a practical point of view, the utility of this approach may be limited by high manufacturing costs.¹⁰²

Carrier or Uptake Enhancers

Carrier molecules and permeability agents has also been used to increase the mucosal absorption of protein drugs.^{102,103} This approach was successfully used to orally deliver hGH, IFN α -2b, and insulin.^{103,104} Hence, enhancement of intestinal permeability increased serum concentrations of hGH, IFN, and insulin as much as 800%. Oral absorption of granulocyte colony stimulating factor (G-CSF) and erythropoietin (EPO) was achieved by covalently coupling DDSs with vitamin B₁₂.^{105,106} In this case, uptake occurred via receptor-mediated endocytosis. This system has possible disadvantages since vitamin B₁₂-mediated delivery is limited by its active transport mechanism along with interference from free vitamin B₁₂ in the host.¹⁰⁷

Prodrugs and Analogs

Altering physicochemical properties of DDSs seems to be the easiest approach to increase efficacy, but it requires the synthesis of new chemical entities.^{102,108,109} Changes can be made in lipophilicity, charge, molecular size, solubility, configuration, isoelectric point, chemical stability and affinity to carriers to enhance absorption and systemic circulation. Two specific approaches were made for oral peptide delivery using chemical modification of peptide amide bond to enhance intestinal permeability and the design of compounds bearing nonpeptide templates.¹⁰⁸

Targeting Optimal Absorption Sites

Targeting specific absorption site(s) and dosage via DDS modification (e.g., lipid vesicles, colloidal carrier systems with and without mucoadhesive polymers) are other approaches to improve protein absorption. It has also been suggested that identification of the optimal absorption site for a given peptide or protein is the first step toward the design of a DDS to maximize uptake.⁵⁶ Regional variations in intestinal penetration barriers to peptides may result in regional differences in absorption. For instance, M cells located on the dome epithelium of gut-associated lymphoid tissue are known to sample macromolecules from the ileum through an endocytic pathway.¹¹⁰ Controlling the characteristics of DDS to deliver proteins and large peptide drugs to M cells has been attempted. However, this method has had variable success.^{96–98}

Targeting Intestinal Transporters

Recent advances in molecular biology techniques have made it possible to study the structure, function and distribution of cellular transporters of the mucosa. Based on molecular characterizations of membrane transporter specificities, and kinetics, the modification and targeting of specific transporter(s) is a promising strategy for DDSs to improve bioavailability and tissue distribution.^{111,112} However, the utility of these transporters are limited by the size of molecules that can be delivered.¹⁰⁶ For example, the most permissive transporter, the bile acid transporter, is limited to peptides <400 Da. Hence, strategies to improve the interaction of nanoparticles with adsorptive enterocytes and M cells of Peyer's patches can be classified into those utilizing specific binding to ligands or receptors and nonspecific adsorptive mechanisms.

Adhesive Carrier Systems

Various colloidal systems have been studied for absorption of DDSs, such as sub-micron emulsions, lipid suspensions, liposomes, and polymeric nano- and microparticles. Controversy still exists on the factors that govern GI uptake, including size, size distribution, consistency, hydrophobicity, and surface properties of colloidal carriers.¹⁰⁷ Prolonged contact of nanoparticles with absorptive cells may be achieved using bioadhesive materials. Bioadhesion has also been followed by particle uptake in a second step.¹¹³ Hence, biomaterials with both adhesive and protective properties might be desirable for oral protein drug delivery to insure drug stability and bioavailability.

In general, GI tract absorption of macromolecules and particulate materials involves either paracellular or endocytic pathways. The paracellular route of absorption is accessible in

<1% of the mucosal surface area. Using polymers such as chitosan,¹¹⁴ starch⁵⁷ or polyacrylate¹¹⁵ can increase the paracellular permeability of macromolecules. Endocytic absorption occurs by either receptor-mediated or adsorptive endocytosis. The later process is initiated by an unspecific cell surface interaction due to electrostatic forces.¹¹⁶ Hence, adsorptive endocytosis depends primarily on the size and surface properties of the DDS. If the surface charge is positive or uncharged, then it will provide an affinity to adsorptive enterocytes through hydrophobic interactions. Whereas if the DDS has a negative surface charged and is hydrophilic, it will have an even greater affinity for adsorptive enterocytes and M cells.

Liposomes

The use of liposomes has been largely abandoned as oral DDSs due to poor stability under the diverse physiological conditions typically found in the GI tract.¹¹⁷ While homogeneous lamellar 100 nm liposomes were taken up by M cells,¹¹⁸ similar studies have led to the overall conclusion that liposomes are ineffective as vehicles for oral vaccines. However, mucoadhesive liposomal systems prepared by coating negatively (phosphatidyl choline) or positively charged (salicylic acid) lipid suspensions with mucoadhesive polymer solutions, such as chitosan and carbopol, showed some success in intestinal absorption of protein drugs, for example, insulin and calcitonin.¹¹⁹

Nanoparticles

The surface area of the human mucosa extends to ~200 times that of skin. The histological architecture of the mucosa is designed to efficiently prevent uptake of particulate matter from the environment. One important strategy to overcome the GI barrier is to deliver therapeutic proteins in a DDS, such as nanoparticles, that are capable of enhancing interaction and uptake by the epithelium of the GI tract. Nanoparticles, as defined by solid particles, with size in the range of <200 nm, allow encapsulation of the protein drugs inside a matrix that protects them from enzymatic and hydrolytic degradation. Various biomaterials of polymers, lectins, etc, can be employed to make nanoparticles using techniques of emulsion polymerization, interfacial polymerization, emulsification evaporation, solvent displacement, desalting, emulsification and diffusion.¹²⁰ Solvent displacement and salting-out have received increasing attention because they provide less stress to protein drugs. The physicochemical properties of nanoparticles and their behavior on exposure to physiological media are mediated by their chemical structures and surface characteristics.¹⁰⁷

The development of suitable nanoparticle carriers remains a challenge due to the fact that the bioavailability of these molecules is limited by the physiology of the epithelial barriers of the GI tract and susceptibility to digestive enzymes. Fortunately, the formulation of polymeric nanoparticles allow for encapsulation of bioactive molecules that protects against enzymatic and hydrolytic degradation. For instance, it has been found that insulin-loaded nanoparticles preserve insulin activity and produce blood glucose reduction for up to 14 days following the oral administration.

MACRO- AND NANO-PARTICLE INDUCERS OF IMMUNITY AND TOLERANCE

Oral Vaccines

Peptide and protein encapsulation in DDSs has been applied to several oral vaccination applications. Moreover, mucosal vaccination, and more specifically oral vaccination would lead to lower production and administration costs. Compared to systemic administration, mucosal vaccination targets the common mucosal immune systems and avoids pain as well as the many of the risks associated with injections. Vaccination at the site of the potential infection is highly desirable to obtain a local mucosal defense. Indeed, >95% of all the pathogens enter via mucosal routes. Locally produced secretory IgA constitutes >80% of all antibodies produced in the host and are considered to be among the most important protective humoral immune factors.^{121,122} Furthermore, a fascinating feature of the common mucosal immune system is that administration of an antigen at one mucosal site can lead to the generation of immune responses at distant mucosal sites. Finally, mucosal immunization has the potential to elicit an immune response against infectious diseases for which current parenteral vaccines either have a low efficiency or minimally effective, such as vaccines against HIV and tuberculosis.^{123,124} In the scope of oral vaccination, it is particularly interesting to favor the uptake of antigen-loaded DDSs by M cells. There is a consensus that Peyer's patch M cells represent a key portal site for some bacteria, viruses and prions to subsequently initiate mucosal immunity. To this end, several strategies have been employed to deliver vaccines by this route.

Eldridge et al.¹²⁵ asserted that microspheres <5 µm in diameter were transported by M cells for mucosal immunization. As a result, numerous microparticulate systems were developed for oral immunization. Many of the polymeric biodegradable microparticles have been composed of PLA or PLGA. Many vaccine antigens have been successfully encapsulated in PLGA microparticles without altering their structural and immunologic integrity.^{126,127} In general, ovalbumin, peptides, bacterial toxoids, inactivated bacteria and, more recently, plasmid DNA entrapped in PLGA microparticles has been shown to induce both mucosal and systemic immune responses following oral or intragastric administration.^{128–132}

While many antigens have been successfully delivered, it is important to mention that protein denaturation can occur during encapsulation in PLA or PLGA polymers, due largely to exposure to organic solvents, elevated temperatures and aqueous organic interfaces.¹³³ Latex and PLGA particles (<500 nm) may be taken up better than the particles 1–5 µm.¹³³ However, there is no compelling evidence that nanoparticles are more effective than microparticles in oral delivery of vaccines. Up to now, only a few studies have examined the capacity of biodegradable nanoparticles to induce mucosal immunity after oral administration.

Jung et al.¹³⁰ used poly vinyl alcohol-co-PLGA to reach a high level of tetanus toxoid (TT) loading by adsorption. Particles given *per os* to mice induced significant TT-specific IgG and IgA immune responses, when compared to intra peritoneal administration of antigen. Particle size was found to significantly affect the induction of antibody production; smaller

particles induced higher titers. In addition, cholera toxin B subunit (CTB) entrapped in submicron particles (~400 nm) caused comparable immunogenicity than the potent oral adjuvant, cholera toxin.¹³⁴ The influence of particle size on immune response after oral delivery of BSA entrapped in 200, 500, and 1000 nm PLGA particles have also been studied. Despite the literature showing extensive intestinal absorption of nanoparticles, high antigen-specific serum IgG antibody levels are routinely observed following oral administration of 1 μ m particles, compared with particles 200–500 nm in size.

PLGA nanoparticles containing *Helicobacter pylori* lysates stimulate antigen-specific mucosal and systemic immune responses and induce Th2-type responses.¹³¹ However, antibody titers of groups immunized with *H. pylori*-loaded PLGA nanoparticles were lower than particles immunized containing free *H. pylori* protein associated with the CTB. Fattal et al.¹³⁵ showed protection of mice against following oral administration of *Salmonella typhimurium* antigen-encapsulated PLGA particles. Oral administration of *Bordetella pertussis* antigen-entrapped PLGA microparticles or nanoparticles was shown to protect against respiratory challenge.¹³⁶ This study demonstrated that a single oral dose of encapsulated *B. pertussis* fimbria could confer protection.

However promising, there are few commercially available oral vaccines. Although particle uptake by M cell has been repeatedly demonstrated in rodents, it remains uncertain whether this will be the case in man.¹³⁷ Two rather disappointing Phase I oral vaccine trials using PLGA have been conducted the last 10 years. Orally administrated *E. coli* colonization factor antigen II (CFA II) entrapped in PLGA microspheres add only 30% efficacy.¹³⁸ A significant increase of anti-CFA II IgA and IgG antibody secreting cells in human volunteers, following oral administration of water *E. coli* CFA II-entrapped PLGA microspheres.¹³⁹

Group B *Streptococcus vaccine* (GBS) is the leading bacterial cause of neonatal sepsis and meningitis. Although antibiotics have decreased the severity of this infection, the best long-term solution lies in the development of effective vaccines. The GBS capsular polysaccharide is a major target of antibody-mediated immunity. The feasibility of producing a GBS vaccine having the ability to produce both a local (i.e., IgA) immune responses and systemic humoral responses that are capable of active and transplacental passive immunization was investigated using an oral DDS. Inactivated GBS antigen was encapsulated in poly (D,L-lactic-co-glycolic acid) by a water-in-oil-in-water (w/o/w) multiple emulsion technique along with immunostimulatory cytosine phosphate guanosine (CpG) motif adjuvants.¹⁴⁰ Immunization of female mice with these microparticles containing GBS type III polysaccharide and CpG adjuvant resulted in significantly higher GBS antibody responses, as compared to nonencapsulated GBS polysaccharide or PLGA-encapsulated GBS polysaccharide vaccine without the addition of the CpG.

Tetanus, caused by tetanus toxin, is considered a significant health problem worldwide, with approximately one million new cases occurring each year. Mice were immunized with TT-encapsulated sulfobutylate-grafted PLGA nanoparticles by oral and nasal route.¹³⁰ Encapsulated TT and *Haemophilus influenzae* type b capsular polysaccharide conjugated to TT (Hib-T) in PLGA microspheres were evaluated for their humoral immunogenicity in

mice. A single injection of these microencapsulated vaccines elicited high antibody levels, which persisted for several months. The antibody levels were similar or superior to those elicited by conventional formulations of Alum-adsorbed TT or soluble Hib-T conjugate vaccine.¹⁴¹

Diphtheria is a communicable disease caused by *Corynebacterium diphtheriae*, which colonizes the mucosa and forms a pseudomembrane at the infection site. This pathogen produces diphtheria toxin, which is responsible for the typical systemic toxemia. Fortunately, anti-diphtheria toxoid (DT) antibodies can protect against diphtheria. DT has been encapsulated in various types of PLA and PLGA microspheres by spray drying and coacervation.¹⁴² Recently, poly-epsilon-caprolactone (PCL)-PLGA blend and co-polymer nanoparticles were used to orally immunize against diphtheria by encapsulating DT.¹⁴³ *In vitro* studies using Caco-2 cells revealed higher uptake of the relatively hydrophobic PCL nanoparticles in comparison to polymeric PLGA, PLGA-PCL blend or co-polymer nanoparticles. Hydrophobic DT nanoparticles induced the highest serum IgG antibody responses when delivered by intranasal route.

Cholera is an acute intestinal infection caused by *Vibrio cholerae* and produces an enterotoxin causing copious, painless, watery diarrhea that can quickly lead to severe dehydration and death. Inactivated *V. cholerae* was successfully entrapped in the PLG microspheres by double emulsion method with trapping efficiencies up to 98%.¹⁴⁴ The immunogenic potential of *V. cholerae*-loaded microspheres was evaluated in adult mice by oral immunization in comparison to *V. cholerae* solution. Results indicated that following oral co-administration of these microspheres, *Vibrio*-specific serum antibody responses were induced with vibriocidal activity.

Oral DNA-Based Vaccines

Plasmid DNA can be encapsulated in nanoparticles with significant retention of biological function, after oral delivery in polymers can elicit systemic and mucosal antibody responses to encoded antigens. Oral administration of chitosan nanoparticles (200 nm) complexed with DNA coding for a dominant peanut allergen elicited antigen-specific secretory IgA and serum IgG2a titers.¹⁴⁵ Similarly, oral feeding of DNA-loaded chitosan nanoparticles can raise immune responses against native dust mite allergens in mice, whereas intramuscular immunization alone did not. Nanoparticles might also facilitate mucoadhesion and DNA uptake by host cells to enhance transfection efficiency. Bivas-Benita et al. compared the potential of chitosan nanoparticles (~500 nm) loaded with *Toxoplasma gondii* GRA1 encoding DNA plasmid (pDNA) or chitosan microparticles loaded with recombinant GRA-1 protein to elicit GRA-1-specific immune responses after intragastric administration using different prime/boost regimens.^{145,146} Interestingly, the GRA1 DNA vaccine resulted in higher anti-GRA1 antibody levels. These results showed that oral delivery of DNA-based vaccines using chitosan carriers efficiently induced immune responses to expressed protein. The type of immune response, however, may largely depend on the prime/boost regimen and the type of vaccine used. A single oral immunization of PLGA nanoparticles (~500 nm) containing rotavirus VP6 DNA was sufficient to elicit rotavirus-specific serum IgG and IgM as well as intestinal IgA responses.^{147,148}

Antigen-Loaded Particles Induce Oral Tolerance

Some polymers are able to elicit an immune response alone when administered orally, such as chitosan, which support a Th2/Th3-biased microenvironment at the mucosal level, in absence of antigen.¹⁴⁹ Systemic unresponsiveness to orally delivered antigens (oral tolerance) may adversely affect oral vaccination or, conversely, could be used as a therapy for individuals that respond to innocuous antigens (e.g., food allergens, transplantation antigens or commensal bacteria).¹⁵⁰ Oral administration of free antigens has been recognized as a method to induce antigen-specific peripheral tolerance.¹⁵¹ Oral tolerance is mediated by two mechanisms that depend on the dose of administered antigens.^{152,153} Repeated administrations of low doses of antigen can induce active suppression. This mechanism functions by expanding antigen-specific regulatory T cell population that actively suppress T helper responses to antagonize pro-inflammatory responses.¹⁵⁴ In contrast, higher doses of antigen induce T cell clonal deletion and/or anergy, characterized by both antibody and cell-mediated immune response inhibition.¹⁵⁵

Biodegradable microparticles, first promoted for vaccine development, now appear attractive as inducers of oral tolerance. Kim et al.¹⁵⁶ showed that a single administration of PLGA nanoparticles containing type II collagen (CII) could induce oral tolerance more efficiently than repeated oral administrations of intact CII. Type II collagen-encapsulated in 300 nm PLGA particles was detectable in Peyer's patches, by microscopy 14 days after stable oral administration.¹⁵⁶ This regimen significantly reduced the incidence and severity of arthritis, serum IgG anti-CII antibodies, and CII-specific T cell proliferation as compared with controls. Similarly, newborns are prone to milk allergies that can be prevented by inducing oral tolerance to β -lactoglobulin. This major allergenic protein was encapsulated in PLG microspheres by w/o/w multiple emulsion technique. Oral administration of these microspheres drastically reduced the amount of protein required to reduce specific anti- β -lactoglobulin IgE response.¹³⁵ A single feeding of 5 μ g of encapsulated β -lactoglobulin tolerized BALB/c mice to subsequent challenge. The tolerogenic dose was 10000-fold less than the dose of soluble antigen alone.

ORAL DELIVERY OF THERAPEUTIC PEPTIDES AND PROTEINS

Insulin

Insulin is the most important regulatory hormone in the control of glucose homeostasis. WHO reports indicated that more than 50 million people around the world suffering from diabetes require daily parenteral injections of insulin. For the treatment of Type I diabetes, insulin is administered typically by three injections per day. An insulin DDS for long-term therapy of this disease would be well received, as this system could alleviate daily injections and possibly improve patient compliance. Insulin has been incorporated into the hydrogel microparticles for oral delivery.¹⁵⁷ Upon exposure to an acidic environment, <10% of insulin is released from the microparticles. However, when the pH of the surrounding medium rises to physiological pH in the small intestine, the insulin trapped inside the gel network is rapidly released. PEG chains in this network serve to maintain the biological activity of the insulin by preventing binding to the ionizable backbone of the encapsulation matrix.^{78,158} The effectiveness of this system for delivering insulin was evident from the

improved hypoglycemic effect on oral administration.¹⁵⁹ Oral administration of insulin-loaded in poly isobutylcyanoacrylate nanocapsules caused a dramatic reduction of blood glycaemia in diabetic rats.¹⁶⁰ It was later shown that these nanocapsules were absorbed by intestinal epithelial cells.¹⁶¹ However, much of the nanocapsules were degraded upon transport across M cells. Adding to this disappointment, Cournarie et al.¹⁶² underlined the high variability in insulin transport across the intestinal barrier.

Oral administration of nanosphere-based insulin delivery systems comprised of polyfumaric anhydride and PLG maintained normoglycemia in the face of a glucose challenge.¹⁶³ Oral administration of chitosan–insulin nanoparticles (50 U or 100 U/kg) were effective at lowering serum glucose levels of streptozotocin-induced diabetic rats.¹⁶⁴ Most likely, this effectiveness can be attributed to the local effect of insulin availability in the intestine.¹⁶⁵ Finally, Alonso co-workers tested the efficacy of insulin-loaded chitosan–glucomannan nanoparticles following oral administration to normal rats. This carrier system was able to elicit a delayed hypoglycemic response, 14 h post-administration.

Perhaps the delay in clinical studies of the oral insulin delivery systems have been deferred by the high variability of the insulin concentration delivered to the blood. Moreover, high doses of insulin will be required due to its low bioavailability for oral delivery. To increase availability, insulin has been encapsulated in blends of PEG along with PLA and PLG by a w/o/w multiple emulsion technique with entrapment efficiencies of 56 and 48% for PLG/PEG and PLA/PEG, respectively.¹⁶⁶ Insulin-loaded microspheres were capable of controlling the release of insulin for 28 days with in vitro delivery rates of 0.94 and 0.65 mg of insulin per mg of particle per day in 4 days and with a steady release rate of 0.4 and 0.43 mg of insulin per mg of particle per day over 4 weeks, respectively. In addition, the extensive degradation of PLG/PEG microspheres over 4 weeks as compared to PLA/PEG blends resulted in stable particle morphology along with reduced fragmentation and aggregation of associated insulin.

These studies may not translate to clinical oral delivery. Only a small portion of insulin orally administered has been shown to reach the blood stream, mainly due to extensive degradation of the protein in the GI tract. Further, insulin's size and hydrophilicity limits its transport across the intestinal epithelium. No specific transport mechanism is present for the passage of insulin cross the intestinal cell monolayer. For many years, researchers have tried to find a solution to these problems and in effect increase the oral bioavailability of insulin. The use of permeation enhancers, protease inhibitors chitosan coatings to stabilize the protein and improve cellular permeability, entrapment of insulin within microparticles and protein modification to resist proteolytic attack are additional creative approaches to orally deliver insulin.^{157,167–172}

Other Peptide and Protein Drugs

Calcitonin has been delivered using oral DDSs. When calcitonin was incorporated in nanoparticles, oral absorption was enhanced in rats and consequently calcium concentration in blood was decreased, compared to oral administration of a calcitonin solution.¹⁰⁷ Mucoadhesive (chitosan) polymeric nanospheres containing calcitonin were also shown to reduce blood calcium 12 h after administration at doses of 125 IU/Kg and 250 IU/ Kg.^{119,173}

Chitosan nanoparticles have also been used to deliver hydrophobic peptides such as ciclosporin A.¹⁷⁴ Chitosan nanoparticles orally administered to beagle dogs provided an improved absorption compared to the currently available ciclosporin A microemulsion (Neoral®). Finally, chitosan particles were used to encapsulate the erythropoietin gene resulted in a rapid increase of hematocrit that was sustained for >1 week.¹⁷⁵

Das and Lin¹⁷⁶ developed a double-coated (Tween-80 and PEG 20000) poly butylcyanoacrylate (PBCA) DDS for oral delivery of dalargin. While the mechanism of PBCA nanoparticle transport of dalargin from the GI tract and release to brain was not elucidated, the study noted a significant dalargin-induced analgesia with double-coated PBCA nanoparticles compared to single-coated particles. The lack of comparative studies makes it difficult to determine the most efficient formulation to orally deliver therapeutic peptides or proteins. To this end, the core of nanocapsules (i.e., liquid vs. solid) has no effect on oral bioavailability of calcitonin.^{173,177}

Chitosan-coated nanoparticles resulted in less (27%) serum calcitonin levels, when compared to uncoated particles. Chitosan-modified with PEG improved the stability of nanocapsules in GI fluids and reduced nanocapsule cytotoxicity. Therefore, modulating the degree of chitosan (i.e., charge) PEGylation modified the stability, cytotoxicity, and enhanced absorption of the particles. This new carrier seems to be close to the ideal carrier combining an adequate size (160–250 nm), moderate encapsulation efficiency (44–50%), stability and mucoadhesion. The translation of these animal studies to human application may not be so straightforward. Unfortunately, the inaccuracy of the dose delivered, and bioavailability greatly limit using DDSs for oral delivery of peptides.

Prolidase deficiency results in chronic intractable skin ulcerations, particularly of lower limbs. To counter this problem, recombinant prolidase was encapsulated in PLGA microspheres by w/o/w multiple emulsion technique.¹⁷⁸ Microencapsulation stabilizes enzymatic activity and resulted in active peptidase release *in vitro* and *in vivo*. Although this was not orally delivered this opens the doors for enzyme replacement therapy through oral protein DDSs.

Interferon α (IFN α) is used in the treatment of chronic hepatitis C virus infection. A novel microsphere delivery system was developed to encapsulate recombinant IFN α in calcium alginate cores surrounded by poly DL-lactide-polyethylene glycol (PLPEG) by w/o/w multiple emulsion technique.¹⁷⁹ Core-coated microspheres stabilized IFN α in the PLPEG matrix. Importantly, the extent of burst release reduced to 14% in core-coated microspheres from 31% in conventional microspheres, highlighting this new approach for water-soluble macromolecular drug delivery. Perhaps this formulation approach can be used in the future for orally delivery of protein therapeutics.

Orally Administered Antibodies

There is evidence to indicate that a fraction of orally administered antibodies can survive passage through the human gastrointestinal tract and retain structural characteristics and immunologic activity. This raises the possibility of treating GI infections by passive immunization using orally administered immunoglobulin. GI infections are important causes

of morbidity and mortality, particularly in developing nations. Passive immunization is currently under experimental and clinical evaluation, and results are encouraging.

Infectious agents such as rotavirus, *E. coli*, *V. cholerae*, *Clostridium parvum*, and *H. pylori* are known to cause gastroenteritis. Severe diarrhea is a major complication and if untreated is life threatening. Evidence seems to indicate that gastroenteritis of most microbial origins may be treated by passive immunization and may not necessarily require attenuated or killed pathogens for vaccination. Guarino et al.,¹⁸⁰ successfully treated rotavirus-induced diarrhea in infants using a nonspecific human serum immunoglobulin. In addition, the shorter duration of diarrhea was associated with a shorter length of rotavirus shedding. Similar results have been shown using rotavirus-specific immunoglobulin isolated from bovine colostrums rather than nonspecific immunoglobulin to treat cases of infant gastroenteritis.¹⁸¹

In other studies, several HIV-infected patients with *C. parvum*-induced diarrhea were successfully treated with specific bovine colostrum immunoglobulin.^{180,182,183} Those who are pre-disposed to gastrointestinal infections, such as children and adults with severe immunodeficiency diseases¹⁸⁴ and recipients of bone marrow transplants¹⁸⁵ may also benefit from passive immunization. Interestingly, the degradation of immunoglobulin in the GI tract of patients with bone marrow transplants is impaired because of the destruction of the intestinal mucosa resulting from the transplant preparation regimen, high gastric pH, rapid intestinal transit, limited oral intake and antibacterial therapy which reduces flora.^{185,186}

REGULATORY CONSIDERATIONS FOR ORAL PROTEIN DELIVERY

Preclinical and toxicological studies must be performed in accordance with guidelines set by the FDA to eliminate formulations that are too toxic for human or animal use and to indicate whether an oral DDS, for example, biodegradable nanoparticles or microspheres containing protein drugs are effective and safe. To gain FDA approval for any oral DDS formulation, it is necessary to consider the presence of residual solvents and polymers that might remain after delivery as well as preclinical and toxicological studies. Virtually all DDS processes require the use of an organic solvent such as dichloromethane or ethyl acetate for maintaining polymer solubility during fabrication. These solvents may pose significant health risks for long-term exposure. Acceptable residual amounts of these solvents may vary among regulatory agencies. For example, the International Conference on Harmonization (ICH) guideline for permissible dichloromethane is 6 mg/day unless it can be shown that the residual solvent is released in a sustained fashion for several days.

The FDA requires the safety and biocompatibility of all polymeric materials used for medical and dental applications to be established prior to use. The tests used to establish safety will depend on the type of device, the drug to be delivered, and its application. *In vivo* and *in vitro* testing of polymeric materials should be designed to investigate the polymer mucosal interface reactions, effects on subsurface tissue, and systemic effects. After oral delivery, bioabsorption studies would begin with animals at predetermined time periods along with mucosal tissue isolation and preparation for immunohistochemical or cytochemical analysis. Scoring systems have been used based on the number of specific cell

types within a specific area of detected DDS to effectively compare the biocompatibility of polymers.^{187–189}

Skin patch tests are common tests for delayed type hypersensitivity evaluation.¹⁹⁰ Hence, acute toxicity could generally be measured by applying a test material comprised of the encapsulated contents onto shaved intact or abraded skin. Various biological parameters such as body weight, mortality, and gross pathological evaluation would also be assessed includes multiple doses over longer periods of time to recognize both acute and chronic toxicity of DDS components.¹⁹¹ Evaluation of biocompatibility of polymers via tissue culture techniques are based on analysis of cellular growth, division, enzyme levels, and synthesis of important macromolecules.^{192,193}

CONCLUSIONS AND FUTURE PROSPECTS

The GI tract has formidable physiological and chemical barriers that will pose several challenges for oral DDS. However, significant progress has been made with each of these obstacles. The development of composite formulation methods, which improves bioavailability yet meets regulatory requirements for reproducibility, intra- and inter-subject variability, and manufacturing cost will be difficult; however, the potential of this emerging field is promising.

Despite considerable research efforts and impressive progress made in recent years, the feasibility of biodegradable DDSs for therapeutic protein or vaccine delivery systems remains open to debate. Micro and nano encapsulation techniques have evolved to allow for the incorporation of sensitive proteins able to resist the harsh environment of the mucosa. It seems that the w/o/w multiple emulsion technique is the most advanced. Indeed, biodegradable polymers (e.g. PLGA) have been used for such DDSs, with well-known degradation properties. An area requiring study is analytical characterization of encapsulated proteins. Advanced methods for protein characterization will be needed to definitively solve real and perceived problems of DDS protein stabilization in DDSs. Further, the development of methods to correlate *in vitro* with *in vivo* protein release would advance the field and increase the rate of development of new DDSs. More collaborative interactions between immunologists, biochemists pharmacologists, and physiology specialists are required to understand protein release and uptake. The future success of biodegradable oral DDSs will primarily depend on the commitment of academia and industry to develop new strategies to orally deliver therapeutic proteins in efficient and cost effective DDSs.

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Figure 1.

Protein drug delivery. An ideal oral protein drug delivery system (DDS) should provide multi-functionality for targeting and controlled release. This in turn will yield improved therapeutic drug index, lower toxicity, and targeted delivery of protein drugs in a site-specific fashion by multiple routes of administration, including *per os*.

Figure 2.

Schematic transverse section of intestinal epithelium and follicle-associated epithelium (FAE) depicting M cell transport of particles or pathogens. Particles can be transported by (I) passive transcellular transport (through the enterocyte). Active transcellular can occur with modifications to enable carrier-, fluid phase-, or receptor-mediated transcytosis (e.g., B₁₂-conjugate). (II) Paracellular transport (between adjacent cells) with or without tight junction enhancers. Only small (<200 Da) hydrophilic molecules are absorbed through this pathway. (III) Particles can be absorbed by M cells of FAE found in Peyer's patches. These particles are susceptible to phagocytosis and degradation by cells of the host immune system (e.g., macrophages (MΦ), immature dendritic cells (iDC), mature DC (mDC)) that present antigens to T cells and B cells. Subsequently, components of the particles are transported to the intestinal lamina propria by these transport mechanisms and are delivered to the periphery by lymphatic and/or vascular endothelium.