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Bile acid transporter mediated endocytosis of oral bile acid conjugated nanocomplex

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ABSTRACT

The development of highly funtional and orally available nanoparticles is the ultimate goal in nanoparticle delivery. Various functional nanoparticles have been studied to that end but there has yet to be an oral nanoparticle that can be successfully applied. Here, we describe for the first time a novel bile acid conjugated nanoparticle that can be selectively absorbed by bile acid transporters in the small intestine. The bile acid conjugate nanoparticles that were first treated with enterocytes were successfully attached to the cell surface and then internalized inside the cells. We show that bile acid based interaction between a nanoparticle and its transporter induces its endocytosis and cellular uptake. This feature of cellular activity, described here for the first time, could be well utilized in the uptake of nanoparticles or macromolecules inside epithelial cells for oral delivery. In animal studies, bile acid conjugated self-assembling nanocomplexes successfully interacted with bile acid transporters in the ileum and were subsequently taken up into the epithelial cells. Considering the importance of orally deliverable nanoparticles, this nanotechnology using bile acid conjugation and transporter mediated endocytosis could be a crucial method for the successful application of various nanoparticles.

1. Introduction

Nanoparticles (NP) or nanocomplexes(NC) are regarded as important carriers of various drugs in the body [1,2], hence their usefulness in drug development and potential in clinical therapy are well recognized [3]. Over the last decades, various nanoparticles have been developed as biologics, genes, drugs and biomaterials [4–6], but few have been successfully applied in the clinic [7,8]. For example, the use of doxorubicin liposome (Doxil) and albumin-paclitaxel nanoparticles (Abraxane), among the few drugs currently available in the market, is limited to a narrow set of indications [9–11].

For a long time, the modality of oral delivery has been noted for its applicability in healthcare therapeutics, particularly for its effectiveness in solving problems related to the toxicity of nanoparticles. However, while oral formulations of nanoparticles have been shown to be effective in animal experiments, few have yet to be tried in human trials [12–14]. Thus the possibility that oral nanoparticles can be used in painless, safe and convenient administration, coupled with the undeniable demand in the market underwrites the strong rationale behind efforts to develop them as drugs and therapeutic materials.

The development of oral nanoparticles is extremely challenging due to biological barriers in the body [15]. First of all, there is the intestinal epithelial barrier in the GI tract [16,17]. Then the harsh environment of the small intestine and the large size of nanoparticles both make easy absorption of nanoparticles difficult [18], necessitating a novel strategy or biotechnology to improve the absorption efficiency [19]. Until now, polymer based, positively charged or chitosan-based nanocomplexes have mainly been used for the oral delivery of nanoparticles or nanocomplexes [20–24]. Recently, however, the Johns Hopkins hospital research group has shown that clinically applicable oral nanoparticles can be

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developed with improved penetrating ability [25–27]. An additional group at MIT has described the delivery of active transport mediated oral nanoparticles that targeted the neonatal Fc receptor (FcRn) in the GI tract [28].

Among the various delivery modalities introduced so far, none has explored the notion of active delivery of nanoparticles using bile acid and its transporters. A bile acid, which is a ligand of bile acid transporter, has been used for nanoparticle or microcapsule formulation in many studies since its excellent function has been discovered [29,30]. In the body, the apical sodium-dependent bile acid transporter (ASBT) in the small intestine transports bile acids into epithelial cells for bile acid recycling [31]. Unlike receptor mediated endocytosis, the notion of absorption via transporter mediated nanoparticles has never been considered as it is difficult for nanoparticles to be transferred across the small pores of transporters [32]. However, when bile acids are conjugated to nanoparticles, the bile acids modify the surface of nanoparticles, effectively improving the affinity with epithelial cells in the small intestine. Uptake by bile acid transporter-mediated nanoparticles is theoretically possible through drug delivery system because macromolecules like peptides or polysaccharides can be successfully absorbed in the GI tract.

On the other hand, it is essential that a clinically available oral nanoparticle should not have any toxicity or accumulation problems. For that reason, US Food and Drug Administration (FDA) approved biomaterials such as heparin and protamine as safe biomaterials to be used for oral nanoparticle formulation [33–35]. Heparin is one of the most negatively charged biomaterials that interacts very strongly with protamine, whose positive charge is half of that in the arginine [36]. Together heparin and protamine can form a stable self-assembly nanocomplex via electrostatic interaction in various physiological conditions [33,37]; this non-hydrophobic nature of bonding is especially important as it indicates that the interaction is not affected by van der Waals force.

In our previous studies, orally active heparin conjugates were developed by using bile acid recycling system, where macromolecules including unfractionated heparin (UFH) and low molecular weight heparin (LMWH) were conjugated with deoxycholic acids for successful absorption in the GI tract [38–40]. When orally administered, these macromolecules were mediated by apical sodium-dependent bile acid transporters (ASBT) naturally present in the small intestine [31,41]; it further showed that they could overcome several biological barriers, exhibiting remarkable interaction with epithelial cells in the small intestine [42–44] and indicating possibilities for effective oral delivery modalities employing nanoparticles or nanocomplexes [30,45,46].

In the present study, we describe a novel nanoparticle that can be designed using functional biomaterials, such as heparin, protamine and bile acids, and orally delivered through the bile acid transporter mediate uptake. Considering the importance of bile acids and their transporters in the absorption process, the use of such nanocomplexes could enhance the delivery of biomolecules across mucus barriers. Furthermore, this study also reveals following distinctive characteristics of the key nanoparticle: (i) it is made of FDA approved biomaterials which can be controlled for its charge and surface; (ii) it has a clear absorption mechanism with bile acid transporters for oral delivery, and (iii) it has clear elimination process without nanoparticle accumulation problems. This is a first study that has ever shown that an orally administered bile acid conjugated nanocomplex can be transported across the epithelial cell membrane in the intestine. In the study, bile acids were conjugated with heparin or protamine to form self-assembling nanocomplex formulations of different charges and properties, which were transported via bile acid transporter mediated endocytosis. No evident exocytosis was observed, which indicates that the absorbed nanocomplexes remained in the epithelial cells for a few days until they were eliminated through the natural turnover of epithelial cells. In summary, this nanotechnology could be used to deliver drugs via bile acid transporters without the accumulation problem, offering as a distinctively efficient modality that can facilitate the oral delivery of various biomolecules.

2. Materials and methods

2.1. Materials

Low molecular weight heparin (Nadroparin, fraxiparin calcium, M.W. 4.5 kDa) was obtained from the Nanjing King-Friend Company. (Nanjing, China). Deoxycholic acid (>98%), ethylenediamine (>99.5%), fluorescein 5-isothiocyanate (>90%), N-hydroxysuccinimide (98%), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (99%) and protamine sulfate were purchased from Sigma chemical Co. (St. Louis, MO, USA). Methanol (>99.9%) and N,N-dimethylformide (DMF, 99.8%) were purchased from Merck (Darmstadt, Germany). DMEM high glucose medium, fetal bovine serum, penicillin–streptomycin and trypsin-EDTA were obtained from GIBCO (Grand Island, NY). Amininated cyanine 5.5 (Cy5.5-amine, 95% in HPLC-MS) and cyanine 5.5 N-hydroxysuccinimide ester (Cy5.5-NHS, 95% in HPLC-MS) were bought from Lumiprobe (Hallandale beach, FL). Eight-week-old male C57BL/6 mice were purchased from Orient Bio (Seoul, Korea). All reagents were used without further purification.

2.2. Synthesis of bile acid conjugates

Low molecular weight heparin (LMWH; M.W. 4.5 kDa) and protamine sulfate (M.W. 4.0 kDa) were used with or without chemical modification. LMWH and dexoycholic acid (DOCA) conjugate were synthesized as described previously. [47,48]. Briefly, N-deoxycholylethylamine (EtDOCA) was prepared from DOCA by reacting with N,N′- dicyclohexylcarbodiimide (DCC) and ethylenediamine. The amine group of EtDOCA was conjugated with the carboxylic groups of heparin in distilled water with excess amount of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and N-hydroxylsuccinimide (NHS). On the other hand, DOCA (400 mg) was activated with DCC (272 mg) and NHS (152 mg) in tetrahydrofuran (40 mL) to prepare a protamine and DOCA conjugate. The synthesized succinimidyl deoxycholate was purified by filtration and washing with cold n-hexane. The protamine sulfate has a primary amine group which can react with the activated DOCA at the end of its structure (N-terminal). NHS-activated DOCA was reacted with protamine in DMF/distilled water co-solvent, and then precipitated in acetone and methanol. All products were confirmed by TLC and NMR as in the previous studies [48,49]. The purity was determined to be > 98% in both RP-HPLC and TLC. The molecular structures of LMWH, heparin-DOCA conjugate, protamine and protamine-DOCA conjugate were drawn by ChemBioDraw Ultra 12.0 (Cambridge Soft Corporation). The structures of ASBT were generated from the protein data bank (protein data bank [PDB] code, 2AWR and 3ZUY). All of the structures were visualized by PyMOL 1.7.0.1 (DeLano Scientific) [31].

For dye-conjugation, firstly, cyanine 5.5 or fluorescein isothiocyanate (FITC) was conjugated to heparin conjugates or protamine sulfate to visualize the nanocomplex. FITC directly reacted with heparin or protamine sulfate in a DMF/distilled water cosolvent. The conjugation with aminated cy5.5 was carried out by EDAC/NHS reaction in distilled water. After precipitating in the methanol and acetone cosolvent, the products were dialyzed for 2 days using a dialysis membrane with the M.W. cutoff of 2000 Da, and the solution was lyophilized by freeze-dryer. The purity of the product