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Review article

Biodegradable nanoparticles for oral delivery of peptides: is there a role for polymers to affect mucosal uptake?

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Abstract

Numerous authors have demonstrated uptake of micro- and nanospheres, consisting of natural or synthetic polymeric materials from the gastrointestinal tract over the past two decades. The exploitation of particulate carrier systems for the delivery of peptides and other hydrophilic macromolecules via the oral route remains a challenging task due to morphological and physiological absorption barriers in the gastrointestinal tract. This review examines recent progress in the field of nanoparticle uptake from this site of administration. Since most studies have been performed with poly(styrene) particles of different sizes relatively little is known about both the effect of physicochemical particle properties critical for absorption after peroral application, and the mechanisms of gastrointestinal particle uptake. Apart from particle size, type and composition of the polymers used for micro- or nanoencapsulation are crucial for an uptake and transport across mucosal barriers. Factors such as particle surface charge and hydrophilic/hydrophobic balance of these polymeric materials have not been investigated systematically since adjustment of these particle properties is almost impossible without synthetic modification of the polymers. The current findings will be reviewed and compared to those obtained with nanoparticles consisting of a novel class of charged comb polyesters, poly(2-sulfobutyl-vinyl alcohol)-graft-poly(D,L-lactic-co-glycolic acid), SB-PVAL-g-PLGA, allowing adjustment of physicochemical nanoparticle properties with a single class of polymers.

Keywords: Nanoparticle; Intestinal absorption; Mucosal vaccination; Biodegradable polyester

1. Introduction

Significant advances in biotechnology and biochemistry have led to the discovery of a large number of bioactive molecules and vaccines based on peptides, proteins and oligonucleotides. The development of suitable carrier systems remains a major challenge for the pharmaceutical scientist, especially after oral application/vaccination since bioavailability is limited by the epithelial barriers of the gastrointestinal tract and gastrointestinal degradation by digestive enzymes. Polymeric nanoparticles (NP), defined as solid particles with a size in the range of 10–1000 nm, allow encapsulation of the drugs inside a polymeric matrix, protecting them against enzymatic and hydrolytic degradation. Recently it was shown that oral application of NP containing insulin reduced blood glucose levels in diabetic rats for up to 14 days [1], stimulating research into colloidal carrier systems for oral administration of peptides considerably.

Not only enhancement of oral peptide bioavailability, but also the concept of mucosal vaccination has become more prominent in recent years. Oral and nasal dosage forms improve patient compliance and facilitate frequent boosting, necessary to achieve a protective immune response. Epithelial surfaces are the port of entry for many viral or bacterial pathogens, and mucosal immunity can be regarded as an important protective barrier.

One limitation for NP as oral delivery systems is the requirement that particles need to be absorbed from the gastrointestinal tract with a sufficient rate and extent from the gastrointestinal tract (GIT). Physiological factors affecting NP absorption and their interdependence with physicochemical properties of the polymeric carrier system is not well understood and will be discussed in more detail below.

The traditional role of polymers in colloidal drug delivery systems has been that of an inert excipient. Since these materials are absorbed into the organism additional requirements need to be considered, such as biocompatibility and biode-

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gradability. Neither aspect has been characterized in detail and both could be problematic in chronic therapy. Moreover, physicochemical properties of polymers influence the formation of NP regarding size and encapsulation efficiency. Therefore, new biomaterials combining all these considerations might be of general interest for the design of nanoparticulate carrier systems for mucosal peptide delivery.

2. The mucosal surface as a potential site for NP uptake

Human mucosae extend to a surface area of at least 200 times that of the skin, amounting to more than 400 m^2 in adults [2]. The GIT provides a variety of physiological and morphological barriers against protein or peptide delivery, such as (i) proteolytic enzymes in the gut lumen (e.g. pepsin, trypsin, chymotrypsin) (ii) proteolytic enzymes at the brush border membrane (endopeptidases), (iii) the mucus layer, (iv) the bacterial gut flora and (v) the epithelial cell lining itself [3]. The histological architecture of the mucosa is designed to efficiently prevent uptake of particulate matter from the environment.

2.1. Cellular components of the mucosal barrier

Mucus-covered enterocytes (Fig. 1), representing the largest population of cells in the mucosa of the small intestine (ca. 90%), are arranged as a single cell monolayer at the gastrointestinal surface. They control the absorption of nutrients, electrolytes and fluids and function as barrier to the environment.

Although the penetration of potentially toxic materials and harmful pathogens is restricted, the mucosal surface is not a completely impenetrable barrier. The absorption of macromolecules and particulate matter seems to be facilitated by different mechanisms [4,5]. The immunological role of the mucosa requires a communication between the exterior and the interior of the GIT and enabling immune reactions by different mechanisms, e.g. enterocytes can be stimulated to produce various cytokines and interleukins and dendritic cells present antigens via MHC complexes to subendothelial T cells [6].

In intestinal tissues highly specialized enterocytes, the membranous microfold (M) cells, collect antigens and macromolecules (Fig. 1) from the gastrointestinal surface. From a morphological point of view, M cells, derived from crypt cells [7], differ from absorptive enterocytes by (i) underdeveloped microvillous and glycocalyx structures, (ii) the presence of apical microfolds, (iii) increased intracellular vacuolization and (iv) absence of mucus. Absorptive enterocytes and M cells constitute the follicle-associated epithelia (FAE). The FAE shuttle colloidal antigens, small bacteria and viruses, via a transcytotic mechanism to lymphoid microcompartments which are in close contact with their covering epithelia [6]. Solitary lymphoid nodules are widespread along the entire intestine and tend to increase in number in the ileum as oval aggregates in the antimesometrial gut wall, the so-called Peyer's patches [8].

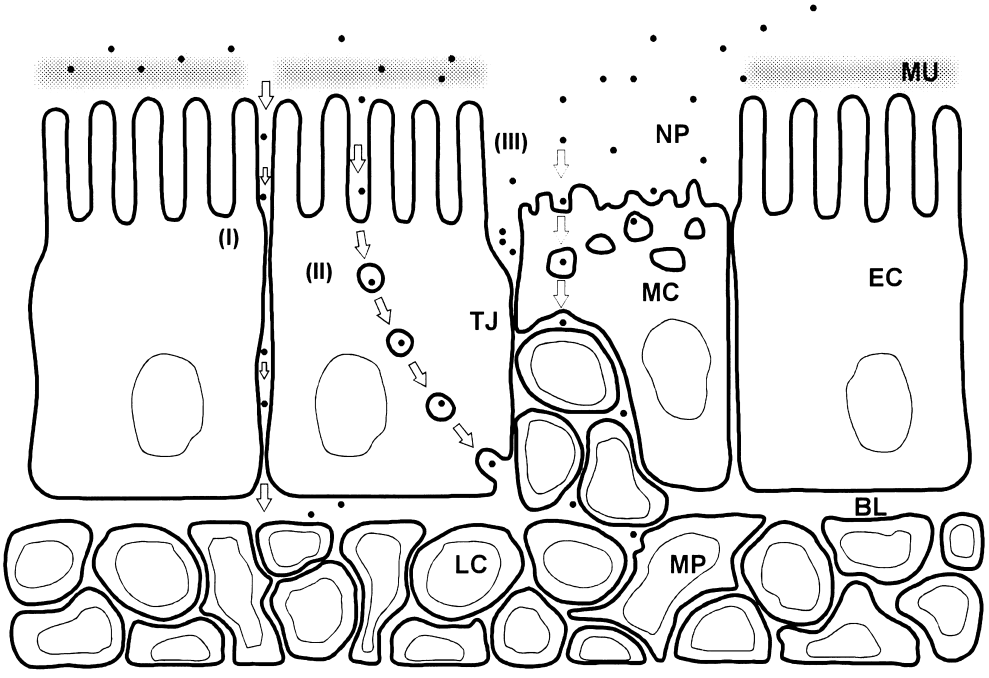

Fig. 1. Schematic drawing of mucus (MU) covered absorptive enterocytes (EC) and M cells (MC) in the small intestine. Lymphocytes (LC) and macrophages (MP) from underlying lymphoid tissue can pass the basal lamina (BL) and reach the epithelial cell layer which is sealed by tight junctions (TJ). Possible translocation routes for NP are (I) paracellular uptake, (II) endocytotic uptake by enterocytes and (III) M cells.

This physiological transport mechanism is thought to be of particular relevance for colloidal carriers, such as NP.

2.2. Gastrointestinal absorption mechanisms of macromolecules and particulate materials

The physician Herbst was the first to describe the passage of small particles through the intestinal mucosa after feeding starch to dogs as early as 1844. The appearance of starch particles in blood and urine was attributed to a damage of the intestinal mucosa, until Volkheimer [9] attributed the passage of particulates through the mucosa of the small intestine to a physiological process. This phenomenon was then designated as 'persorption' and was thought to be the result of the peristalsis. There is still much controversy surrounding the mechanism of particle transport across the epithelial barrier [10]. For the transport of macromolecules and particulates through the gastrointestinal epithelium, different pathways can be distinguished.

2.2.1. The paracellular route for absorption of NP

Since the paracellular spaces, sealed by tight junctions, contribute less than 1% of the mucosal surface area and the pore diameter of these junctions was reported to be < 10 Å, significant paracellular transport of macromolecules and particles is an unlikely event (Fig. 1, I). Paracellular permeability for peptides can be enhanced, however, by polymers, such as chitosan [11], poly(acrylate) [12,13] or starch [14]. These polymers are thought to enhance paracellular transport of peptides such as insulin by charge mediated polymer binding to epithelia, resulting in a structural reorganization of tight junction-associated proteins (chitosans) or by a reduction of free extracellular Ca^{2+} concentration (polyacrylates). In addition, the inactivation of intestinal proteolytic enzymes, located in the gut lumen (e.g. trypsin) or at the brush border (aminopeptidases) contributes to their absorption promoting effects.

These hypothetical mechanisms for increasing the paracellular permeability have been derived mostly from cell culture models, and their relevance for the in vivo situation is unclear. Uptake of NP > 50 nm cannot be easily explained by a temporary widening of tight junctions. Also, the absorption of microparticles as proposed by Volkheimer needs further clarification [15].

2.2.2. The endocytotic pathway for absorption of NP

Endocytotic processes are characterized by pinching of membrane vesicles from the plasma membrane, followed by an internalization of the engulfed extracellular materials. The endocytic pathway is controlled by a series of highly complicated and iterative molecular sorting events which result in the transport of membrane vesicles to subcellular compartments [16], for instance, the delivery of molecules to lysosomal compartments. Of particular interest are transcytotic processes at mucosal surfaces by which macromolecules or particles, internalized at the apical plasma membrane of the epithelial cells, are transported to the contralateral plasma membrane and released to the basolateral compartment (Fig. 1, II–III) [17]. During endocytotic processes various types of membrane vesicles have been observed, ranging from the actin-dependent formation of phagosomes, involved in particle uptake, to smaller clathrin-coated vesicles, responsible for the internalization of extracellular fluid and receptor-bound ligands [16].

Receptor mediated endocytosis (RME) is an active transport mechanism, requiring receptors at the apical cell membrane. After specific ligand binding to the receptor, an endocytotic process is initiated, which is saturable as well as energy- and time-dependent. For a recent review see Refs. [16,18]. Two different pathways have been described, namely (i) clathrin-dependent RME and (ii) potocytosis, which is based on clathrin-independent RME. While clathrin coated vesicles show diameters of 60 nm, potocytotic vesicles have been described with sizes up to 500 nm. In some cases RME may be followed by transcytosis to subepithelial compartments, e.g. uptake and translocation of maternal IgG antibodies from the neonatal GIT.

By contrast, adsorptive endocytosis does not require specific ligand-receptor interactions at the cells surface. This process is initiated by an unspecific physical adsorption of material to the cell surface by electrostatic forces, H-bonding or hydrophobic interactions [19] and is followed by an invagination of the local plasma membrane, forming intracellular vesicles. Similar to RME this process occurs predominantly at the base of microvilli. Adsorptive endocytosis is energy-dependent, saturable and can lead to either intracellular processing or a transcytotic transport of the engulfed macromolecules or particles. Since specific receptors are not required, adsorptive endocytosis depends primarily on size and surface properties of the adsorbed material. Therefore, this mechanism may to have potential for oral delivery of macromolecules and colloidal carriers, although its efficiency remains to be demonstrated.

2.3. Mucosa-associated lymphoid tissues (MALT)

The mucosa is protected by various non-immunologic (pH, proteolytic enzymes, mucus, gut-associated microflora) and highly specialized co-operating immunologic mechanisms. In healthy human adults, this local immune system contributes almost 80% of all immunocytes which are localized in the different mucosa associated lymphoid tissues (MALT) [20]. The gut-associated lymphoid tissues (GALT) are composed of widespread lymphoid microcompartments, e.g., the Peyer's patches in the small intestine, the appendix and solitary follicles in the large intestine [21]. After transepithelial transport the antigens are processed by antigen presenting cells and presented to T-lymphocytes which can provide T-cell help to primed B cells in the same region. The primed B cells leave the MALT via the lymphatics and enter the blood stream. Finally, the B cells reach different distant mucosae, where they differentiate

into plasma cells. The typical humoral immune response at the mucosal surface is characterized by the production of secretory immune globulin A, sIgA. Apart from the humoral immune response, T-lymphocyte-mediated cytotoxicity, natural killer activity and antibody-dependent cell mediated cytotoxicity may also play an important role [22]. The GALT has important ramifications for oral administration of NP. For one, chronic oral application could induce an immune response against carrier and/or drug. Secondly, delivery of antigens to the GALT might be exploited to induce mucosal immunity after oral vaccination.

2.4. Methods to study the interaction of NP with gastrointestinal tissues

Similar to transport studies of low molecular weight drugs, investigations of intestinal particle uptake can be performed using different models under in vivo [8,23], in situ [24], ex vivo [25] or in vitro conditions [26,27]. Animal models have been utilized routinely to quantitate the oral bioavailability of drugs applied in nanoencapsulated form, mostly by comparison with an intravenous dose. Also the evaluation of tissue or body distribution of NP requires studies in animal models [28].

In vivo studies both in experimental animals and humans [29] have been conducted to obtain information of intestinal tissue distribution of NP. They distinguish between the cellular site of absorption, enterocytes or M cells, and the influence of the mucus layer. The results are characterized by very high intra- and inter-species variabilities. These variations in NP uptake are often attributed to the number of Peyer's patches, which are known to vary considerably in number with animal species and age. Mouse [30], rat [31], guinea pig [32], rabbit [33] and pig [25] models have been chosen to study intestinal NP absorption and the observed extent of absorption varies considerably [30,31]. Therefore, an 'ideal' animal model has not emerged so far and extrapolation to humans remains to be demonstrated.

Quantitation of NP uptake after oral administration in animal models is difficult and different analytical methods have been employed, including light-, fluorescent- and electron microscopy, radiometry and fluorometry, or fluorescence activated cell sorting (FACS). For a detailed review see Ref. [26].

Microscopy, especially confocal laser scanning microscopy and transmission electron microscopy, are valuable tools to demonstrate the presence and localization of NP in gastrointestinal tissues. Quantification using microscopic techniques is, however, problematic since NP need to be counted in microscopic slides after tissue preparation from different sites along the GIT. Therefore, only a semi-quantitative picture of the NP distribution in GIT tissue is obtained. Preparation of slides and counting of NP is prone to artifacts, especially for small NP with sizes <500 nm. Reliable quantitative data are difficult to obtain by microscopic techniques and mass balance studies have been carried out using radioactively labeled NP. While assessment of particle uptake using radio-labeled NP has the advantage of being very sensitive, instability of the label can generate false results and no information on NP localization is obtained except with gamma scintigraphy.

Particle quantification using fluorescently labeled NP by means of both fluorimetry and FACS is rapid, simple and sensitive. Quantification is only possible if a calibration curve, prepared in the same way as the samples, is used. Since these techniques involve a destructive step, they usually do not discriminate between intracellular and surface located NP.

Transepithelial transport is often regarded as the rate-limiting step for NP absorption after oral application. Therefore, in vitro or ex vivo models of the epithelium can be useful for studying carriers for mucosal delivery of drugs, e.g. excised gut tissue or cell culture models. Although excised gut tissue allows investigations of NP interactions with mucus, enterocytes as well as FAE, this method is subject to the viability of such preparations and extrapolations need to be carefully validated.

Cell culture models of the intestinal epithelium have been used recently to study NP interactions with epithelia. The Caco-2 cell line, a model for studying transport and metabolism of low molecular weight drugs, gained most interest to assess the binding and uptake of NP to intestinal epithelium. Caco-2 cells share feature of both absorptive intestinal cells (glycocalyx, microvilli, tight junctions) and M cells (lack of mucus, the ability to transport virus proteins [34] or bacteria [35] to submucosal layers).

A major advantage of cell culture models is their potential to allow both quantitation and visualization of NP transport and localization on a cellular level using a noninvasive technique. Moreover, cyctotoxic effects can be assessed by monitoring the transepithelial resistance, membrane damage (release of cytosolic lactate dehydrogenase) or metabolic interactions (determination of mitochondrial dehydrogenase activity). Cell integrity and cytotoxicity are especially important parameters when evaluating intracellular NP uptake, since toxic side effects of the carrier system are known to increase epithelial permeability and can lead to misinterpretations.

The extent and size dependence of NP uptake into Caco-2 cells and their translocation have been discussed in the literature. While Frey et al. [36] reported that the glycocalyx of fully differentiated Caco-2 cells impedes internalization of NP with 5 nm size, translocation through intact Caco-2 monolayers in the order of ca. 20% were reported by Hillery et al. [37]. Moreover, Desai et al. [38] as well as McClean et al. [39] observed a distinct uptake of large NP and microspheres with mean diameters from 1 μm (Desai: 15%) up to 10 μm (Desai: 6%), without any loss of monolayer barrier function. The uptake mechanism for NP in this size range, exceeding the microvilli dimensions by far, is still unknown.

Recently, Kerneis et al. [40] reported the conversion of Caco-2 cells into a M-cell like cell culture model, after co-

cultivation with lymphocytes from Peyer's patches. This in vitro model could be very interesting for studying in vitro interactions between NP and M cells. The reproducibility of the M-cell model and its physiological and histological properties need to be established and validated before extrapolations to other methods can be made.

3. Gastrointestinal carrier systems for peptides

One strategy to overcome the gastrointestinal barrier is the association of the drug with a synthetic colloidal carrier system. This concept provides an improved drug stability against enzymatic degradation in the harsh intestinal environment due to the protecting polymer matrix. Furthermore, prolonged contact of NP with absorptive gastrointestinal cells may be achieved using bioadhesive polymers. Adhesion of colloidal carriers to the mucosal epithelium (bioadhesion) could be followed by NP uptake in a second step. Therefore, biomaterials with both adhesive and protective properties would be desirable for oral peptide delivery systems.

Various colloidal carrier systems have been studied for absorption enhancement of peptides, such as sub-micron emulsions, lipid suspensions, liposomes [41], iscoms [42] as well as polymeric nano- and microparticles [43]. Controversy still exists on physicochemical factors governing gastrointestinal uptake, including size, size distribution, consistency, hydrophobicity and surface properties.

Liposomes, which have been frequently investigated, have been abandoned due to insufficient stability under in vivo conditions [44]. The concept of solid polymer NP was proposed and pioneered by Speiser and coworkers [45] and since 1976 various nano-encapsulation techniques using a wide range of polymers have emerged. Important manufacturing techniques and polymers are summarized in Table 1.

Proteins and antigens can be encapsulated into the NP and/or adsorbed to the surface by physical or chemical mechanisms. Formulation of colloidal polymeric carriers is often anything but straightforward. In the case of the w/o/w double emulsion techniques, most appropriate for the microencapsulation of proteins, homogenization using ultrasonication or high-speed turbines must be employed to obtain dispersions in the required size range. Many proteins are sensitive to high shear stress and are destroyed during NP preparation. The localization of the protein after NP preparation is also often unknown. Therefore, novel methods based on solvent displacement and salting out have received increasing importance [46,47], because they provide less stress to protein drugs. A systematic modification of polymers as (described in Table 1) in order to optimize them for NP processes has not been performed.

3.1. Targeting of NP to epithelial cells in the GIT using ligands

Several strategies have been proposed to improve the efficiency of NP absorption. Targeting strategies to improve the interaction of NP with absorptive enterocytes and M cells of Peyer's patches can be classified into those utilizing specific binding to ligands or receptors and those based on non-specific/adsorptive interactions.

3.1.1. Lectins

The surface of enterocytes and M cells display cell-specific carbohydrates, which may serve as binding sites for colloidal drug carriers, containing appropriate ligands. Certain glycoproteins, lectins, bind selectively to this type of surface structure by specific, receptor-mediated mechanisms. Different lectins have been studied to enhance oral peptide absorption [48,49]. Bean lectin (phaseolus vulgaris), which also, facilitates NP internalization in absorptive enterocytes, is not suitable because it induces strong systemic immune responses after oral administration [50]. Tomato lectin, less toxic than bean lectin and also known to show an uptake in cell Caco-2 cell culture, was intensively investigated [51,52]. Unfortunately, it was found to bind preferentially to the gastrointestinal mucus [53] and lesser extent to M-cell or enterocyte surfaces.

Table 1
Summary of NP manufacturing techniques and employed polymeric materials [46,47]

Technique	Material
Emulsion polymerization	Poly(alkyl methacrylate)
	Poly(alkyl cyanoacrylate)
	Poly(styrene)
	Poly(vinylpyridine)
	Poly(acroleine)
	Poly(glutaraldehyde)
Interfacial polymerization	Poly(alkyl cyanoacrylate)
	Poly(lysine) derivatives
Emulsification evaporation	Poly(lactic acid)
	Poly(lactide-co-glycolide)
	Poly(ε-caprolactone)
	Poly(β-hydroxybutyrate)
	Ethyl cellulose
Solvent displacement	Poly(alkyl methacrylate)
	Poly(lactic acid)
	Poly(lactide-co-glycolide)
	Poly(ε-caprolactone)
Salting out	Cellulose acetate phthalate
	Poly(alkyl methacrylate)
	Ethyl cellulose
	Poly(lactic acid)
	Poly(lactide-co-glycolide)
Emulsification diffusion	Poly(lactic acid)
	Poly(ε-caprolactone)
Desolvation, denaturation[a]	Albumin
	Casein
	Gelatin
	Alginate
	Chitosan
	Ethyl cellulose

[a] Induced by heat, pH changes, salts, organic solvents, complexing with macromolecules, sonification or chemical crosslinking.

The development of lectin coupled or coated carriers for specific GIT targeting turned out to be difficult for several reasons. Firstly, the glycosylation pattern of M cells shows significant variations in affinities between both animal species and investigated gut region [54]. Secondly, little is known about the lectin binding pattern in the human gastrointestinal tract and identification of suitable target molecules is a time-consuming undertaking. Thirdly, lectin binding to enterocyte microvilli was shown to destroy the brush border [55]. Overall, the concept of using lectins to enhance NP uptake in the GIT is still in its infancy and more work is necessary to demonstrate the viability of this approach also for humans.

3.1.2. Vitamin B-12

Vitamin B-12 absorption from the gut under physiological conditions occurs via receptor-mediated endocytosis. An essential requirement is the presence of the intrinsic factor, a 60 000 g/mol mucoprotein. Intrinsic factor is formed in the mucus membrane of the stomach and binds specifically to cobalamins. The mucoprotein complex reaches the ileum, where resorption is mediated by specific receptors. The ability to increase oral bioavailability of various peptides (e.g. granulocyte colony-stimulating factor, erythropoietin) and particles by covalent coupling to vitamin B-12 has been extensively studied by Russell-Jones and coworkers [50,56,57]. Possible disadvantages of the vitamin B-12 mediated carrier systems are the limited capacity of this active transport mechanism and the interference with vitamin B-12 absorption in chronic therapy. From the data obtained so far, extrapolation to humans remains speculative and requires additional investigations.

3.2. Absorption enhancement using nonspecific interactions

Increased NP internalization can not only be achieved by utilizing specific ligands, but also by nonspecific interactions of the colloidal carrier systems with epithelia. The absorption of various NP prepared from different polymers has frequently been studied addressing factors, such as size, surface charge and hydrophobicity.

3.2.1. Size dependence of NP absorption

Many studies regarding size effects of NP absorption by intestinal epithelia have been performed using poly(styrene) standard particle suspensions of defined size distributions (Table 2). Jani et al. [8,28] observed that particles with mean diameters of 50 and 100 nm showed a higher uptake in the rat intestine than larger particles. The NP 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 NP were detected in the intestinal mucosa and GALT. In the case of 500 nm NP only 10% were localized in intestinal tissues. NP $>$ 1 μm in diameter yielded only little uptake and exclusive localization in Peyer's patches. Although NP $>$ 3 μm were found occasionally in FAE they showed no passage to associated lymphoid tissues.

Summarizing numerous absorption studies of poly(styrene) NP in intestinal tissues there seems to be agreement concerning several facts: (i) NP $<$ 100 nm show higher extend of uptake by absorptive enterocytes than NP $>$ 300 nm. (ii) The uptake of NP $<$ 100 nm by the follicle-associated epithelia is more efficient than uptake via absorptive enterocytes. (iii) Uptake of NP $>$ 500 nm by absorptive enterocytes is an unlikely event. (iv) Only NP $<$ 500 nm reach the general circulation. (v) The size-dependent NP passage to mesenteric lymph nodes is still the subject of controversy.

Findings similar to those of Jani and Sass were reported by Desai et al. [65] for the absorption characteristics of NP prepared from poly(lactic-co-glycolic acid), PLGA. The uptake of 100 nm NP in the rat intestine was significantly increased compared to larger particles of 1 and 10 μm. While nearly identical uptake rates were observed in the Peyer's patch regions and the enterocytes for 100 nm NP, larger NP were only detected in the Peyer's patches. Therefore, size is an important parameter controlling the internalization of NP into epithelia of the GIT and as a rule of thumb, sizes smaller than 500 nm are required.

3.2.2. Hydrophobicity and surface charge

Apart from particle size, NP surface properties seem to influence the uptake by intestinal epithelia (Table 3).

Uptake of NP prepared from hydrophobic polymers seems to be higher than from particles with more hydrophilic surfaces. Poloxamer coating of poly(styrene) NP caused a decrease of gastrointestinal uptake in vivo [73]. Moreover, hydrophobic poly(styrene) NP seem to have a higher affinity for M cells than for absorptive epithelia. Less hydrophobic biodegradable PLGA NP show interactions with both cell types [69]. These results are in accordance with observations of Norris and Sinko [27] who investigated the in vitro mucus permeability of NP consisting of polymers of varying hydrophobic/hydrophilic balance. They found that in contrast to more hydrophilic NP, hydrophobic poly(styrene) beads showed poor mucus penetration.

The affinity of charged colloidal carriers to intestinal tissues is the subject of much discussion. Carboxylated poly(styrene) NP show significantly decreased affinity to intestinal epithelia, especially to M cells, compared to positively charged and uncharged poly(styrene) NP [8]. On the other hand, Mathiowitz et al. [74], who used NP consisting of negatively charged poly(anhydride) copolymers of fumaric and sebacic acid, observed highly increased particle adhesion to the cell surfaces. These NP, detected in paracellular spaces, enterocytes and Peyer's patches, increased the absorption rates of encapsulated dicoumarol, insulin and plasmid DNA. The results are in accordance with results of other authors investigating the affinity of negatively charged poly(acrylic acid) NP, to intestinal epithelia [13].

In summary, these results support the following conclu-

Table 2
Size-dependent NP absorption by the gastrointestinal tract

Type of particle	Size range	Model	Result	Ref.
Poly(styrene) NP	500 nm–1 μm	Rat	Absorption of 500 nm NP in M cells. Absorption time is dependent on particle size	[58,59]
Poly(styrene) NP	50 nm–3 μm	Rat	Higher uptake of small NP. NP > 100 nm did not reach the bone marrow, and NP 300 nm were absent from blood	[28]
Poly(styrene) NP	2–9 μm	Mouse	2 μm NP in lymph nodes 9 μm NP retained in GALT. Extent of uptake depends on dose and fed state of the animals	[23]
Poly(styrene) NP	50 nm–1 μm	Rat	Extent of uptake is size-dependent. Smaller NP show faster absorption and organ distribution than larger NP	[60,61]
Carboxylated poly(styrene) NP	100–900 nm	Rabbit, rat	Accumulation of 900 nm NP at FAE, 100 nm NP were found along the serosal surface. Higher extent of uptake in rabbit	[62]
Poly(styrene) NP	150 nm–10 μm	Rat	Better uptake of 0.5 μm NP than 3.0 μm NP. 10 μm NP are retained in the mesenteric lymph nodes	[63]
Poly(styrene) NP	2–20 μm	Rat	All NP accumulated in GALT. Only 2 μm and 6 μm NP are transferred in the nodal tissues	[64]
BSA-loaded poly(lactide-co-glycolide) NP	100 nm–10 μm	Rat (intestinal loop)	Uptake of smaller NP is 15–250-fold higher. FAE show 2–200-fold higher uptake than normal intestinal tissue	[65]
OVA-loaded poly(lactide-co-glycolide) NP	600 nm–26 μm	Mouse	Uptake into FAE increased with increasing NP size up to 11 μm, thereafter decreased and became zero with NP > 21 μm	[66]
Poly(lactide-co-glycolide) NP	1 μm–10 μm	Rat (intestinal loop)	Uptake of small NP by FAE. No uptake of larger NP. Small NP were recovered in the liver, lymph nodes and spleen	[67]
Poly(lactide-co-glycolide)NP	100 nm–10 μm	Caco-2 cell culture	NP uptake is size-, concentration-, time-, temperature-dependent. Smaller NP show better uptake than larger ones	[38]

sions. (i) Uncharged and positively charged NP consisting of hydrophobic poly(styrene) provide an affinity to FAE as well as to absorptive enterocytes, whereas (ii) negatively charged poly(styrene) NP show only low affinity to any type of intestinal tissues. (iii) Negatively charged NP from more hydrophilic polymers show highly increased bioadhesive properties and are absorbed by both M cells and absorptive enterocytes. Negative charges on the NP surface are not the only requirement, a combination of both NP surface charges and increased hydrophilicity of the matrix material seem to affect the gastrointestinal uptake in a positive sense.

3.2.3. Systemic appearance of NP after oral administration

A retrospective analysis of literature data concerning the total amount of NP absorbed after oral administration and the systemic appearance or organ distribution turned out to be very difficult. Doses, application schedules, vehicles for NP administration, NP itself as well as animal models and analytical methods vary considerably. Therefore, it is not surprising that the literature on the extent of intestinal absorption of NP is not consistent. Extents of absorption vary from a few ppm up to nearly 50% (Table 4).

Although uptake of NP through the gut wall by different

Table 3
Influence of particle surface characteristics on gastrointestinal NP absorption

Type of particle	Size range	Model	Result	Ref.
(a) Poly(styrene), (b) carboxylated poly(styrene) NP	100 nm–3 μm	Rat	Better uptake of (a) compared to (b)	[8]
(a) Poly(styrene), (b) poly(methyl methacrylate), (c) poly(hydroxy butyrate), (d) poly(lactide)s, (e) poly(lactide-co-glycolide)s, (f) ethyl cellulose, (g) cellulose acetate hydrogen phthalate, (h) cellulose triacetate NP	1–10 μm	Mouse	Good uptake of (a–g), no uptake of (h–j). Uptake was specific to FAE and restricted to NP < 10 μm. The majority of NP < 5 μm were transported through the efferent lymphatics within macrophages	[68]
(a) Poly(styrene), (b) poly(lactide-co-glycolide) NP	500 nm	Rabbit	Binding by (b) to the FAE is an order of magnitude lower than that of (a)	[69]
Carboxylated poly(styrene)	870 nm	Rat	Speed and extent of uptake increased with volume and tonicity of suspending vehicle	[70]
(a) Blank poly(styrene) NP and poly(styrene) NP coated with (b) poloxamer 407	60 nm	Rat	(a) showed good uptake. (b) inhibited uptake in the small intestine and reduced uptake from the large intestine	[71]
Amidineated, sulfatylated, carboxylated carboxyl modified poly(styrene) NP,	10 nm–2 μm	Intestinal mucin (Ussing chambers)	Permeability varied according to the surface characteristics. PS-amidine NP (less hydrophobic) had a higher permeability than other NP	[27,72]
Poly(styrene) NP coated with different poloxamers	60 nm	Rat	Adsorption of surfactants onto NP inhibit uptake in the small intestine and shunted to the large intestine. Total uptake was decreased compared to blank NP	[73]
Gold tracer, dicumarol, insulin and plasmid DNA-loaded poly(fumaric-co-sebacic acid) NP	700 nm	Rat (inverted sac)	NP show adhesion to mucus and traversed FAE and normal epithelium. Transfer of drugs to the circulation	[74]

mechanisms and gastrointestinal tissues seems to be uncontested, the usefulness of this phenomenon to increase oral bioavailability of macromolecular hydrophilic drugs remains to be demonstrated. In the case of poorly absorbed drugs with a small therapeutic window the high variability of drug absorption may be problematic. Furthermore, agreement exists that intestinal absorption of NP is significantly smaller than that of conventional drugs. High costs of peptides and small extents of NP absorption may render these carriers commercially unfeasible.

Particle size as well as particle properties have been recognized as crucial parameters for bioadhesion to and adsorption from gastrointestinal tissue. NP surface charge and the hydrophilic/hydrophobic balance have an especially important impact on gastrointestinal NP absorption.

An optimal carrier will combine positive aspects as outlined above but minimize problems such as biocompatibility and biodegradability. Therefore, NP made from a novel type of polymer were prepared. These biodegradable polyesters may be advantageous for gastrointestinal NP delivery, as they allow both the adjustment of hydrophilic/hydrophobic balance and the possibility to introduce different degrees of substitution using charged groups.

4. SB-PVAL-g-PLGA NP for gastrointestinal protein delivery

Novel biodegradable polyesters for colloidal protein carriers were synthesized with this concept in mind. By grafting short lipophilic, poly(lactic-co-glycolic acid) chains onto charge-containing, hydrophilic backbones, namely sulfobutylated poly(vinyl alcohol) (PVAL), novel biodegradable comb polyesters (Fig. 2) were obtained [82,83].

The PLGA chain composition as well as chain lengths can easily be varied to affect the balance of hydrophilic and hydrophobic domains. Moreover, the PLGA chain number can be varied by the molecular weight of the backbone,

Table 4
Extent of absorbed particles, systemic appearance or organ distribution after gastrointestinal NP absorption

Type of particle	Size range	Model	Absorption	Ref.
Poly(methyl methacrylate) NP	100 nm	Rat (bile cannulated)	10–15%	[75]
Poly(styrene) NP	600–750 nm	Rabbit (intestinal loops)	~5%	[24]
Poly(styrene) NP	1.1 μm	Rat, rabbit	39%	[33]
Poly(styrene) NP	1 μm	Rat, rabbit	5%	[28]
Poly(styrene) NP	2–9 μm	Mouse	0.01%	[23]
Poly(styrene) NP	500 nm	Rabbit	M cells ~5–10 NP/45 min	[76]
Poly(styrene) NP	60 nm	Rat	10%	[77]
TiO2 NP	500 nm	Rat	11.9%	[78]
Poly(styrene) NP	2 μm	Rat	1.78	[79]
Poly(styrene) NP	500 nm	Rat	4.28%	[71]
Poly(styrene) NP	1 μm	Rat	0.2–50 ppm	[31]
Poly(styrene) NP	50 nm, 500 nm, 1 μm	Rat	18%, 8%, 1%	[80]
Poly(lactide co-glycolide) NP	1–5 μm, 5–10 μm	Rat	12.7%, 0.11%	[67]
Poly(lactide co-glycolide) NP	100 nm, 500 nm, 1 μm, 10 μm	Rat (intestinal loop)	PP[a] 30–49% and NPP[b] 28–35%, PP 0.1% and NPP 0.06–0.3%, PP 1.5–7.5% and NPP 0.01%, PP 0.8–1.3% and NPP 0.3–0.5%	[65]
Poly(lactide) NP	800 nm	Rat	5.68%	[81]

[a] Peyer's patch.
[b] Non-Peyer's patch.

whose degree of charge modification offers further possibilities to modify NP properties.

By means of a modified solvent displacement technique [84], small negatively charged NP with narrow size distribution were prepared from the SB-PVAL-g-PLGA. The amphiphilic character of these polymers and the blocked structure (PLGA chains combined with a charged macromolecular backbone) lead to NP with a core–corona-like structure [84]. Controlled polymer precipitation is followed by rearrangement of the lipophilic and hydrophilic domains. This results in an arrangement with an inner polyester core and an outer backbone coating. It was possible to adjust NP surface charge and hydrophilicity by the initial degree of backbone charge modification as well as by the PLGA chain lengths. Negative surface charges seem to be one structural prerequisite of colloidal carriers, leading to significant bioadhesion on the mucosa of the small intestine [74,85,86]

Data summarized in Table 5 demonstrate that NP-cell association depends on the type of polymer used for particle preparation. Uncharged hydrophilic NP (non-modified PVAL-g-PLGA, SB(14)-PVAL-g-PLGA) showed the least bioadhesion. The use of uncharged more hydrophobic NP (poly(styrene), PLGA) resulted in a moderate increase of cell affinity. Bioadhesion of highly charged SB(43)-PVAL-g-PLGA NP was twice as high as for PLGA and poly(styrene) NP [87].

Summarizing the bioadhesion data, the following can be concluded. (i) In the case of NP prepared from comb polymers with SB-PVAL backbones an increasing degree of negatively charged groups increased the affinity to intestinal Caco-2 cells. Interestingly, SB-PVAL-g-PLGA NP with low surface charge reduced cell affinity compared to uncharged PVAL-g-PLGA NP. (ii) Comparing minor charged NP of polymers with different hydrophobicity (SB(14)-PVAL-g-PLGA vs. PLGA and PVAL-g-PLGA vs. poly(styrene)), more hydrophobic NP showed increased bioadhesion.

Negative surface charge and hydrophobicity increased the affinities of NP to Caco-2 monolayers, whereas the anionic character seems to be the most critical parameter.

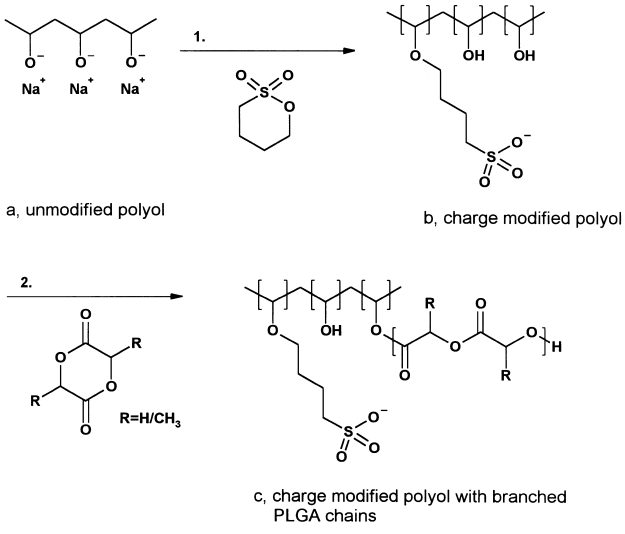

Fig. 2. Synthesis pattern and chemical structures of the novel branched biodegradable comb polyester SB-PVAL-g-PLGA.

Table 5
In vitro Caco-2 cell association of NP consisting of different polymers [92]

Particle	Size (nm)	ζ-Potential (mV)	Cell-associated fraction[a] (%)
Poly(styrene) NP	90 ± 5	−12.3	21.1 ± 3.2
Poly(lactide-co-glycolide) NP	112 ± 22	−32.3	19.4 ± 1.1
PVAL-g-PLGA NP	119 ± 15	−14.9	15.7 ± 1.1
SB(14)-PVAL-g-PLGA NP	116 ± 19	−34.1	12.1 ± 0.7
SB(26)-PVAL-g-PLGA NP	109 ± 6.4	−41.7	23.4 ± 0.7
SB(43)-PVAL-g-PLGA NP	104 ± 9.3	−56.4	39.1 ± 1.4

[a] Initial NP dose per monolayer surface: 0.76 μg/mm^2.

4.1. Uptake of SB-PVAL-g-PLGA NP

The high monolayer association with negatively charged SB(43)-PVAL-g-PLGA NP is mainly attributed to an intracellular uptake after 80 min of incubation as shown in Fig. 3. At earlier time points (40 min) NP were predominantly located at the cell surfaces (Fig. 3a). After 80 min, NP fluorescence was detected in intracellular compartments, especially the perinuclear and was also observed within the filter membrane pores below the cells, suggesting a transcytotic transport mechanism (Fig. 3b).

During these experiments cell viability (membrane, metabolic and tight junction integrity) was not impaired by NP concentrations of up to 2.5 mg/ml and incubation times of 200 min compared to the buffer controls.

These data, especially the membranous and metabolic integrity of the cells, demonstrate both the excellent mucosal compatibility of the novel comb polyesters and the fact that NP bioadhesion and uptake were not due to toxic effects.

4.2. Protein adsorption onto SB-PVAL-g-PLGA NP

Loading of NP by a high degree of adsorption of proteins due to the charge interactions between the surface located SB groups and proteins [88] is another interesting feature. Adsorptive loading might prove interesting since the preparation of protein loaded NP, commonly using double emulsion techniques, can be critical due to inactivation of proteins. Moreover, for mucosal vaccination, dispersion of an antigen in a polymeric matrix may be unfavorable since the entrapped antigen is not accessible for presentation to T lymphocytes after the uptake until its release from the polymeric matrix.

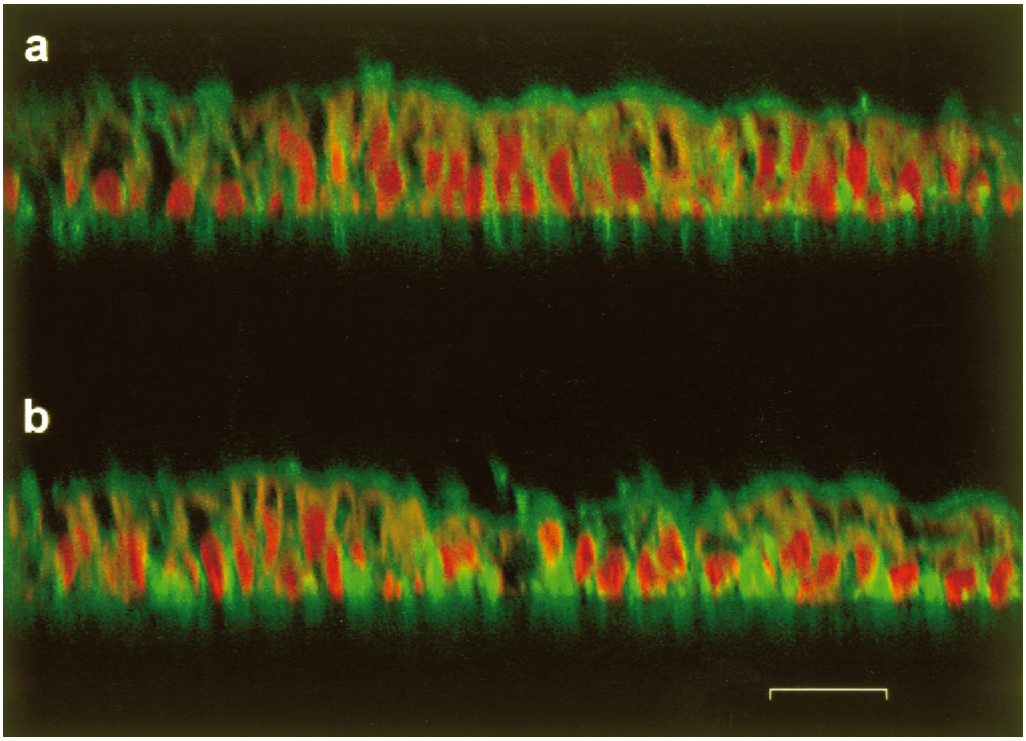

Fig. 3. Uptake and translocation of coumarin 6-labeled SB(43)-PVAL-g-PLGA NP (104 nm) using Caco-2 monolayers (Z-sections, confocal laser scanning microscopy) after 40 (a) and 80 min incubation (b) at 37°C. Formalin fixed monolayers were counter-stained with PI to visualize cell nuclei (red fluorescence). Scale bar = 20 μm.

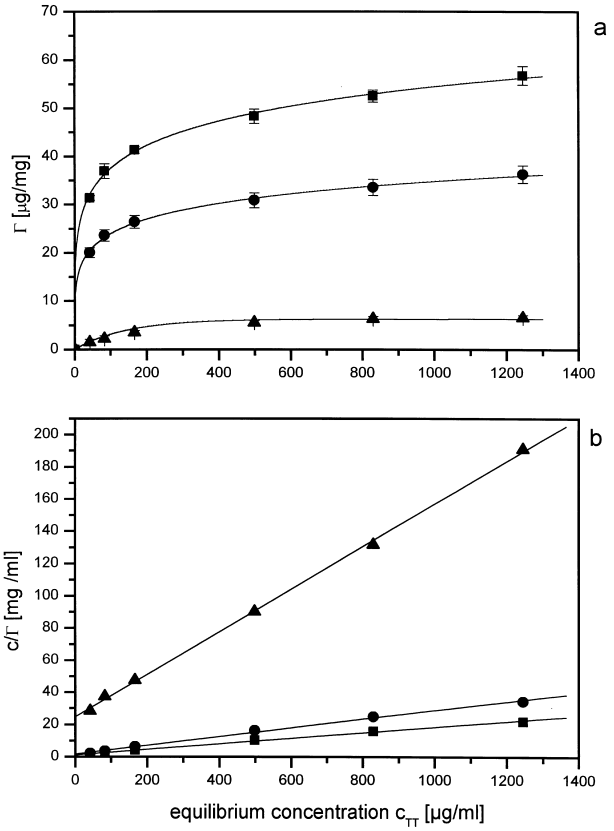

Fig. 4. (a) Adsorption isotherm for tetanus toxoid on different polymeric nanoparticles. (b) Adsorption isotherms have been fitted using Langmuir's equations. ■, SB(43)-PVA-g-PLGA; ●, SB(27)-PVA-g-PLGA; ▲, PLGA.

To evaluate this concept, adsorption studies with the model protein, tetanus toxoid (TT), have been performed. The adsorption isotherms for TT on SB(43)-PVAL-g-PLGA NP, SB(27)-PVAL-g-PLGA NP and PLGA50 NP are given in Fig. 4a. The adsorption isotherms are of the high-affinity type and have been reported to be typical for flexible non globular proteins previously [89]. The antigen adsorption data was fitted to the langmuirian type of adsorption isotherm over the concentration rate studied (Fig. 4b). The results for linear PLGA agree closely with literature [90]. The fits show also the different affinities of TT to the different polymeric surfaces. Whereas only slight differences were detectable for negatively charged surfaces, the affinity of PLGA NP is distinctly decreased, indicating the importance of the charged sulfobutyl groups on the particle surface.

4.3. Protein delivery by means of SB-PVAL-g-PLGA NP

The interactions of protein loaded SB(43)-PVAL-g-PLGA NP with Caco-2 cells were studied using immunogold staining as detection method. Human serum albumin (HSA) was used as a model protein since its adhesion properties to Caco-2 cells are known to be rather low.

As shown in Fig. 5, the protein could be localized inside Caco-2 cells and at cell surface. Free HSA did not show any cell association. The protein NP adsorbate seemed to be endocytosed at the basal area of the microvilli, leading to localization in upper cell compartments. Some spots at deeper cell compartments also suggested a transcellular transport of HSA. Moreover, the in vitro immune reactions suggested that the protein remained intact during cellular incubation with the SB(43)-PVAL-g-PLGA NP as well as during cellular uptake, an essential requirement for delivering protein pharmaceuticals.

4.4. Immune response to orally administered SB-PVAL-g-PLGA NP in mice

Based on the results of TT adsorption studies and HSA NP uptake in Caco-2 cell culture, the ability of antigen loaded

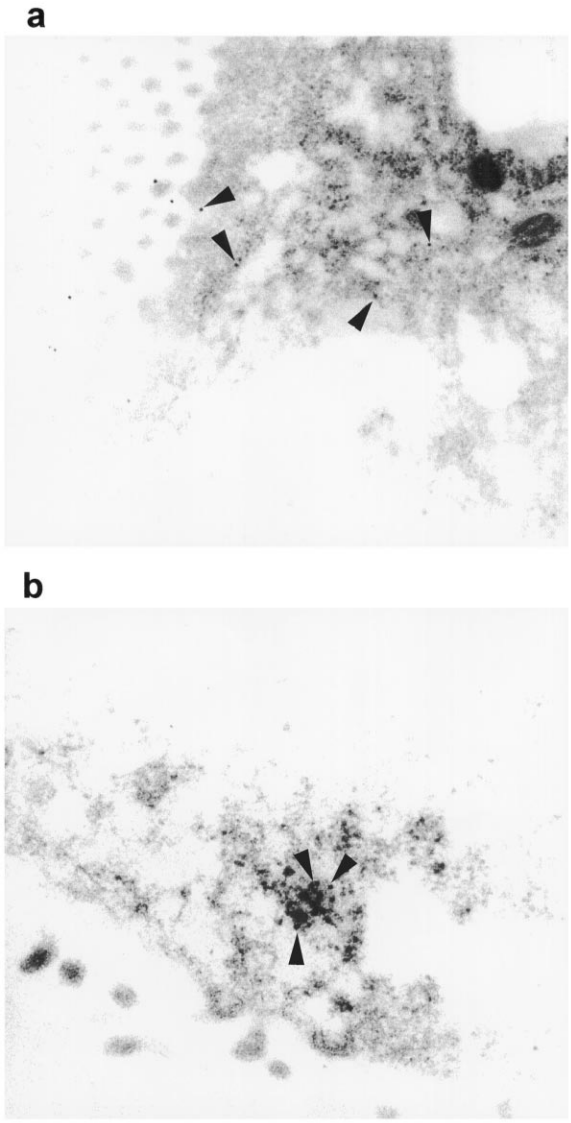

Fig. 5. Immunogoldstaining TEM (anti-HSA-gold, 15 nm) to visualize albumin distribution after coadministration with SB(43)-PVA-PLGA NP to Caco-2 monolayers: NP-mediated surface adhesion and intracellular distribution (arrows) of HSA (a: ×60 000; b: ×100 000). The invagination of the plasma membrane at the base of the microvillus (a, middle) forms a vesicle-indicated endocytotic uptake.

SB(43)-PVAL-g-PLGA NP to induce an immune response after oral application was investigated in a mouse model.

Four weeks after the first immunization with TT loaded SB(43)-PVAL-g-PLGA NP IgG as well as IgA antibody responses were detectable, as shown in Fig. 6. They were significantly increased in comparison to the blood samples before vaccination. Oral vaccination using TT NP induced only weak IgG titers (0.1% of those obtained for the i.p. applicated Tetanol). Serum IgA titers were increased to nearly twice as much as those induced by Tetanol. Application of pure antigen solutions did not induce significant titers. By converting soluble TT into a particulate species through adsorption, an adjuvant effect is achieved. An improved uptake by M cells and an enhanced processing by antigen presenting cells of particulate materials has been proposed to be due to the similarity of an antigen coated particle to original pathogens; e.g. small bacteria or viruses [91,92]

5. Conclusions

Two decades of research have demonstrated that NP uptake by gastrointestinal tissues is a reality and oral delivery of high molecular weight drugs, such as polypeptides, proteins and oligonucleotides, associated with a colloidal carrier might become feasible under certain conditions.

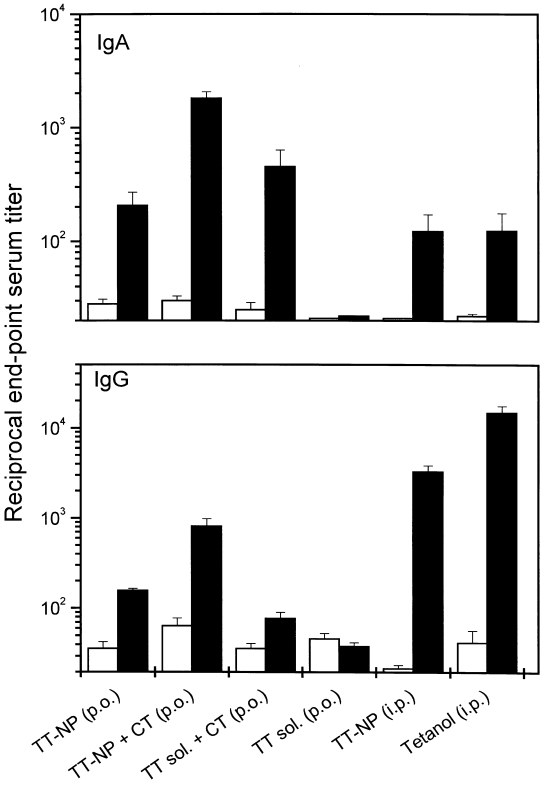

Fig. 6. Immune responses to aluminum adsorbed tetanus toxoid (Tetanol), tetanus toxoid-loaded SB(43)-PVA-g-PLGA nanoparticles (TT-NP) and free tetanus toxoid (TT sol.) with and without coadministration of cholera toxin (CT). Three immunizations, either by oral (p.o.) or intraperitoneal (i.p.) application were carried out at weeks 1, 2 and 3. Blood samples were collected 1 week before (hollow bars) as well as 6 weeks after immunization (black bars) and assayed for serum IgG titers as well as IgA titers by enzyme-linked immunosorbent assay.

It is widely accepted that NP properties, e.g. size, surface charge and hydrophobicity, affect their intestinal absorption characteristics. Smaller NP show a higher extend of uptake than larger ones via both FAE and absorptive enterocytes. The size dependence of the passage to mesenteric lymph nodes is discussed controversially. From poly(styrene) NP it is known that lipophilic uncharged and positively charged NP provide affinity to absorptive tissues, while negatively charged NP show only low adhesion to and uptake from any type of intestinal tissue. Negatively charged NP from more hydrophilic polymers, on the other hand, provide enhanced bioadhesive properties and are absorbed by both M cells and absorptive enterocytes.

A combination of surface charges and matrix hydrophilicity seems favorable, the available polymeric carrier systems for oral macromolecular delivery still need further improvement. Novel biodegradable SB-PVAL-g-PLGA NP offer some interesting properties to overcome several problems of polymeric NP. PLGA chain lengths, number and composition can be varied as well as nature and degree of charge modification to adjust polymer properties to all the needs of a matrix material for an optimal colloidal carrier for oral protein delivery. New biomaterials combining different physicochemical properties to affect peptide absorption through NP have shown some promise but require further work to realize their potential.

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