The biological challenges and pharmacological opportunities of orally administered nanomedicine delivery

1. Abstract

Introduction: Nano-scale formulations are being developed to improve the delivery of orally administered medicines, and the interactions between nanoformulations and the gastrointestinal luminal, mucosal and epithelial environment is currently being investigated. The mucosal surface of the gastrointestinal tract is capable of trapping and eliminating large particles and pathogens as part of the natural defences of the body, it is becoming clearer that nanoformulation properties such as particle size, charge, and shape, as well as mucous properties such as viscoelasticity, thickness, density, and turn-over time are all relevant to these interactions. However, progress has been slow to utilise this information to produce effective mucous-penetrating particles. Areas covered: This review focuses on delivery method of nanomedicines both into and across the gastrointestinal mucosal surface, and aims to summarise the biological barriers that exist to successful oral nanomedicine delivery and how these barriers may be investigated and overcome. Expert commentary: Despite successes in the laboratory, no nanotechnology-enabled products are currently in clinical use which either specifically target the intestinal mucous surface or cross the epithelial barrier intact. New nanomedicine-based treatments of local diseases (intestinal cancer, inflammation, infection) and systemic diseases are advancing towards clinical use, and offer genuine opportunities to improve therapy.

2. Introduction

Oral delivery is currently the most common and the most accepted route of drug administration used in patient treatment. This is for several reasons, including the convenience of oral administration, the low degree of invasiveness compared to other routes,

and the fact that the human intestine is particularly suited for the absorption of small molecules with certain physiochemical properties. The human intestinal epithelium consists mostly of enterocytes which express microvilli on the apical exterior, increasing the mean total mucosal surface of the digestive tract interior to around 32-400 metres^2, depending on the methods used to assess the surface [1,2]. Despite the obvious advantages of intestinal absorption as a method of drug delivery, there are a number of critical considerations when attempting to exploit this route. These include i) the stability of the medicine in the luminal fluid; ii) the drug solubility in the various pH and contents found in the intestinal tract; iii) the mucous and cell membrane as a barrier for drug absorption; iv) the activities of first pass drug elimination processes in both the intestine and liver [3]. These issues can often be overcome by the application of design rules, such as Lipinski's rule of five (No more than five hydrogen bond donors, no more than ten hydrogen bond acceptors, a molecular mass less than 500, a log P of less than 5) and its various iterations. Rational screening of drug candidates in this way can reduce the attrition inherent in drug development programmes, but an additional approach is to utilise nanoformulation technologies for improving drug delivery.

Nanomedicine involves the utilisation of particles or emulsions within the size range of 1-999 nanometres and is being researched to improve drug dissolution, protect drugs from the luminal environment, release drugs in a more controlled manner, and exploit the various particle-specific anatomical, physiological and molecular machinery of the gastrointestinal system [4]. The most promising types of nanoformulations used for drug delivery include the particle-based systems such as inorganic nanoparticles, solid drug nanoparticles (SDNs), solid lipid nanopartcles (SLNs), polymeric nanoparticles and dendrimers, and the non-solid-

based systems such as nanoemulsions (NEs), liposomes and micelles [5]. Of note, the only nanoformulations currently used clinically for oral administration are SDNs.

Targeted oral nanoformulations are being developed which can directly bind to the intestinal mucous and the epithelial surface for local release of drug, as well as improve the dissolution of drugs in the gastrointestinal fluid [6-9]. In addition, studies are working to develop orally administered nanoparticles which can reach the systemic circulation intact. These goals require radically different strategies to be successful and will encounter specific barriers, depending on the intended lifespan and end goal of the nanoparticle. This review will focus on these different biological barriers encountered by nanomedicines following oral administration. The authors will also investigate into how scientists have attempted to overcome these barriers, and in some cases how barrier mechanisms have been exploited for improving the effectiveness of oral nanomedicine delivery. Additionally, the authors have also briefly assessed the limitations of assessing nanoparticle intestinal absorption using *in vitro* methodologies traditionally used for small molecules.

3. Barriers to the oral absorption of nanoparticles

If an orally administered nanoparticle is to reach the systemic circulation intact, numerous barriers both physical and chemical must be overcome if the particle is to avoid destruction or undesirable modification. In sequential order following administration, the barriers that will be encountered include i) the oral, gastric and intestinal fluid environment; ii) the mucous surface of the stomach and intestine; iii) the epithelial cells of the intestine (Table 1). If the target of the nanoparticle is the mucosal surface, as is the case when treating gastric *H. pylori* infection, it would not be required for the nanoparticle to be able to traverse the epithelial cell

88 layer. Further still, if the purpose of the nanoparticle is to increase the dissolution rate of
89 poorly soluble drugs then there would be no requirement to design the particles to cross either
90 mucous or cells. Effectively, the design challenge facing formulation scientists becomes more
91 complex when nanoparticles are required to overcome additional barriers. This may explain
92 why all oral nanomedicines currently used in clinical therapy were developed to improve the
93 dissolution of poorly soluble drugs, as described in Section 6. In Sections 3.1 to 3.3 the
94 authors describe the physical and chemical properties of each barrier, how these properties
95 can impede the ability of nanoparticles to reach the systemic circulation, and how scientists
96 have attempted to overcome these barriers when designing nanoscale delivery systems.

97

98 3.1 The oral, gastric and intestinal fluid environment

99 Once administered orally, nanoparticles enter a fluid environment which has the potential to
100 influence the properties of the nanoparticles. Relevant factors include: i) enzymes in the
101 saliva, stomach fluid and intestine fluid; ii) emulsifiers in bile iii) food components; iv) the
102 pH environment of the stomach and intestine; v) gut motility; vi) disease state. Although not
103 easily visualised as a physical barrier to nanoparticle absorption, the fluid environment of the
104 gastrointestinal system can change rapidly and dramatically, and this may have a significant
105 knock-on effect on the absorption, toxicity and efficacy of orally-administered nanoparticles.

106 Many of the excipients present in lipid-containing nanoparticulate systems are esters, and
107 therefore it is unsurprising to find that these systems can be hydrolysed by the various
108 lipolytic enzymes in the gastrointestinal fluid [10]. Nanoparticles can be utilised to protect
109 drugs and macromolecules from intestinal enzymatic degradation. For example, diabetes
110 treatment using orally-administered insulin is impeded due to intestinal protease activity [11]

and nanoparticle formulations have been developed to protect the insulin from this degradation [12,13]. This demonstrates that nanoparticles can be used to improve the intestinal stability of contained pharmaceutical ingredients, but it is also important to understand how the nanoparticles themselves are affected by intestinal enzymes, the levels of which can fluctuate greatly depending on feeding state.

Bile salts are water-soluble, amphipathic end products of cholesterol metabolism. Formed in the liver, bile acids are excreted into the small intestine via the bile duct and pancreas, where they help solubilise and promote the absorption of dietary lipids [14]. This process of lipid solubilisation can also impact on the stability of lipid-containing nanoparticles. When the objective is keep lipid nanoparticles intact in the intestine, natural bile salts are a potentially disruptive factor, causing irreversible degradation. However, when the objective is to adjust the dissolution rate of lipid nanoparticles in the intestine for the purposes of controlled drug release, bile salts can be of potential benefit when included as an excipient of the nanoparticle. A study by Oblich et al investigated the effect of different surfactants on the degradation rate of SLNs by lipase, and found that SLNs containing cholic acid sodium salt degraded around 50% more rapidly than SLNs containing poloxamer 407 [15]. This study suggested that SLN degradation rate in the intestine is adjustable by utilising the correct combination of surfactants, and that bile salts could be used to increase the rate of SLN breakdown.

Digestion of food in the gut produces amino acids, di- and tri-peptides, lipids and carbohydrates which are potentially capable of binding to, and altering the properties of, nanoparticles [16]. Proteins are known to form together with nanoparticles to create coronas [17] although very few studies have looked at the formation of coronas made from food components. A study by Di Silvio et al showed that coronas were formed around magnetite

nanoparticles when co-incubated with digested bread *in vitro*, and furthermore that when these corona-nanoparticle complexes were isolated they were more readily taken up than corona-free particles into Caco-2 intestinal cells [18]. In a similar study by Lichtestein et al, poly (acrylic acid)-coated silver nanoparticles co-incubated with food digestion products (a mixture of proteins, fatty acids and carbohydrates) showed 40% higher accumulation in Caco-2 cells compared to when no food digestion products were included [19]. It conclusion from these *in vitro* studies is that food components can interact with nanoparticles, and this interaction can lead to alterations in nanoparticle physicochemical properties and their ability to accumulate in cells. The majority of *in vitro* nanoparticle cell accumulation studies do not take this factor into consideration, and this may be detrimental when developing nanoparticles for oral administration.

3.2 The mucous of the gastrointestinal system

The mucous layers of the body contain a complex mixture of carbohydrates, lipids, proteins, water, salts and biological debris. The most common constituents of mucous are mucin proteins 1-40 MDa in size, and the constituents, macro-rheology and micro-rheology of mucous has been extensively reviewed previously [20,21]. Mucous acts as a physical barrier against foreign particles, including pathogens, toxins and environmental particles, while allowing passage of selected gases, ions, nutrients, small molecules and certain proteins. The chemical attributes are similar for the various mucous layers present on the body [22], and the mucosal surfaces most relevant to current nanoparticle delivery research include those at the surfaces of the mouth, lungs, nasal passage, eyes, vagina and gastrointestinal system. This review focuses on the mucosal barriers of the gastrointestinal system, which includes the

158 distinct mucosal environments of the stomach, intestine and colon. However, other mucosal
159 barriers are also occasionally referred to, due to relevant investigations on rheological and
160 nanoparticle-interacting properties being performed on alternative mucosal sites and also due
161 to the lack of research performed directly on gastric and intestinal mucous in humans. Of
162 particular note, a number of studies have been conducted using cervico-vaginal mucous due
163 to the relative simplicity of mucous collection.

164 The mucous systems of the human stomach, intestine and colon have distinct attributes and
165 functions, and exist in radically different luminal environments. The acidity of the contents of
166 the human stomach is, at its highest, generally around pH 1.5-2 in fasted healthy subjects
167 [23]. This level of acidity would damage an unprotected stomach wall, and indeed this
168 clinical scenario leads to the formation of gastric ulcers [24]. To prevent this, the mucous in
169 the stomach acts as an effective buffer zone and, combined with the excretion of neutralising
170 bicarbonate molecules by the epithelial cells, results in a near-neutral pH adjacent to the
171 apical cell surface of the stomach [25]. The stomach has a two-layered mucus system: the
172 *firmly-adherent mucous* layer, which consists of transmembrane-spanning mucins of between
173 100 and 500 nm in length [24], and the *loosely-adherent mucous* layer, which consists of
174 much larger mucins of up to several micrometres in length and is not covalently attached to
175 the gastrointestinal cells. The mucus, when subjected to shear force, forms a slippage plane
176 between two surfaces with an unstirred layer immediately adjacent to the epithelial cell
177 surfaces in the glycocalyx. The contents of the gut are coated by the shed mucous from the
178 *loosely-adherent mucous* layer and are lubricated as they move along the intestine. The
179 mucous layer of the small intestine is considerably thinner than the equivalent found in the
180 stomach and large intestine, and this can be attributed to the small intestine being the primary
181 site of absorption and that barriers impeding this process would be detrimental. The small

intestine also does not show a consistent two-layered mucous system as is found in the other regions [21], showing instead a consistent loosely-adherent layer but only sporadic patches of adherent layers. Glycosylation patterns present on the proteins of the small intestine mucous barrier mimic the glycosylation present on the cellular surface of the intestinal epithelium. Many bacteria bind naturally to glycosylated proteins for cellular entry, therefore the mucous acts as an alternative interaction site able to trap bacteria before cellular entry can be achieved [26]. In addition, small intestine mucous contains adsorbed lipids which can impede bacteria via non-specific hydrophobic bonding [27,28]. Trapped bacteria are then subjected to excreted antimicrobial molecules and can subsequently be shed back into the intestinal fluid along with the *loosely-adherent mucous* layer [29]. As with the stomach, the mucous system of the large intestine consists of a *loosely-adherent* and *firmly-adherent* layer. The thickness of the *firmly-adherent* layer is several hundred micrometers in humans, and is continually renewed from the epithelial cells with a half-life of around an hour [30]. The density of the *firmly-adherent* layer is very high, with the intention of keeping intestinal bacteria from reaching the epithelial cells. Conversely, the *loosely-adherent* layer of the large intestine is around 3-4 times less dense than the *firmly-adherent* layer, and is made to harbour the commensal bacteria.

As mentioned previously, gastrointestinal mucous represents a potential barrier for penetration of large particles, and this includes nano-scale formulations designed to deliver therapeutic or diagnostic materials to the intestinal epithelial surface or the systemic circulation [31-35]. It must be accepted that, except in the cases where nanoparticles are used purely for luminal drug dissolution enhancement, nanoparticles will need to encounter the gastrointestinal mucous in some capacity. There is continuing research into the development of nanoparticles capable of interacting with the gastric and intestinal mucous, with mixed

success, and it is important to distinguish between muco-adhesive particles and muco-penetrative particles, both of which will be discussed in this section of the review.

3.2.1 Muco-adhesive nanoparticles

As the name suggests, muco-adhesive nanoparticles are capable of adhering to mucosal layers of the body. There are two common rationales for using mucoadhesive nanoparticles of orally-administered therapy. Firstly, to increase the residence time of the particles at the site of absorption, which in the case of oral therapy is usually the small intestine. This is expected to increase the fraction of drug absorbed by allowing the intestinal absorption process to occur over a longer period. Secondly, adherent particles can be used to target the treatment of local diseases of the stomach, intestine and colon. If an orally administered particle does not have the ability to adhere to mucous, it is likely to pass directly through the gastrointestinal tract without any significant contact with the epithelial cell layer [36].

Mucous can potentially bind to nanoparticles via various physicochemical mechanisms, hydrophobic interactions [37] and, in the case of cationic nanoparticles, via electrostatic impedance caused by anionic groups on mucins contained in the mucous [38]. In addition to these factors, the entanglements between mucins and other constituents create a “mesh” that can physically block particles larger than the mesh pore size. Although also influenced by surface chemistry and lipid exposure, endogenous and fabricated structures under 60 nm generally do not show impedance by mucous [39]. Instead, they are capable of passing through the barrier at speeds comparable to movement through water following Brownian diffusion theory, provided that the molecules do not interact physicochemcally with the mucous [40]. To investigate this further, Olmsted et al measured virus movement through

mucous and found that Norwalk virus (38 nm) and human papilloma virus (HPV) (55 nm) both diffused in human cervical mucus at the same rate as they do in water, whereas herpes simplex virus (180 nm) moved through human cervical mucus around 1000-fold slower than through water. In a study investigating the movement of nanoparticles through porcine intestinal mucous, pore size was determined using electron microscopy to be 211 ± 7 nm, and nano-sized latex beads above this size were unable to pass through the mucous [41]. These restrictions to macromolecule movement through mucous have led some researchers to conclude that nanoparticles above a certain size will not be able to traverse the intestinal mucous barrier intact. Confusingly, a study has been reported where larger nanopartlesparticles (200 nm and 500 nm) were able to overcome the mucous pore size limitation when particles were coated with polyethylene glycol [42]. Indeed these larger particles showed around 50-fold improved cervicovaginal mucous penetration when compared to 100 nm particles that lacked the polyethylene glycol coating. This indicates that mucous pore size is a potential limiting factor for absorption of larger particles, but that methods other than particle size reduction may be available to overcome this issue, potentially by altering the pore sizes present in the mucous.

Nanoparticles movement through the mucous barrier can also be impeded by mucous shedding. If nanoparticles adhere to the *loosely-adherent mucous* layer they risk rapid clearance, as this layer is continuously being shed and replaced from the stomach and intestine surface. To investigate this, Tirosh *et al* investigated the migration of adhesive (polycarbophil) and nonadhesive (Eudragit RL-100) particles in the rat intestine and found no difference in retention times [43]. The adhesive particles predominantly bound to the *loosely-adherent mucous* layer, and were found to be coated in mucous "plugs" following discharge from the perfused rat jejunum. To overcome this phenomenon, particles in other studies have

been designed to naturally adhere to the *firmly-adherent mucous* layer, which resides below the loose layer and is not readily shed. Particles synthesised from common polymers, such as polylactic acid (PLA), polylactic-co-glycolic acid (PLGA) and polyacrylic acid (PAA) are able to adhere to the intestinal mucous via hydrophobic interactions, hydrogen bonding, entanglement, or more commonly a combination of these factors [32]. The exact influences that particle properties have on mucous interactions are still poorly understood but are worthy of investigation to further validate and optimise this delivery strategy.

3.2.3 Muco-penetrative nanoparticles

Considering the complications associated with using muco-adherent nanoparticles, research has been undertaken to create muco-penetrative particles capable of traversing the mucous layer and reaching the epithelial layer intact. Attempts have been made to design mucous-penetrating nanoparticles containing "mucous permeation enhancers", also known as mucolytic agents, capable of degrading the mucous layer, in a process analogous to that used for treatment of cystic fibrosis [33]. This strategy is particularly useful for allowing access of peptide-based treatment to the epithelial cell layer, and has been reviewed previously [44]. Another strategy used to create mucous-penetrating nanoparticles is to coat particles with "stealth" excipients which avoid mucous interactions. In mouse studies, vaginal mucous penetration of carboxylic acid–coated, fluorescent polystyrene nanoparticles was increased when particles were coated with polyethylene glycol, and the extent of mucous penetration was greater when the polyethylene glycol was of a shorter chain length and was more densely packed on the particle surface [45]. The authors hypothesised that the longer polyethylene glycol chains were physically restricting the movement of the particles through the mucous. Encouragingly, the particles coated with polyethylene glycol had a longer residence time in

277 the vaginal mucous than did the conventional particles. Furthermore, particles coated with
278 polyethylene glycol which contained acyclovir protected 53% of mice from artificially
279 introduced HCV-2 infection, compared to only 16% protected by soluble acyclovir. In a
280 separate study, bovine serum albumin was used to coat silicon oxide nanoparticles and this
281 was shown to reduce the interactions of the particles with mucous derived from cow
282 submaxillary glands [46]. Bovine serum albumin is a negatively-charged macromolecule and
283 it was hypothesised that this would reduce electrostatic interactions with the similarly
284 negatively-charged mucins present in the mucous. Although these studies emphasise the
285 potential of mucous-penetrating nanoparticles for improving treatment delivery, these studies
286 focussed on mucous located at the site of the vagina and the submaxillary glands,
287 respectively, and further investigations are required to establish whether this strategy of
288 penetrating mucous is viable in the intestinal environment. Of note, the optimal properties
289 that are emerging for designing a muco-penetrative particle (dense, hydrophilic coat and
290 negative charge) are unlikely to suit nanoparticles which require to be endocytosed into the
291 intestinal epithelial cells.

292 Many investigations have been undertaken to develop improved treatments for *Helicobacter*
293 *pylori* (*H. pylori*) gastric infection and this provides a good case study for the development of
294 both muco-adhesive and muco-penetrative nanoparticles for treating localised mucosal
295 infections. *H. pilori* is a Gram negative bacteria associated with 95% of duodenal ulcers, 80%
296 of stomach ulcers and an increased risk of the development of stomach cancer in humans [47-
297 49]. *H. pylori* resides predominantly within the stomach, where it shrouds itself deep within
298 the *firmly-adherent mucous* layer and is capable of attaching to gastric epithelial cells [50].
299 One of the key barriers that the bacteria must overcome to achieve this is to penetrate the
300 gastric mucous. It achieves this by producing urease, which catalyses hydrolysis of gastric

urea to yield ammonia and carbon dioxide, thus elevating the pH of its gastric environment. This pH elevation also induces a dramatic decrease in the viscoelastic properties of the gastric mucous, allowing the bacteria to travel freely towards the gastric epithelial surface [51]. Current therapy for *H. pylori* eradication consists of a triple-drug regimen including two antibiotics (clarithromycin combined with either metronidazole or amoxicillin) and a proton pump inhibitor [52]. Eradication success is not high, with nearly 25% of patients showing continued infection despite treatment [53]. It can be hypothesised that treatment failure occurs due to a possible combination of factors that are unfavourable to drug action, such as the development of drug resistance, the high acid environment and low residence time within the target organ (the stomach), and the presence of a large, dense mucous layer protecting the bacteria from stomach contents [54]. As such, it has been hypothesised that a formulation able to deliver drugs into the gastric mucous for maximum and prolonged local exposure, whilst protecting the drug from the acidic pH, would constitute an optimal oral treatment for *H. pylori* (Figure 1a and 1b). Several delivery mechanisms have been investigated for improving treatment, including floating tablets [55], microspheres [56], beads [57], gels [58], and nanoparticles (Table 2). Muco-adhesive nanoparticles have been developed to increase the residence time of antibiotics in the stomach, thus increasing the likelihood of bacterial eradication [59-61]. More recently, Thamphiwatana *et al* designed nano-scale (around 100 nm with a zeta potential of -54 mV) liposomal linolenic acid (lipoLLA) residues for eradication of *H. pylori* [62]. Results demonstrated that liposomes readily attach to *H. pylori in vitro*. When mice were orally administered 1.2 mg of fluorescently labelled lipoLLA, around 6% and 3% of the dose was retained within the mucous of the stomach four hours and twenty four hours post dose, respectively. These data also demonstrated a significantly improved antimicrobial efficacy of LipoLLA in reducing *H. pylori* bacterial load in mouse stomach compared with other treatment regimens, including the standard triple therapy. Muco-

326 penetrative nanoparticles have been created using chitosan and either heparin [63] or sodium
327 alginate [64] to allow a system of reaching the bacteria at the gastric epithelial surface. Other
328 studies have been undertaken to optimise *H. pylori* treatment by encapsulation of multiple
329 treatments in one particle [65] and the development of nanoparticles with pH-responsive
330 degradation [63]. All such design projects need to consider how particles interact with the
331 gastric mucous, which remains the crucial target site and this remains an exciting area of
332 exploration with the potential to also uncover novel mechanisms and modalities for treatment
333 of diseases both within the gut and systemically.
334

335 ## 3.3 The intestinal epithelial cell barrier

336 The small intestine has a large surface area estimated at 30-300 m^2 [2] whereas the large
337 intestinal has considerably lower surface area due to the lack of microvilli, which suggests
338 that the absorption of nanoparticles is more likely to occur at the small intestinal surface. The
339 epithelial cells of the small and large intestine predominantly consist of absorptive
340 enterocytes, with mucous-secreting goblet cells and other immunological and hormone-
341 producing cells also present at lower levels. Microfold cells (M cells) are also present on the
342 intestinal epithelium and usually cluster together to form Peyer's patches. M cells allow for
343 the uptake of particles directly into the immunological system of the gut [66]. Once intact
344 nanoparticles have passed through the mucous layers surrounding the intestinal wall, particles
345 must either remain within the mucous, spontaneously break down to release contents, or be
346 able to penetrate the epithelial cells barrier. As is the case for all intestinally-absorbed
347 substances, nanoparticles penetrate the epithelial cell barrier by either the transcellular
348 (through the cell) or paracellular (between cells) pathways. Sections 3.3.1 to 3.3.3 investigate
349 these pathways in more detail.

3.3.1 The permeability of the cell membrane and tight junctions

Bile salts, in addition to their ability to promote solubilisation of lipid-based nanoparticles in the intestinal fluid [67]. It has been demonstrated that bile salts enhance the epithelial transport of hydrophilic drugs via the paracellular route and that of hydrophobic compounds via both the paracellular and transcellular routes [68]. The mechanisms by which bile salts achieve this are not fully understood, but investigations have suggested that bile salts can interact with membrane-bound phospholipids and incorporate with the cell membrane to form hydrophilic reverse micelle pores [67], can bind to calcium channels which control the paracellular route [69], and can inhibit the activity of membrane-bound drug efflux transporters [70].

Tight junctions are multi-protein complexes which form a physical, selectively impermeable seal between the cell of the epithelium of biological barriers, including the gastrointestinal system [71]. Four key proteins have been identified in the formation of tight junctions: junctional adhesion molecule [72], tricelluin [73], occuldin [74], and members of the claudin family [75]. The junctions are expressed at the apical pole of the epithelial cells and act as a barrier to the diffusion of solutes through the intercellular space and recruit various cytoskeletal as well as signalling molecules at their cytoplasmic surface. Additionally, the junctions are able to impede the paracellular transport of macromolecules, including nanoparticles. To overcome this barrier, certain substances have been tested to reversibly open tight junctions, temporarily allowing movement of macromolecules between the cells and giving direct access to subepithelial tissue and blood vessels. Sonaje et al found that chitosan nanoparticles increased the paracellular movement of insulin through a Caco-2 monolayer [76]. Tight junction opening was confirmed by staining the intracellular space

with lanthanum and it was shown that tight junctions closed rapidly following removal of chitosan. Importantly, these observations were confirmed in an animal biodistribution study. Other studies have shown similar results using chitosan nanoparticles [77], suggesting that chitosan represents a safe permeation enhancer and is a potentially effective carrier for oral protein delivery.

3.3.2 Endocytosis and exocytosis mechanisms

Endocytosis is a general mechanism found in all cells that transport membrane proteins and extracellular material in membrane vesicles into the cell interior [78]. The endocytic pathways consist of two distinct mechanisms: phagocytosis, which occurs only in certain immunological cells, and pinocytosis, which can occur in virtually all mammalian cell types and is most likely the dominant endocytic mechanism involved in particle uptake in the intestinal epithelium. Pinocytosis, or the process of "drinking" particles into the cell, is further divided into clathrin-mediated, caveolae-mediated, independent and macro pinocytosis, as shown in Figure 2 (reviewed in [78,79]). Each of these mechanisms appearing to have non-synonymous preferences of particle size, charge, shape, modulus and surface chemistry [80]. However, the extent and mechanisms of endocytosis of each particulate delivery system into cells, including into the enterocytes of the intestinal epithelium, is still poorly understood and still require investigation on a case-by-case basis. Recent investigations into the endocytosis of nanoparticles are given in Table 3. Following endocytosis into the intestinal epithelial cells, nanoparticles can either remain inside the cell unchanged, break down to release their contents intracellularly, or undergo exocytosis (Figure 2). If the objective is that internalised nanoparticles reach the systemic circulation intact, it is essential that particles are exocytosed from the basolateral membrane of the

epithelial cell. However, despite its importance there has been little effort made to investigate the exocytosis of nanoparticles. Receptor-mediated transcytosis is a process where vesicular transport of macromolecules can occur from one side of a cell to the other, and is mediated by ligand-receptor interactions. Importantly, receptor-mediated transcytosis avoids the intracellular degradation and recycling stages present in other endocytic pathways. Lectins are a group of carbohydrate-binding proteins known to be involved in cell recognition and adherence processes, and this property has led to the development of lectin-conjugated nanoparticles for targeted oral drug delivery [81-83]. Other ligands have been similarly utilised for delivery of both drugs and gene therapies, such as invasions [84]. A potential issue found with the active targeting approach is that the ligand-bound particles appear to have a reduced ability to diffuse across the mucous layer [85,86]. At present, no orally administered nanoformulation has been accepted for clinical use which has also been shown to reach the systemic circulation intact. Therefore, investigators should determine if oral administration is a feasible approach to delivering intact nanomedicines to the systemic circulation, or if simpler methods (ie intravenous or intramuscular injection) are preferred.

3.3.3 M-cell-mediated transcytosis

M cells are specialized epithelial cells which transport antigens from the intestinal lumen to cells of the immune system: soluble macromolecules, small particles, and even entire microorganisms are transported via this route. The reasons for this process are to initiate an immune response in the case of harmful organisms and to develop a tolerance in the case of food antigens [87,88]. M cells are concentrated in specific regions of the intestinal epithelium called Peyer's patches, of which there are around a few hundred in the ileum of adults and a decreasing number with increasing age [89]

As M cell-mediated transcytosis is capable of transporting larger molecules and is also able to bypass lysosomal degradation, there have been attempts to exploit this route for enhancing nanoparticle cellular uptake. To demonstrate this *in vitro*, increases in monolayer permeation of over one thousand-fold have been observed in studies measuring movement of 200-500 nm polystyrene particles, when cell monolayers have been altered to include M cells [90]. Emerging literature is demonstrating the importance of certain material characteristics for effective targeting of particles to Peyer's patches. For example, a particular suitability of neutral particle charge [91] and the enhanced uptake observed following the coating of particles with proteins derived from intestinal bacteria [92,93]. The endogenous process of M-cell-mediated pathogen uptake has been exploited by producing vaccines coupled to cholera toxin [94]. These chitosan-bound particles were more readily transcytosed by M cells, via the ganglioside GM1 receptor. Another example of exploiting the endogenous role of M-cells is the use of yeast capsules containing nanoparticles [95].

Uniquely in the gut, M cells provide an exploitable access route to the gastrointestinal-associated lymphoid tissue (GALT), thus bypassing the requirement of particles to enter the portal vein and undergo first-pass liver clearance before reaching the systemic circulation. It is not fully understood how this would affect the distribution of nanoparticles, and further investigations are required to determine if, for orally administered nanoparticles, the GALT is a more desirable entry point than the portal vein. Despite encouraging results *in vitro*, there has been limited success in exploiting M cell-mediated nanoparticle transcytosis in humans. Firstly, there are a very limited number of Peyer's patches in the human gut [89] and the total number of M cells on the intestinal surface is estimated at only one millionth the number compared to other epithelial cells [96]. Secondly, the maximum kinetic activity of an individual Peyer's patch is potentially too low for exploitation. As the primary role of M cells

is to sample gut contents and not to act as a large-scale transportation route for macromolecules, transcytosis capacity is expected to be low. This was emphasised in a study by Pappo et al, which investigated the uptake of polysetyrene nanoparticles by individual Peyer's patches from rabbit intestine. The study found that the activity of all combined patches only led to 0.02% of the nanoparticles being internalised [97]. Thirdly, it is not clear whether nanoparticles are efficiently transported from the Peyer's patches to the systemic circulation. Polystyrene nanoparticles have been found to be associated with dendritic cells at the Peyer's patches up to fourteen days, following uptake into the patches [98].

4. Limitations in using *in vitro* and *in vivo* models in nanoparticle oral absorption studies

Once nanoformulations have been created and are ready for assessment *in vitro*, it is important to assess the degradation rate of formulations in a variety of biologically relevant matrices. In addition to this, there are several *in vitro* methods that have been developed to assess cell membrane permeation which are directly relevant to intestinal absorption. A simple high-throughput screening technique is the parallel artificial membrane permeability assay (PAMPA), where drug movement through a lipid-infused artificial membrane is measured. Different lipid combinations can be utilised, including more complex layers consisting of phospholipids [99]. Using PAMPA does come with disadvantages such as the absence of drug transporting proteins and, particularly relevant for nanoparticles, the absence of endocytosis mechanisms and a mucous layer. Several cell line-based systems are available to assess for potential intestinal permeability of compounds, including the measurement of drug movement through single layers of MDCKII cells or, more commonly in drug

discovery, Caco-2 [100] cells cultured on permeable supports. The Caco-2 monolayer permeability system includes intestinal drug transporter expression, although to varying levels compared to *in vivo* expression [101], and has been demonstrated to allow for endocytic pathway-mediated cell entry [102], and therefore provides a more realistic scenario than PAMPA when screening drugs. However, this system is potentially unsuited to screening nanoparticle movement across a cell monolayer. Firstly, Caco-2 cells do not produce a mucous layer. Secondly, no M cells are present on a Caco-2 monolayer. As explained in the previous section, the mucous layer provides a potential barrier to large particles entering the intestinal epithelial cells, and M cells allow access of large particles into Peyer's Patches. In an attempt to address these issues, a more complex "triple culture" system has been developed. In this system, HT29-MTX cells, which produce a mucous layer on their apical surface, are seeded alongside Caco-2 cells to form a mixed population monolayer. Additionally, RajiB immunological cells can be introduced to the basolateral chamber, resulting in differentiation of a proportion of Caco-2 cells to M cells. This system has been used to investigate the effect of polystyrene particle size on cellular permeation potential [103].

When investigating nanoparticle-induced tight-junction opening *in vitro*, Caco-2 cells monolayers are a commonly used model [104]. However, Caco-2 cells do not express the exact set of proteins associated with tight junction formation *in vivo* [105]. Of note, claudin-5 is present in human jejunum tight junctions and claudin-8 is present in human large intestine, but neither proteins are expressed in Caco-2 cells. Linnankoski et al compared the porosity and pore size of tight junctions in human intestinal wall and in commonly utilised epithelial cell lines used to represent the intestinal epithelium [106]. Compared to intestinal tissue tight junctions, Caco-2 and MDCK-II cells both showed smaller tight junction porosity and 2/4/A1

493 cells showed larger pore size. The effects of these differences are unknown but it should be
494 acknowledged that the tight junctions formed by commonly used cell lines may not fully
495 represent those found *in vivo*.

496 The pancreatic enzymes present in the intestinal fluid are often not included during *in vitro*
497 assessment of nanoparticle Caco-2 uptake experiments, even though these enzymes have the
498 potential to break down nanoparticles. To avoid this issue, physiologically-relevant intestinal
499 buffers can be created which include pancreatic enzymes [107]. Simulated intestinal fluid
500 (SIF) is described in the USP and contains pancreatin at 10 mg/mL, whereas other commonly
501 used intestinal buffers such as fasted state simulated intestinal fluid (FaSSIF) and fed state
502 simulated intestinal fluid (FeSSIF) contain no enzymes. In addition to the luminal enzymes,
503 the enterocytes of the small intestine express multiple digestive protease enzymes which are
504 active at the apical surface and are potentially relevant to peptide-containing-nanoparticle
505 stability. Caco-2 cells show no or poor expression of several of these protease enzymes, as in
506 the cases of transmembrane protease serine 4 and dipeptidyl-peptidases 4 [108] and therefore
507 any interactions between these enzymes and nanoparticles will be poorly represented in *in*
508 *vitro* studies utilising Caco-2 cells.

509 *In vitro* models are beginning to be established to investigate many aspects of orally absorbed
510 nanomedicines and their interactions with intestinal mucous. However, current *in vitro*
511 systems cannot fully replicate the complexities observed *in vivo*. In order to breach the gap
512 between *in vitro* models and clinical application, assessment in pre-clinical species is
513 required. Despite the advantages of using a more complex biological system, there are
514 inherent difficulties in extrapolating data from pre-clinical species to predictions in human.
515 The gastrointestinal tract, when compared between species, have shown significant
516 differences, such as in pH [109] and transit time [110]. There is also significant variation in

517 the lymphoid structures present in the GI tract. Lymphocyte-filled villus are present in human
518 and rat whereas cryptopatches are only found in mice [111]. Rodents do not produce
519 intestinal mucin to the same extent as is observed in humans, and this most likely reduces the
520 barrier properties to successful drug delivery and nanoparticle distribution [112].
521 Additionally, rodents have a significantly higher density of Peyer's patches in the intestine
522 when compared to humans and this should be considered when optimising oral absorption via
523 this pathway [113]. In addition to anatomical differences, there are important variances
524 between species in the expression and/or activity of metabolic enzymes and transporters
525 relevant to intestinal absorption [114-117].

526

527 # 5. Conclusions

528 There exist great possibilities for nanotechnologies to develop controlled and targeted drug
529 delivery systems by exploiting the anatomical, physiological and molecular processes at the
530 gastrointestinal mucosal surface. However, progress has been slow, better mechanistic
531 understanding is required, and as a result no nanotechnology-enabled products are currently
532 in clinical use which specifically target the mucous layers of the stomach or the intestine.
533 Similarly, no orally-administered nanoparticles clinically in use have been designed to allow
534 the particle to gain entry to the systemic circulation intact. Compared to small molecules, the
535 relationship between nanoparticle characteristics and intestinal absorption potential are
536 poorly understood. In order to inform future design strategies, further investigations in this
537 area are warranted and will improve our understanding of any relationships which can be
538 utilised for addressing treatment and bioavailability goals.

539 Ideally, oral nanoformulations which aim to gain entry to the systemic circulation will be able
540 to avoid being degraded in luminal environment, avoid being trapped and cleared by the
541 *loosely-adherent mucous* layer, will be able to penetrate the mucous to reach the intestinal
542 surface where transcytosis of the particle can then take place. Alternative routes past the
543 epithelial cells, such as paracellular movement and via M-cells, also exist. When the desired
544 outcome is local release of drug at sites along the gastrointestinal system, such as is the case
545 with *H. pylori* eradication, adherence of nanoparticles to the *firmly-adherent mucous* layer
546 would be advantageous, as would a controlled release strategy. Many global research efforts
547 are now focused upon harmonising progress from technology development and disease
548 understanding, and an inter-disciplinary approach to development in this area is warranted.

549

550 6. Expert Commentary

551 Despite the potentially large clinical advantages and continued research activity in the
552 development of nanotechnology-based oral drug delivery products, it cannot be ignored that
553 there has been a disappointingly small number of new products that have progressed to use in
554 humans. Furthermore, all oral nanomedicines currently used in clinical therapy are SDNs
555 with the objective of improving the dissolution rate of poorly soluble drugs. The
556 immunosuppressant sirolimus, which has very low aqueous solubility, is available as an oral
557 solution but also as an SDN tablet (Rapamune®, licenced 1999 in USA, 2001 in EU). The
558 tablet is generally preferred for ease of use and exhibits a ~20% increase in oral
559 bioavailability compared with the oral solution, with potential for further improvements on
560 the current formulation [118]. In a similar scenario, the antiemetic aprepitant has very low
561 solubility at low pH and oral administration of traditional formulations shows poor

562 bioavailability and high inter-patient pharmacokinetic variability. An SDN formulation of
563 aprepitant (Emend®, licenced in 2003) was created and resulted in an improved oral
564 absorption. Food intake can result in altered and/or variable drug absorption, and SDN
565 formulations have been developed to negate this effect in the cases of the antihyperlipidemic
566 fenofribate (Tricor®, licenced in 2004) and the anticancer drug megestrol acetate (Megace®
567 ES, licenced in 2005).

568 To rectify this situation, the authors believe that several developmental barriers exist which
569 need to be addressed, such as the lack of validated *in vitro* assays for assessing
570 nanoformulation pharmacokinetics, the unsuitability of standard *in vivo* intestinal absorption
571 models, the lack of interest from companies to reformulate potentially unprofitable generic
572 treatments, poor scientific understanding of the technology, and the difficulty in convincing
573 regulatory bodies of the safety of intestinal mucous-targeting and mucous-altering
574 nanomedicines. Regarding the issue of safety, a primary role of mucous is to protect exposed
575 surfaces from foreign entities, and very little is known about how orally administered
576 nanoformulations can affect this role. In an *ex vivo* experiment using human cervicovaginal
577 mucous, Wang et al found that the level of mucous penetration of 1 μm muco-inert particles
578 was increased ~10-fold following pre-treatment of the mucous with 200 nm mucoadhesive
579 nanoparticles [119]. The authors hypothesised that the mucoadhesive particles were eliciting
580 this effect by bundling mucin fibers together through polyvalent adhesive interactions, and
581 suggested that this may lead to greater exposure of mucosal cell barriers to foreign particles,
582 including pathogens and other potentially toxic nanomaterials. In addition, mucous
583 penetrating nanoparticles, which actively degrade mucous, are likely to have a similar effect.
584 Indeed, this has been demonstrated *in vivo*. N-acetyl-L-cysteine (NAC), a commonly used
585 mucolytic agent, was apparently shown to cause a 6-fold increase in the absorption of 3.2 μm

586 polystyrene particles in both the Peyer's patches and mesenteric lymph nodes in a ligated rat
587 intestine model [120], although this data does not support the belief that M cells can only
588 endocytose particles of around 1 μm in size and below [121]. In another study, a 30%
589 depletion of mucus by pilocarpine in an *ex vivo* rat intestinal absorption model showed a 3-
590 fold increase in E. Coli translocation [26]. If this were to translate to an increased infection
591 risk in humans, there would be serious safety issues concerning the use of mucous-targeting
592 nanoparticles which has remained relatively unaddressed in current studies. Of particular
593 concern would be the use of mucous-targeting treatments in immune-compromised patients,
594 such as in the use of anti-HIV drugs and cancer chemotherapy. Future research should be
595 undertaken to investigate this risk, and to understand whether it is possible to use mucous-
596 targeting particles to improve oral medicine delivery without increasing exposure of the
597 intestinal surface to pathogens present in the intestine.

598

599 # 7. Five-year view

600 Ideally, within five years it is feasible that a mucous-targeted and potentially mucous-
601 modifying nanoformulation will have been accepted for clinical use by the relevant
602 regulatory bodies. There will be additional trials investigating the use of SDNs and other
603 nanoformulations for improving the dissolution and bioavailability of poorly soluble drugs, as
604 this remains the only strategy that has led to clinically accepted oral nanomedicines. A recent
605 example is the success of using SDN reformulation to increase the bioavailability of the
606 antiretrovirals lopinavir and efavirenz in animal studies [4,122] which is now being assessed
607 in Phase 1 trials. Through improved assay design and large scale screening programmes,
608 there will be an increased understanding of how the physicochemical properties of

609 nanoparticles affect mucous binding and endocytosis. General “design rules” are currently
610 being established which will only become more sophisticated over time [37]. Improvements
611 in technology, such as in the use of “lab-on-a-chip” microfluidic set-ups, will potentially help
612 to increase the predictive ability of *in vitro* experiments to measure movement of
613 nanoparticles across the gut [123,124]. Similarly, the growing wealth of information
614 regarding the influence that physicochemical characteristics play on nanoparticle
615 biocompatibility and safety is allowing improved design and rationale for their development
616 and preclinical assessment. From all this, a start is being made in establishing standard rules
617 which associate nanoparticle characteristics with toxicity, including immunological issues
618 [125].

619

620 # 8. Key highlights

621 • Nano-scale oral formulations are being developed to allow delivery of poorly
622 absorbed medicines such as peptides, macromolecules and certain small drug-like
623 molecules.
624 • The mucous layer of the gastrointestinal tract is capable of trapping pathogens and
625 eliminating large particles, including nanoparticles, as part of the natural defence
626 system of the body.
627 • The interactions between nanoformulations and the gastrointestinal mucous layer are
628 poorly understood.
629 • Nanoformulation properties such as particle size, charge, and shape, as well as
630 mucous properties such as viscoelasticity, thickness, density, and turn-over time are
631 all relevant to these interactions but definitive design rules have not been established.

- *In vitro* and animal models traditionally used in drug development are often unsuitable for assessing the potential of nanoparticles to enhance the oral bioavailability of treatment.
- Research should focus on developing reliable screening methods which take into consideration the influence of the intestinal mucous on nanoparticle absorption
- A goal of this research should be the establishment of a "design blueprint" for nanoparticles that are capable of traversing the gastrointestinal epithelium, hence enabling oral delivery of drugs that are currently poor oral absorption or are limited to administration by injections.
- The ultimate goal is that absorption-enhancing nanoformulations will be accepted into the drug market.

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	pH	Volume (mL)	Radius (cm)	Transit time (h)	Firmly adherent mucous (μm)	Loosely adherent mucous (μm)
Stomach	1.3	46.56	NA	0.25	75-170	97-158
Duodenum	6	41.56	1.53	0.26	16 ± 3	154 ± 39
Jejunum	6.2-6.4	276.5	1.29-1.45	1.67	15 ± 2	108 ± 5
Ileum	6.6-7.4	214.65	0.82-1.13	1.29	29 ± 8	447 ± 47
Colon	6.8-6.8	97.82	2.41-3.39	16.76	116 ± 51	714 ± 109

Table 1. Physiological characteristics of the sections of the gastrointestinal system. The pH, volume, radius and transit time data are the data used in the GastroPlus physiologically based pharmacokinetic modelling software for simulated fasted human subjects [126]. The thickness of loosely and firmly adherent mucous was obtained from rats [127] and is a meta-analysis of several studies giving the mean thickness ± standard deviation or, in the stomach, thickness range.

Excipients	Drug	Notes	Reference
Muco-adhesive nanoparticles			
Ethyl cellulose, methyl cellulose	α-mangostin	Controlled drug release at higher pH	[59]
Poly (lactic-co-glycolic acid)	Clarythromycin, acetohydroxamic acid	Enhanced in vitro H. pylori kill	[60]
Poly (lactic-co-glycolic acid)	Clarythromycin	Equal or enhanced eradication effect against clinical H. pylori strains	[61]
Chitosan, alginate	Pexiganan	Greater gastric mucous retention and effectiveness in rodent model	[128]
Chitosan, alginate, gelatin, Poly-γ-glutamic acid	Amoxicillin	Increased drug stability in low pH	[129]
Targeted nanoparticles			
Precirol ATO5®, Miglyol-812®, Tween 60®	Docosahexaenoic acid	Disrupts H. pylori membrane	[130]

Montmorillonite, polyethyleneimine	Metronidazole	Disrupts H. pylori membrane, improved effectiveness in rodent model	[131]
Ureido-conjugated chitosan	Amoxicillin	Targeted to H. pylori urea transporter	[132]
Lpp20 antigen, myristic acid	None	Lpp20 used as template to imprint nanoparticle surface and encourage binding to H. pylori	[133]
Fucose-conjugated chitosan, glutamate	Amoxicillin, clarithromycin, nomeprazole	Increased mucoadhesion and targeting of bacteria	[134]
Muco-penetrative Nanoparticles			
Chitosan, heparin	None	Can penetrate mucous to reach bacteria	[63]
Chitosan, alginate	Amoxicillin	Long-term mucous penetration confirmed using fluorescent labelling	[64]

Table 2. Examples of nanoparticles created to target H pylori infection by employment of various design strategies. Strategies occasionally overlap, although, roughly categorise into the adherence to gastric mucous to prolong drug exposure, the targeting of the bacteria or of the site of bacteria residence deep within the mucous.

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Nanoparticle	Size (dm)	Surface Charge	Cell Types	Summary	Reference
Polystyrene NPs	40 & 200 nm Can be easily synthesised into a broad range of sizes	Positive & negative	Mouse macrophage (J774A.1) Cervical cancer cell line (HeLa) Lung carcinoma cell line (A549) Brain astrocytoma cell line (1321N1)	Endocytosis of polystyrene NPs are mostly caveolin-mediated & clathrin-mediated. Mouse macrophage, cervical cancer, lung carcinoma and the brain astrocytoma cell lines also utilise micropinocytosis, phagocytosis, microtubule cytoskeleton and actin polymerisation mechanisms. Due to their ease of synthesization in a broad range of sizes, polystyrene NPs are favoured in studies surrounding bio-nano interactions.	[135] [136] [137]
Vitamin E-loaded OAE NPs	50, 120, 420 and 730 nm	Negative	Caco-2 cell line Excised rat jejunum	OAE NP endocytic mechanisms are clathrin-mediated and caveolae-mediated endocytosis and micropinocytosis based. It was found that as size increased uptake decreased.	[138]
Folate–phytosterol–alginate NPs	150 nm	Negative	Folate-receptor-overexpressing cancer cells (KB cells)	Folate receptor–mediated endocytosis is the main uptake mechanism of folate–phytosterol–alginate NPs.	[139]

CS NPs	80 nm	Positive	QGY & QGY/Gefitinib cells (established Gefitinib resistant)	Caveolae-mediated endocytosis, clathrin-mediated endocytosis and macropinocytosis are the main mechanisms of CS NP cellular uptake.	[140]
Mannosylated CS NPs	260 nm	Positive	Murine macrophage cell line Human embryonic kidney cell line	In comparison to CS NPs, mannosylated CS NPs can target macrophages and enter via mannose receptor-mediated endocytosis.	[141]
CS/HA-g-PCL NPs	50 – 300 nm	Positive & negative	Esophageal squamous carcinoma cell line (EC109)	CS/HA-g-PCL NPs target tumour cells and enter via CD44 receptor-mediated endocytosis.	[142]
PR9/QD complexes	100 nm	Positive	Lung carcinoma cell line (A549)	Endocytic inhibitors were used to show that PR9/QD complexes enter the cell via classical endocytosis such as caveolae-mediated and clathrin-mediated endocytosis.	[143]

SNA NPs	10 nm	Negative	Endothelial cell line (C166) Lung carcinoma cell line (A549) Mouse fibroblast (3T3) Human keratinocyte (HaCaT)	The 3D oligonucleotide shell promotes the entrance of SNA NPs into cells via lipid-raft–dependent, class A scavenger receptor-mediated and caveolae-mediated endocytosis.	[144]
siRNA-conjugated Au nanoconstructs	13 and 50 nm spheres, and 40 nm stars	Negative	Glioblastoma cell line (U87)	After 24-hour incubation 13nm spherical AuNPs remained dispersed within endocytic vesicles whilst 40nm star and 50nm sphere AuNPs did not. Although the specific endocytic pathway was not determined, the suitability of 40 and 50nm AuNPs for siRNA delivery was highlighted.	[145]
FBS-coated AuNPs	20nm	-	Lung fibroblasts (MRC5)	Endocytic inhibitors demonstrated that FBS-coated AuNPs enter both cell types	[146]

			Chang liver cell line	through clathrin-mediated endocytosis.	

1035 Table 3. Investigations into the endocytosis of various nanoparticles. Abbreviations: Au,
1036 gold; CS, chitosan; CS/HA-g-PCL, chitosan coated polycaprolactone-grafted hyaluronic acid;
1037 dm, diameter; FBS, fetal bovine serum; nm, nanometer; NPs, nanoparticles; OAE, oleoyl
1038 alginate ester; PR9, cell-penetrating peptide; QD, quantum dot; QGY, hepatocellular
1039 carcinoma cell line; siRNA, small interfering ribonucleic acid; SNA, spherical nucleic acid.

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