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Intestinal absorption of fluorescently labeled nanoparticles

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Abstract

Characterization of intestinal absorption of nanoparticles is critical in the design of noninvasive anticancer, protein-based, and gene nanoparticle-based therapeutics. Here we demonstrate a general approach for the characterization of the intestinal absorption of nanoparticles and for understanding the mechanisms active in their processing within healthy intestinal cells. It is generally accepted that the cellular processing represents a major drawback of current nanoparticle-based therapeutic systems. In particular, endolysosomal trafficking causes degradation of therapeutic molecules such as proteins, lipids, acid-sensitive anticancer drugs, and genes. To date, investigations into nanoparticle processing within intestinal cells have studied mass transport through Caco-2 cells or everted rat intestinal sac models. We developed an approach to visualize directly the mechanisms of nanoparticle processing within intestinal tissue. These results clearly identify a mechanism by which healthy intestinal cells process nanoparticles and point to the possible use of this approach in the design of noninvasive nanoparticle-based therapies.

Key words: Nanoparticles; Nanomaterials; Cancer; Endocytosis; Nanotechnology

Background

Therapeutic nanoparticles are colloidal structures with a cargo space for drugs that is segregated from the environment by a hydrophilic, usually polyethylene glycol (PEG), corona that prevents recognition by macrophages and enables long-term circulation in the bloodstream.[1,2] The size of nanoparticles (10-100 nm) permits their extravasation and accumulation in tumor sites; this is known as the enhanced permeability and retention (EPR) effect.[1,2] Passive targeting is based on pathophysiological characteristics unique to solid tumors: hypervascularity, irregular vascular architecture, potential for secretion of vascular permeability factors, and absence of effective lymphatic drainage that prevents efficient clearance of macromolecules.[1,2] Concurrently, the development of nanoparticles has matured so that they have become a major tool in intravenous (*i.v.*) targeted anticancer therapy and in the pharmaceutical industry.[1–5] A cohort of various PEGylated nanoparticles for *i.v.* administration has been explored for cancer imaging and therapy, and has resulted in numerous marketed formulations in various stages of clinical trials.[1,2]

Nanoparticles are not generally administered orally mainly because of physiological obstacles; *i.e.*, from the perspective of cellular drug delivery, access to the cytosolic space of eukaryotic cells is restricted primarily to hydrophobic small drugs with a MW <500, which have relatively high membrane partition coefficients and permeability constants.[4,5] To increase intestinal uptake, nanoparticles can be conjugated with various bioadhesive (*e.g.*, poly(lactic acid (PLA)),[3] P-gp pump-inhibiting (*e.g.*, d-α-tocopheryl PEG succinate (TPGS)),[6] and vitamin[7–11] (*e.g.*, biotin, folic acid, vitamin B_{12}, and transferrin) ligands.

In this work, we employed model micellar nanoparticles consisting of a hydrophobic phospholipid core and a PEG hydrophilic corona, and characterized their physicochemical and *in vivo* drug delivery characteristics. We used these micellar nanoparticles to visualize their processing directly and to elucidate the mechanisms of endocytosis within healthy intestinal tissues for the first time.

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Methods

Micellar nanoparticles characterizations

Physico-chemical characteristics of phospholipid based PEGylated micellar nanoparticles linked to vitamin (biotin) via either amid or disulfide bonds were investigated. Nanoparticles were imaged with a cryo-TEM (JEOL 2100 TEM, Vironova, Sweden). Proton (^{1}H NMR) NMR spectra were recorded with a Bruker ARX 300 MHz spectrometer in deuterated chloroform as a solvent. Nanoparticles labeled with hydrophobic marker coumarin 6 were used for *in vivo* studies and confocal imaging of rat ileum cross-sections. Fluorescent HPLC analysis with a fluorescence detector was used to develop analytical method for distinguishing nanoparticle linked and free hydrophobic fluorescent marker molecules. HPLC analysis was conducted under isocratic conditions at ambient temperature using a reversed-phase column (Alltima™ C18 column 5 μ, 250 × 4.6 mm, Grace Division).

Animal studies

All animal experiments were approved by the Animal Ethics Committee, Institute of Medical and Veterinary Science (Adelaide SA, Australia), Project No. 35a/12. Animals were treated humanely, and all procedures employed are in accordance with the Animal Ethics Committee, Institute of Medical and Veterinary Science (Adelaide SA, Australia) guidelines.[12]

In vivo absorption study

Groups of six male Sprague Dawley rats weighing 330 ± 30 g were used for each absorption study. Each group was dosed with one of the three fluorescent nanoparticle formulations (the ratio coumarin 6:nanoparticles was 1:10) at the same dose (0.5 ml of 1 mg/ml nanoparticle dispersion in phosphate buffer) by oral gavage under lightly inhaled anesthesia (isoflurane to effect); *i.e.*, the nanoparticles were dispersed in phosphate buffer and administered as 0.5 ml of a 1 mg/ml dispersion. The rats were cannulated in the right jugular vein under isoflurane inhaled anaesthesia and allowed to recover.

The cannulated rats were fasted overnight (14 ± 1 h) before each oral dosing and were given access to food 4 h after each dose, but water was accessible at all times. Blood samples (0.2 ml) were collected from the jugular vein at the designated time intervals, and the cannula was flushed with an equal volume of heparinized normal saline (50 U/5 ml) to prevent blood clotting. An aliquot of 100 μl of plasma was vortex-mixed with 200 μl acetonitrile and centrifuged at 11,963 ×*g* for 10 min to remove proteins before the HPLC analysis, as described before. The pharmacokinetic parameters were determined using the PC software, WinNonlin® Standard Edition Version 4.1 (Pharsight Corp.) using a noncompartmental model.

Collection of rat ileum

Groups of six male Sprague Dawley rats weighing 330 ± 30 g were euthanized humanely by CO_2 inhalation, and the ileum was collected and stored at −70 °C in CryoStor™ solution until use.

Fluorescence imaging of intestinal cross-sections

Four-centimeter pieces of everted rat ileum were dually stained with LysoTracker Red and coumarin 6 labeled nanoparticles. Pieces of rat ileum were withdrawn from the buffer at predetermined time intervals (every 2 min during the first 30 min, 1 h, and 2 h). In the endocytosis inhibition experiments, samples were incubated for 1 h with 10 μg/ml chlorpromazine, 1 μg/ml filipin III, or 1 μg/ml lovastatin + methyl-β-cyclodextrin before incubation with the test nanoparticles. Cross sections were imaged using a confocal laser scanning microscope (Leica TCS SP5, DMI6000B inverted microscope).

Results

Nanoparticles characterization

We investigated the *in vivo* oral absorption of three types of nanoparticles (Figure 1, *A*): basic nanoparticles, unmodified nanoparticles without a targeting ligand, and nanoparticles linked to a biotin-targeting ligand via either amide or amide–disulfide–

Figure 1. Nanoparticle synthesis and characterization. **(A)** 1: unmodified nanoparticles 1,2-distearoyl-sn-glycero 3-Phosphoethanolamine-n-[amino(polyethylene glycol)-2000] ammonium salt (DSPE–PEG2000–NH_2); formula kindly supplied by Avanti Polar Lipids, Inc. and reproduced with permission; 2: Nanoparticles conjugated to biotin via peptide bonds 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[biotinyl(polyethylene glycol)-2000] ammonium salt (DSPE–PEG2000–biotin); formula kindly supplied by Avanti Polar Lipids, Inc. and reproduced with permission; 3: Diagram of the conjugation reaction between DSPE–PEG2000–NH_2 and succinimidyl 2-(biotinamido)-ethyl-1,3'-dithiopropionate (formula reproduced from Thermo Fisher Scientific with permission) that results in a polymer conjugated to biotin via disulfide bonds (DSPE–PEG2000–SS–biotin). **(B)** ^{1}H NMR spectra of model nanoparticles. Blue: ^{1}H NMR of DSPE–PEG2000–NH_2: δ 0.87 (t, 6H, (–CH_3)$_2$), 1.25 (s, 60H, H from the hydrophobic chain), 1.58 (s, water from the solvent), 1.9 (s, blunt peak from heteroatoms NH_2), 2.2 (m, 4H, CH_2CO–), 3.2 (m, 2H, CH_2NHCO–), 3.3 (m, 2H, CH_2-NH–), 3.6 (s, 176H,–OCH_2CH_2–), 3.8 (m, 2H, CH_2O–), 3.9 (m, 2H, CH_2OP–), 4.2 (m, 2H, CH_2OPO–), 4.3 (d, 2H, CH_2OCO–), 5.2 (s, 1H, CHCO–), 6.5 (s, CONH–). Red: ^{1}H NMR of DSPE–PEG2000–biotin: δ 0.87 (t, 6H, (–CH_3)$_2$), 1.25 (s, 60H, H from the hydrophobic chain), 1.45 (m, 2H,CH_2–biotin chain)), 1.6 (s, water from the solvent), 1.7 (m, 4H, CH_2 biotin chain), 2.15 (t, 2H, biotin), 2.2 (m, 4H,–CH_2CO–), 2.7 (d, 1H, CH_2-S- biotin), 2.9 (m, 1H, CH_2S-biotin), 3.1 (m, 1H, biotin), 3.2 (m, 2H, CH_2NHCO–), 3.3 (m, 2H, CH_2-NH–), 3.6 (s, 176H,–OCH_2CH_2–), 3.8 (m, 2H, CH_2O–), 3.9 (m, 2H, CH_2OP–), 4.2 (m, 2H, CH_2OPO-), 4.3 (d, 2H, CH_2OCO–), 4.4 (m, 1H, biotin), 4.5 (m, 1H, biotin), 5.1 (s, 1H, CHCO–), 5.8/6.7 (s, –CONH–). Green: ^{1}H NMR of DSPE–PEG2000–SS–biotin: δ 0.87 (t, 6H, (–CH_3)$_2$), 1.25 (s, 60H, H from the hydrophobic chain), 1.45 (m, 2H,CH_2–biotin chain)), 1.6 (s, water from the solvent), 1.7 (m, 4H, CH_2 biotin chain), 2.15 (t, 2H, biotin), 2.2 (m, 4H,–CH_2CO–), 2.37 (t, 2H, CO-CH_2–), 2.7 (d, 1H, CH_2-S-biotin), 2.9 (m, 1H, CH_2S-biotin), 3.1 (m, 1H, biotin), 3.15 (t, 2H, –CH_2-S–), 3.2 (m, 2H, CH_2NHCO–), 3.3 (m, 2H, CH_2-NH–), 3.38 (t, 2H, –S-CH_2), 3.39 (t, 2H, –CH_2-NH–) 3.6 (s, 176H, –OCH_2CH_2–), 3.8 (m, 2H, CH_2O–), 3.9 (m, 2H, CH_2OP), 4.2 (m, 2H, CH_2OPO–), 4.3 (d, 2H, CH_2OCO–), 4.4 (m, 1H, biotin), 4.5 (m, 1H, biotin), 5.1 (s, 1H, CHCO–), 5.8/6.7 (s, –CONH–). **(C)** TEM images of model nanoparticles dispersed in 0.01 M phosphate buffer pH = 7.4. Upper: DSPE–PEG2000–NH_2. Middle: DSPE–PEG2000–biotin. Lower: DSPE–PEG2000–SS–biotin. Small regions of the images have been band-pass filtered to enhance the contrast (boxed region).