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CHITOSAN NANOPARTICLES FOR ORAL DELIVERY OF RECOMBINANT PROTEINS

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Abstract

Recombinant proteins are almost always injected, and an oral alternative would improve convenience and adherence. Chitosan nanoparticles have been studied as oral carriers for more than two decades because the polymer is mucoadhesive, biodegradable and able to loosen epithelial tight junctions. This review summarises published work on the gastrointestinal barriers that proteins face, the preparation of chitosan particles by ionic gelation and related methods, proposed absorption mechanisms, and chemical modifications that extend activity to intestinal pH. A synthesis table collects representative designs. The review reports no new data. The literature shows measurable pharmacological effects in animals, mostly with insulin, but modest bioavailability, poor standardisation and limited long-term safety evidence still hold back clinical translation.

1. INTRODUCTION

Most protein therapeutics are given by injection. Insulin, growth hormone, interferons, erythropoietin and monoclonal antibodies are produced by recombinant technology, and nearly all are administered subcutaneously, intramuscularly or intravenously [1, 2]. Injection works, but it is unpopular. Patients who need daily or weekly doses report pain, anxiety, lipohypertrophy and missed doses, and the cost of trained administration adds to the burden on health

systems. An oral formulation has therefore been a long-standing goal, with insulin as the prototype because diabetes is common and chronic. Decades of work have shown how hard the problem is. A protein swallowed in a plain capsule is digested, and the small fraction that survives is poorly absorbed, so oral bioavailability of unprotected peptides and proteins is usually reported as far below ten percent and often below one [3, 4, 5].

Nanoparticles made from chitosan entered this field in the late 1990s. A group in Santiago de Compostela showed that chitosan could be crosslinked with the polyanion tripolyphosphate under mild aqueous conditions to give particles of a few hundred nanometres that carry proteins without organic solvents or heat [6, 7]. The idea was attractive for obvious reasons. Chitosan is a cationic polysaccharide derived from crustacean chitin, it sticks to mucus, and it was already known to open the junctions between epithelial cells in culture [8]. Since then, hundreds of papers have described chitosan-based particles for insulin and other proteins, and several reviews have catalogued the approaches [9, 10]. The same group soon reported improved nasal absorption of insulin with these particles, which encouraged attempts to move the system to the gastrointestinal tract [11].

This review brings that literature together for a reader who wants to know what has been shown, what has not, and why progress to the clinic has been slower than the number of publications would suggest. It covers the barriers a protein meets in the gut, the chemistry and preparation of chitosan particles, the mechanisms proposed for improved absorption, chemical derivatives developed to overcome the pH limitation of the native polymer, and the preclinical evidence. It then discusses weaknesses in the field and possible ways forward. No new experiments are reported, and numerical values are quoted only where they are widely reproduced or are properly attributed to a cited source. Where the evidence rests on a single laboratory or a single animal model, that is stated, because the field has a tendency to treat early promising results as settled.

2. BARRIERS TO ORAL PROTEIN DELIVERY

The first barrier is chemical and enzymatic. Gastric acid denatures many proteins, and pepsin begins to cleave those that remain. In the small intestine the pancreatic proteases trypsin, chymotrypsin and elastase continue the work, followed by peptidases on the brush border membrane. Even if a protein escapes digestion, it may aggregate, deamidate or oxidise in the changing pH and ionic environment, and these changes can reduce activity or raise immunogenicity [12, 13]. For a molecule such as insulin, which is small by protein standards and relatively robust, protection from enzymes is one of the easier goals. For larger and more fragile recombinant proteins, maintaining the folded structure through preparation, storage and transit is a separate and harder challenge that is often left untested in published papers.

The second barrier is the mucus layer that covers the intestinal epithelium. Mucus is a hydrated gel of heavily glycosylated mucins, constantly secreted and shed, and it traps particles by steric and adhesive interactions. Particles that stick tightly to mucus are carried away with its turnover and never reach the cells beneath, while those that diffuse freely may pass through but are not retained [14]. Positively charged carriers such as chitosan particles bind strongly to the negatively charged sialic acid and sulfate groups on mucins, which prolongs residence at the surface but can slow penetration to the epithelium [14, 15]. How to balance adhesion against diffusion is not settled, and the answer probably depends on particle size, surface charge and the segment of the gut targeted.

Beyond the mucus lies the epithelium itself. Tight junctions between neighbouring enterocytes restrict paracellular passage to small solutes, and proteins of several kilodaltons or more cannot pass unless the junctions are opened. Transcellular uptake is possible through endocytosis, but it is limited by the small surface area of specialised cells and by lysosomal degradation inside enterocytes [16]. Specialised M cells over Peyer's patches take up particles more readily and are sometimes proposed as an entry route, although they make up a very small part of

the epithelium and their contribution to systemic absorption of proteins is hard to quantify [16, 17]. Together, these features explain why a protein with a molecular mass in the tens of kilodaltons usually reaches the circulation in tiny amounts, if at all.

The pH gradient along the gut adds a further complication to every strategy discussed below. Gastric fluid sits at about pH 1 to 3 in the fasted state, the duodenum is mildly acidic, and the pH rises through the jejunum and ileum to near neutral or slightly above. Protein solubility, particle charge and polymer behaviour all change over this range, so a carrier that performs well at one point may be inactive at another [14, 18]. Chitosan is a good example, since its amino groups are protonated and active in the first part of the journey and largely deprotonated by the time the particle reaches the lower small intestine. Timing also matters. Fed and fasted states, gastric emptying and transit time vary between individuals, which means that a particle meeting the right pH at the right time in one subject may miss it in another.

The consequences for formulation are practical as well as scientific. If only a small percentage of an oral dose is absorbed, the quantity of expensive recombinant protein needed per patient is many times that of an injection, which strains manufacturing capacity and cost [2, 19]. Absorption variability also matters, since an insulin dose that is absorbed to a different degree each time makes glucose control difficult and potentially dangerous. Surveys of oral peptide technologies in preclinical and clinical development note that only a handful of approaches have progressed to late-phase trials, and that most successful candidates rely on strong permeation enhancers rather than on nanocarriers alone [20]. Any case for chitosan nanoparticles has to be made against this background of high bars and modest returns.

Other mucosal routes show what chitosan particles can achieve when the barriers are lower. In the nasal cavity, chitosan nanoparticles loaded with insulin enhanced absorption in animal models compared with the protein in solution, and the effect was linked to mucoadhesion and to transient opening of the epithelial barrier [11]. The nasal mucosa has no strongly acidic fluid, no pancreatic enzymes and a thinner mucus layer, so success there cannot simply be transferred to the gut. The comparison is nevertheless useful, because it shows that the benefits of chitosan in nanoparticle form are real in a friendlier environment and then asks how much of the effect survives the harder conditions of the intestinal lumen.

3. CHITOSAN AND NANOPARTICLE PREPARATION

Chitosan is a linear copolymer of glucosamine and N-acetylglucosamine obtained by alkaline deacetylation of chitin, the structural polysaccharide of crustacean shells and fungal cell walls. Commercial grades differ in degree of deacetylation, usually between about 70 and 95 percent, and in molecular weight, from a few tens to several hundred kilodaltons. These two parameters control almost everything of interest, from solubility to charge density to mucoadhesion [18, 21]. The amino groups have a pKa near 6.5, so the polymer is cationic and water-soluble in dilute acid but loses charge and precipitates as the pH approaches neutrality. This single property explains both the usefulness of chitosan in the stomach and upper intestine and the central difficulty of using it in the more neutral conditions of the lower gut.

Safety and degradability are generally favourable. Chitosan is broken down by lysozyme and by enzymes of the colonic flora into oligosaccharides and sugars that are metabolised or excreted, and toxicity studies in animals have found it to be well tolerated in the doses used for delivery, although effects depend on molecular weight and degree of deacetylation [21, 22]. Cytotoxicity in cell culture tends to rise with charge density, which means that the features that improve permeation also raise concerns at higher concentrations. This trade-off recurs throughout the field and is often glossed over in papers that report only efficacy. A balanced assessment treats chitosan as a tolerable excipient whose dose and grade should be matched to the application, not as a material without risk.

Ionic gelation is by far the most used method of making the particles. A dilute chitosan solution in weak acid is stirred while tripolyphosphate solution is added dropwise, and the multivalent anion crosslinks protonated amino groups to form a network that collapses into nanoparticles [6, 7]. No organic solvent is needed, the process takes minutes at room temperature, and the protein is simply mixed with one of the two solutions beforehand. These virtues explain its popularity. The nanoparticles form only within a narrow window of concentrations and ratios; outside it the system gives clear solution, opalescent suspension or macroscopic aggregates. Studies of the mechanism show that particle formation depends on the interplay of charge neutralisation, chain length and mixing rate, which is why details of the protocol matter [23].

Particle size, charge and polydispersity are all tunable, within limits. Lower molecular weight chitosan and lower concentrations generally give smaller particles, and the mass ratio of chitosan to tripolyphosphate shifts the zeta potential and size distribution [23, 24]. Depolymerisation of chitosan by enzymatic or oxidative routes provides lower molecular weight material, but it also changes viscosity and biological activity, and results from one preparation may not carry over to another [25]. For reporting, a polydispersity index (PDI) is often quoted, and values below about 0.3 are conventionally regarded as acceptable, a convention that is applied loosely to polymeric systems, although light scattering reports only an intensity-weighted average [26]. A narrow distribution is desirable, since larger particles in a broad population can dominate the apparent behaviour.

Protein loading follows from electrostatics as much as from entrapment. Insulin has an isoelectric point near 5.3, so above that pH it carries a net negative charge and associates with the cationic polymer, which raises association efficiency and slows early release. Proteins that are positively charged at the working pH are loaded less efficiently, and may need an anionic partner or a different crosslinking scheme. Encapsulation efficiency is calculated from the difference between total and free protein, but this indirect method says nothing about whether the protein remains folded and active [12, 13]. Reports that include a bioactivity measure, such as an enzyme assay or a receptor binding test on protein released from the particles, are far more convincing than those that give only loading figures.

4. MECHANISMS OF ABSORPTION ENHANCEMENT

Mucoadhesion is the property most closely associated with chitosan. The positively charged amino groups interact with sialic acid and sulfate residues on mucins, and hydrogen bonding and hydrophobic contacts add to the effect [15]. Adhesion lengthens the time a particle spends at the mucosal surface and brings the cargo closer to the epithelium, which should increase the opportunity for uptake. The effect is real in ex vivo experiments, where chitosan particles are retained on intestinal tissue much longer than uncoated polymeric particles. The question is whether more retention translates to more absorption in vivo, given mucus turnover. That link has been shown in some animal studies and not in others, and the divergence may reflect the pH of the segment tested as much as the carrier design.

The most cited mechanism is reversible opening of tight junctions. Early work on Caco-2 monolayers found that chitosan lowered transepithelial electrical resistance and raised the permeability of marker molecules in a dose-dependent and reversible way [8]. Later studies showed that chitosan changes the distribution of tight-junction proteins such as ZO-1 and occludin, and that the effect depends on molecular weight and degree of deacetylation, with higher charge density generally stronger [27, 28]. The consequence is a paracellular route that a protein could in principle use. However, the effect needs protonated amino groups, and so it is strong at the slightly acidic pH of the duodenum and weak or absent at neutral pH. Particles that lose their charge before reaching the epithelium cannot be expected to open junctions.

Transcellular uptake is the second proposed route. Particles in the range of roughly 100 to

300 nm are internalised by endocytosis, and smaller particles have been reported to cross the intestinal epithelium more readily than larger ones in rat models [29]. Uptake by enterocytes, however, tends to deliver the cargo to lysosomes where proteins are degraded, and the fraction that escapes into the circulation by transcytosis is probably small [16, 30]. Models that include mucus-secreting cells in co-culture show lower particle uptake than simple Caco-2 monolayers, a reminder that cell culture can overstate transport [30]. In practice, paracellular and transcellular mechanisms probably operate together, and which one dominates for a particular formulation is seldom established directly.

Protection of the cargo is the third contribution, and it is less discussed. A protein embedded in a polymer matrix is shielded from proteases during the transit through the stomach and upper intestine, provided the particle stays intact, and sustained release from the matrix can maintain a local concentration at the absorption site [16, 31]. The shell-and-core particles discussed later were designed with this in mind. A difficulty is that protection and release oppose each other: a particle tight enough to keep out enzymes may also hold the protein when it should be released. The balance has rarely been measured in conditions that mimic the sequence of gastric and intestinal fluids, and where it has, results vary widely between laboratories using nominally similar formulations.

Peyer's patches deserve a separate mention. These lymphoid follicles are covered by M cells specialised for sampling particles from the lumen, and nanoparticles of suitable size and surface properties are taken up there far more efficiently than by normal enterocytes [16, 17]. For vaccines this route is the intended one. For systemically acting proteins such as insulin, M cell uptake would lead the cargo into lymphatic tissue rather than directly into the blood, and the proportion reaching the systemic circulation is unclear. Some authors have therefore considered chitosan particles more suitable for oral vaccination than for hormone replacement, a view that the existing data do not contradict.

5. DERIVATIVES AND FORMULATION STRATEGIES

The pH problem described above has driven most of the chemistry. Native chitosan is protonated in acid but loses charge at the pH of the jejunum and ileum, where most absorption takes place. The main designs are collected in table 1, which groups representative examples by strategy and records the main reported features. Several studies by one Taiwanese group used particles made of chitosan and gamma-polyglutamic acid, in which the polyacid stabilises the structure at low pH and allows the chitosan shell to be exposed and then to open junctions as the particle enters the intestinal lumen [31, 32]. Experimental work in diabetic rats reported a hypoglycaemic effect from insulin in such particles, an outcome described as a response to a pH-triggered release of the cargo.

Chemical modification of the polymer is the second strategy. Quaternisation of the amino groups gives N-trimethyl chitosan, which carries a permanent positive charge and keeps its solubility and effect on epithelial permeability at neutral pH [33, 34]. In Caco-2 monolayers it lowered electrical resistance and raised the passage of model peptides, with the degree of quaternisation influencing both the effect and the cytotoxicity. These properties make it more potent than the native polymer in the lower intestine, but also raise questions about the safety of more powerful permeation enhancement over long periods. Reviews of chitosan derivatives for gastrointestinal delivery stress the need to balance activity with tolerability rather than simply choosing the strongest enhancer [9, 22].

Other modifications address different problems. Thiolated chitosan carries free sulfhydryl groups that form disulfide bonds with cysteine-rich domains in mucus glycoproteins, so adhesion is considerably stronger than that of the unmodified polymer, and the thiol groups additionally crosslink within the particle to give better stability [35, 36]. Hydrophobic modification, for example with lauryl succinyl groups, produces amphiphilic chitosan that was studied for oral insulin delivery and absorption [37].

Pegylated chitosan nanocapsules were developed for oral delivery of a therapeutic peptide, and the degree of PEG grafting influenced stability in simulated fluids and uptake by cells [38]. Each modification cures one weakness and adds new variables, from batch consistency to regulatory classification of a new excipient.

Table 1: Representative chitosan-based carrier designs for oral or mucosal protein delivery

Design	Reported feature	Ref.
Chitosan and tripolyphosphate ionic gelation	Mild aqueous formation of protein-loaded nanoparticles without organic solvent or heat	[6, 7]
Chitosan and polyethylene oxide early protein carrier	Particles stable in physiological media were proposed as carriers for proteins and vaccines	[6, 7]
Insulin-loaded chitosan nanoparticles rat intestinal studies	Intestinal absorption of insulin improved compared with insulin solution in rats	[39]
Nanoparticles shelled with chitosan pH-responsive composite	Stable in acid, loosening at intestinal pH; tight junction opening and hypoglycaemic effect in rats	[31, 32]
Lauryl succinyl chitosan amphiphilic derivative	Evaluated for oral insulin delivery and absorption; hydrophobic groups assist association	[37]
Chitosan–PEG nanocapsules pegylated carrier	Degree of pegylation influenced stability in simulated fluids and peptide uptake	[38]
N-trimethyl chitosan quaternised	Permanent cationic charge; active on Caco-2 permeability at neutral pH	[33, 34]
Thiolated chitosan thiomer	Disulfide bonds with mucus glycoproteins give stronger adhesion and particle stability	[35, 36]
Native chitosan solution permeation enhancer	Reversible fall in Caco-2 electrical resistance and tight-junction protein redistribution	[8, 28]
Chitosan grade effects molecular weight and acetylation	Transport across Caco-2 cells depended on molecular weight and degree of acetylation	[27]

The distribution of the examples in table 1 is shown in fig. 1. The designs fall unevenly across strategies, with chemically modified chitosans the most numerous, which reflects the effort made to overcome the pH problem. Plain ionic-gelation systems come next, and these are the base against which most modified particles are compared [6, 7, 39]. The pH-responsive composites are few but have the most developed animal data. The chart should not be read as a measure of effectiveness, since it counts published designs rather than results, but it does show where research effort has gone and where fewer comparative data are available, particularly for permeation effects measured with standard cell models [8, 27, 28].

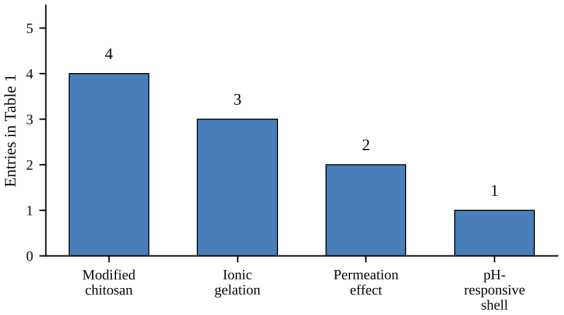


Fig. 1: Number of entries in table 1 by design strategy

Formulation of the final dosage form adds another layer. Particles suspended in water would meet gastric acid, so oral products would probably be filled into enteric-coated capsules or tablets that release the nanoparticles in the small intestine. Enteric coating, mucoadhesive patches and ingestible devices have each been combined with nanocarriers in preclinical work, and reviews of oral peptide technologies suggest that such combinations may be more productive than a single technology on its own [20, 40]. In this setting the particle is one component of a system, not the whole solution, and the contribution of chitosan to the total effect needs separate evaluation, which control experiments omitting the polymer or the coating can provide.

6. PRECLINICAL EVIDENCE AND CHARACTERISATION

Physical characterisation is standard but not always informative. Dynamic light scattering gives hydrodynamic size and PDI, laser Doppler electrophoresis gives zeta potential, and electron microscopy shows shape and a dry diameter that is typically smaller than the

hydrodynamic value because the hydrated polymer collapses [24, 26]. The more telling measurements are those done in biorelevant media. Particles can change size or aggregate when they are moved from acidic dispersion into simulated intestinal fluid with salts and enzymes, and release profiles measured in water can be misleading. Authors who report stability in simulated gastric fluid followed by simulated intestinal fluid, together with the integrity of the released protein, provide much more useful evidence than those who show only a size distribution in water.

In vitro permeability models are the next step. Caco-2 monolayers are widely used, and co-cultures with mucus-secreting HT29-MTX cells add a mucus layer that better represents the barrier a particle must cross [30]. These systems can show whether a formulation lowers electrical resistance, raises marker transport or is taken up by cells, and they are quick and reproducible. They also lack the unstirred layer, peristalsis, bile salts and flowing luminal fluid that shape uptake in living animals, so rankings of formulations from cell culture do not always match animal results [30]. The best studies use at least two complementary models and describe their limits, and treat cell culture as a screen for toxicity and mechanism instead of a prediction of oral bioavailability.

Animal studies, usually in diabetic rats, supply most of the efficacy data. Chitosan nanoparticles loaded with insulin lowered blood glucose after intestinal or oral administration in several reports, with effects appearing hours after dosing and lasting longer than after subcutaneous insulin, and the same pattern was seen with shelled and modified particles [31, 32, 39]. The pharmacological availability relative to injection is generally reported as a modest fraction, and plasma insulin levels, where measured, are low. These findings establish that some protein is absorbed in a bioactive form. They do not show that the amounts are sufficient or consistent enough for clinical use, and direct comparison between studies is difficult because dose, animal strain, fasting state and route differ.

Comparison with other polymeric carriers helps put the chitosan literature in perspective. Poly(lactic-co-glycolic acid) particles are more stable and have a longer record in regulatory submissions, but they are anionic, release protein slowly and expose it to acidic microenvironments as the polymer degrades, which can damage sensitive biologics. Alginate, dextran sulfate and pectin matrices share the advantage of aqueous processing, though they lack the mucoadhesive and permeation-enhancing properties of chitosan [14, 19, 40]. Reviews of oral insulin carriers conclude that no polymer has yet outperformed the others in a systematic head-to-head test, since different studies use different doses, models and readouts [10, 19]. For this reason, the argument for chitosan rests more on its biological properties than on any demonstrated superiority in performance.

A recurring methodological issue is the choice of outcome. Blood glucose is easy to measure but is affected by stress, fasting and handling, and a drop in glucose following oral gavage of a particle suspension may arise from several causes. Plasma assays for the intact protein are more specific, though immunoassays can cross-react with fragments, so confirming that the measured material is intact insulin is desirable. Pharmacokinetic profiles are rarely compared with those of an intravenous or subcutaneous dose in the same animals, which is needed to calculate absolute bioavailability. Without such controls, claims of improved absorption rest on comparison with oral insulin solution, which is an extremely low bar given its near-zero absorption [3, 4, 5].

7. LIMITATIONS AND FUTURE DIRECTIONS

Reproducibility is the most immediate problem. Chitosan is a natural, heterogeneous material and different suppliers, lots and extraction methods yield polymers with different degrees of deacetylation, molecular weight distributions, residual protein and endotoxin [18, 21, 25]. Many papers give only a nominal molecular weight range and a deacetylation percentage from a supplier, and few characterise the polymer they actually used. Because the behaviour of the particles depends so strongly on these parameters, small differences between batches can

produce qualitatively different outcomes. A study that cannot be repeated with a standardised polymer is of limited use as a basis for development, and polymers of pharmaceutical grade, with defined specifications, would reduce this uncertainty.

Stability and scale-up raise further questions. Chitosan and tripolyphosphate particles often aggregate when stored as suspensions and are usually freeze-dried with sugar cryoprotectants, which can change size on redispersion. Manufacturing nanoparticles consistently at scale, while preserving protein activity, is considerably harder than preparing a laboratory batch with a magnetic stirrer [2, 19]. Very few oral nanocarrier products for proteins have reached late-stage clinical testing, and none based on chitosan has become a routine therapy; the analyses of oral peptide technologies explain this by pointing to bioavailability, variability and cost rather than any single technical failure [20, 40]. Realistic development plans need to address these issues from the outset, not once efficacy has been shown.

Long-term safety deserves more attention than it receives. A therapy such as insulin is taken daily for decades, and repeated exposure of the intestinal epithelium to a permeation enhancer that opens tight junctions could, in principle, alter barrier function, increase the entry of luminal antigens or interact with dietary components and other drugs [21, 28]. Short toxicity studies in rodents do not answer this. Future work might therefore include repeated-dose studies with histology and permeability markers, more physiologically relevant in vitro systems, and comparison with other absorption enhancers on the same cargo [4, 20, 30]. Targeted designs that use ligands to direct particles to specific receptors may reduce reliance on junction opening, though these have yet to show clear benefits over simpler systems.

Manufacturing economics will also shape what is feasible. Recombinant proteins are costly to produce, and an oral dosage form that wastes most of the dose may be commercially unattractive unless the protein is cheap and the absorption is predictable [2, 19]. For this reason, the most plausible near-term uses of chitosan particles may be in settings where only small amounts of protein are needed or where local action in the gut is the goal. Systematic comparison of cost per effective dose with injectable products is rare in the nanoparticle literature, and this gap would be worth closing before more elaborate carriers are designed.

8. CONCLUSION

Chitosan nanoparticles address several of the barriers to oral protein delivery at once. They protect the cargo to some degree, adhere to mucus, and, at acidic pH, loosen epithelial tight junctions, and they can be prepared by a simple aqueous process that avoids organic solvents [6, 7, 8]. Animal studies, nearly all with insulin, show that some bioactive protein is absorbed and that blood glucose can fall after oral dosing [31, 32, 39]. The principal weakness of the native polymer is its loss of charge at intestinal pH, and quaternised, thiolated, hydrophobic and pH-responsive derivatives have been developed to compensate [33, 34, 35, 37].

The gap between laboratory demonstration and a usable medicine remains wide. Reported bioavailability is modest, polymer quality is poorly standardised, and long-term safety of repeated tight-junction modulation is largely untested [20, 21, 28]. Better in vitro models, absolute bioavailability measurements, defined chitosan grades and realistic comparison with injectable products would make the evidence more useful [19, 30]. Until then, chitosan nanoparticles are best regarded as a well-studied platform with proven principles and unproven clinical value, and the claims made for them should be weighed with that in mind [2, 40].

9. ABBREVIATIONS

Caco-2-Human colon adenocarcinoma cell line used as an intestinal model, HT29-MTX-Mucus-secreting human colon cell line, PDI-Polydispersity index, PEG-Polyethylene glycol, TPP-Tripolyphosphate, ZO-1-Zonula occludens-1, pKa-Acid dissociation constant, TEER-Transepithelial electrical resistance

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All the authors have contributed equally.

12. CONFLICT OF INTERESTS

Declared none

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