Published in final edited form as:

Nanomedicine. 2015 July ; 11(5): 1169–1178. doi:10.1016/j.nano.2015.02.016.

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.

Graphical Abstract

Highlight of 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: time-dependent visualization and quantification of nanoparticle endocytic uptake through histological cross-sectioning of healthy rat intestinal tissue.

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Competing financial interests: The authors declare no competing financial interests.

Keywords

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.^{1,2,3,4,5} 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^{7,8,9,10,11} (*e.g.*, biotin, folic acid, vitamin B₁₂, 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.

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 (^1H 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 (AlltimaTM C18 column 5 μ , 250 $\times$ 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 units/5 ml) to prevent blood clotting. An aliquot of 100 μ l of plasma was vortex-mixed with 200 μ l 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 CO₂ inhalation, and the ileum was collected and stored at -70°C in CryoStorTM 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 1A): basic nanoparticles, unmodified nanoparticles without a targeting ligand, and nanoparticles linked to a biotin-targeting ligand via either amide or amide–disulfide–amide bonds. The starting polymer-containing amine group was transformed into a disulfide–biotin–modified polymer by a direct reaction using succinimidyl-2-(biotinamido)-ethyl-1,3'-dithiopropionate (Figure 1A). We characterized the chemical and physical properties of the nanoparticles and confirmed their structure using ¹H NMR (Figure 1B). We characterized the morphology of the nanoparticles by transmission electron microscopy (TEM) (Figure 1C).

Quantification of nanoparticles labeled with hydrophobic marker molecules

The hydrophobic cargo core of micellar nanoparticles was labeled with the fluorescent molecule coumarin 6 (Figure 2A). We labeled nanoparticles at coumarin 6:nanoparticle ratios of 0.01:10, 0.1:10, and 1:10 (Supplementary Figure 1B-D). Even after 48 h dialysis and separation using size-exclusion chromatography, high-performance liquid chromatography (HPLC) chromatograms showed clear peaks of the linked and free fluorescent molecules (Figure 2A). A similar trend was observed for all three nanoparticle types (Supplementary Figure 1E,F). For *in vivo* studies, we selected the most sensitive nanoparticle peak detection area at a 1:10 ratio (lower detection limit ~ 0.002 µg/ml), and we disregarded peaks from the free fluorescent marker.

In vivo studies

To study the intestinal absorption, we administered fluorescently labeled nanoparticles orally to rats. After jugular vein cannulation, blood samples were taken at fixed time intervals over 24 h, and the concentration of fluorescent nanoparticles in the plasma was measured by a fluorescence HPLC method. Oral absorption of nanoparticles was studied in a fasted rat model. The mean plasma concentration–time curves following a single nanoparticle dose of mg/kg are presented in Figure 2B,C along with a summary of the corresponding pharmacokinetic data. Bare nanoparticles without the targeting ligand DSPE–PEG2000–NH₂ produced insignificant absorption in fasted rats ($p < 0.05$). Statistical analysis showed that the pharmacokinetic data obtained for the bare DSPE–PEG2000–NH₂ and targeting ligand containing DSPE–PEG2000–biotin did not differ significantly ($p < 0.05$); *i.e.*, the intestinal absorption for both nanoparticle types was negligible. Interestingly,

the DSPE-PEG2000-SS-biotin nanoparticles markedly increased the intestinal absorption in the fasted state, giving a superior oral absorption compared with bare DSPE-PEG2000-NH₂ and peptide-linked DSPE-PEG2000-biotin nanoparticles ($p < 0.05$).

To determine whether lysosomal enzymatic degradation of disulfide bonds is a feature of intestinal intracellular processing, we analyzed the nanoparticle uptake in the everted sacs of rat ileum and compared the uptake to that of unprocessed nanoparticles (Figure 2D). In contrast to the intracellularly processed nanoparticles, the original nanoparticles with intact disulfide bonds formed a complex with FITC-avidin.

The mechanism of nanoparticles endocytosis across healthy intestinal tissue

Receptor-mediated endocytosis is a major mechanism by which cells take up nutrients and signaling molecules, and downregulate receptors on the cell surface.^{13,14,15,16} Because of the highly dynamic nature, how the endocytic network has evolved remains a puzzle. Here, we monitored the time-dependent behavior of the endocytic network for 2 h by incubating pieces of rat ileum first with LysoTracker® Red, which selectively labels lysosomes, and then with nanoparticles labeled with coumarin 6. A series of histological images is presented in Figure 3A-F. Equivalent images of labeling with pure coumarin 6 and bare nanoparticles are included in the Supplementary section for comparison (Figures 2 and 3). Time-dependent fluorescent images show the typical feature of clathrin-mediated endocytosis (CME).^{15,17} To investigate further the nanoparticle intestinal uptake mechanism, we used endocytosis inhibitors (Figure 4). Chlorpromazine prevents the formation of clathrin-coated pits without affecting other endocytic pathways.^{6,18,19} Filipin is a selective caveolae-mediated endocytosis inhibitor that binds cholesterol in the plasma membrane and hence interferes with the formation of caveolae.^{6,17,18} The presence of cholesterol is considered to be essential for the CME and most of the clathrin-independent uptake pathways.^{6,18,19} Cholesterol-dependent endocytosis is usually inhibited by a combination of methyl- β -cyclodextrin, which selectively extracts cholesterol from the plasma membrane, and lovastatin, which blocks cholesterol synthesis *de novo*.¹⁹ Endocytosis was unaffected by filipin (Figure 4B) but was inhibited by chlorpromazine (Figure 4A) and cholesterol-dependent uptake inhibitors (Figure 4C). We sought to determine whether biotin-conjugated nanoparticle uptake in healthy intestinal cells occurs through macropinocytosis.²⁰ To induce competitive absorption, rat ileum tissues were incubated with free biotin and then gradually increasing concentrations of fluorescent nanoparticles (Figure 4D). Macropinosome-like structures were not detected at lower or higher nanoparticle concentrations.

Discussion

Nanoparticles characterization

Several laboratories have reported on the use of PEGylated phospholipid micellar nanoparticles in targeted nanomedicine for cancer.^{21,22,23} Here, we engineered such PEGylated phospholipid nanoparticles with proven ability to solubilize and firmly to retain substantial quantities of water-insoluble anticancer drugs (*e.g.*, m-porphyrin, tamoxifen, taxol, paclitaxel) while keeping the original size distribution close to that of the original “empty” 10–40 nm nanoparticles.²¹ These pegylated phospholipid nanoparticles have a

demonstrated ability to accumulate in cancer tissue via the EPR effect after *i.v.* administration.²²

The ¹H NMR spectrum of DSPE-PEG2000-NH₂ (Figure 1B) was consistent with previously reported spectra for similar nanoparticles having different tail groups.²⁴ Clearly, a blunt peak at 1.9 ppm denoting the presence of a primary amine group was absent from the structure of the biotin-modified micellar nanoparticles, indicating successful conjugation. Unlike the unmodified nanoparticles, the biotin-modified nanoparticles exhibited peaks that originated from the biotin structure within the polymer chain,²⁵ namely proton shifts occurring at 1.45m, 1.7m, 2.15t, 2.7d, 2.9m, 3.1m, 4.4m and 4.5m ppm (Figure 1B). The DSPE-PEG2000-SS- biotin nanoparticles showed additional peaks when compared with the DSPE-PEG2000- biotin nanoparticle spectrum, namely, peaks arising from protons in the -NHCOCH₂CH₂SSCH₂CH₂NHCO- part of the polymeric chain. Triplet peaks from the methylene groups are evident in Figure 1B. The proton shift occurring at 2.37t ppm originates from the methylene group in -NHCOCH₂-, the shift occurring at 3.15t ppm corresponds to -CH₂-S-, and the shifts occurring at 3.38t and 3.39t ppm correspond to the methylene groups in -S-CH₂- and -S-CH₂-CH₂-NH-CO-, respectively (Figure 1B). TEM images (Figure 1C) showed that most of the nanoparticles had acquired a uniform spherical structure. The DSPE- PEG2000-NH₂ nanoparticles contained a high number of small particulate structures (denoted by the dark spots in the images). Most of the particles were approx. 10 nm in size, were granular, and were nonaggregated, but occasional aggregation was also noted. Small regions of the images were band-pass filtered to enhance the contrast (boxed region). Similar morphological considerations apply to DSPE-PEG2000-biotin. The DSPE-PEG2000-SS- biotin nanoparticles comprised mainly two types of structure: particulate structures, 10 nm in size, and wormlike aggregated structures with a size 100–150 nm having different degrees of branching.

Quantification of nanoparticles labeled with hydrophobic marker molecules

Nanoparticles have been quantified in biological media by measuring the fluorescence intensity upon induction of either a covalently or hydrophobically linked fluorescent marker. Here, we describe a method for distinguishing hydrophobically linked and counterpart-free fluorescent marker molecules. Coumarin 6 is a hydrophobic marker commonly used to determine nanoparticle concentrations in various *in vitro* and *in vivo* media, especially in pharmacokinetic, biodistribution, and cell uptake studies.^{3,6,26} These studies are usually conducted under the assumption that the overall fluorescence reflects the nanoparticle concentration. The fact that the overall fluorescence does not reflect nanoparticle concentration is indicated by the observation that during hydrophobic host–guest bonding, the equilibrium between the free and linked molecules always exists.²⁷ To confirm this observation, we labeled nanoparticles at coumarin 6:nanoparticle ratios of 0.01:10, 0.1:10, and 1:10 (Supplementary Figure 1B-D). Even after 48 h dialysis and separation using size-exclusion chromatography, high-performance liquid chromatography (HPLC) chromatograms showed clear peaks of the linked and free fluorescent molecules (Figure 2A). A similar trend was observed for all three nanoparticle types (Supplementary Figure 1E,F). Importantly, the proposed method differentiated free and nanoparticle-linked

fluorescent markers, enabling precise quantitative analysis of the nanoparticles in the biological media.

***In vivo* studies**

Bare nanoparticles without the targeting ligand DSPE-PEG2000-NH₂ produced insignificant absorption in fasted rats ($p < 0.05$). This result is inconsistent with that reported by Benny et al.³ in mice. The authors reported significant intestinal uptake of the bare PEG-PLA 10 nm nanoparticles. Significant intestinal absorption has been reported for larger 100–200 nm PLA-TPGS²⁸ and DSPE-PEG-TPGS⁶ nanoparticles. This could be related to the bioadhesive and P-gp pump-inhibiting characteristics of both PLA and TPGS, which would be reflected in greater cellular permeability.

Statistical analysis showed negligible oral absorption for the bare DSPE-PEG2000-NH₂ and targeting ligand containing DSPE-PEG2000-biotin nanoparticles ($p < 0.05$). This finding is supported by the observation that peptide bonds exhibit poor stability in the harsh intestinal conditions.^{29,30} Interestingly, the DSPE-PEG2000-SS-biotin nanoparticles markedly increased the intestinal absorption in the fasted state ($p < 0.05$). This outcome was surprising because two peptide bonds are present in the structure of DSPE-PEG2000-SS-biotin nanoparticles and are separated by a disulfide bond. Similar modification of proteins to introduce disulfide bonds has been reported to prevent enzymatic cleavage of peptide bonds.^{29,30}

The mechanism of nanoparticles endocytosis across healthy intestinal tissue

It is known that the process of nanoparticle absorption through the intestinal barrier is energy-dependent endocytosis.^{14,15} To identify the mechanism of endocytosis, the most common approach is to monitor nanoparticle transport across an intestinal barrier such as Caco-2 cells or in the rat or mouse everted intestinal sac in the presence of inhibitors.^{6,18} The drawbacks of this approach are twofold. First, it is difficult to elucidate the real mechanism; *i.e.*, there is always the possibility that the inhibitor changes the cellular structure so that it retains a certain amount of nanoparticles. Second, cancer cells, including Caco-2 cells, have specific metabolic needs and consequently exhibit nutritional phenomena not typical for healthy cells such as the overexpression of nutritional ligands and uptake of nutrients from the extracellular space through macropinocytosis.²⁰ Therefore, we sought to determine whether the endocytic uptake of nanoparticles through healthy intestinal tissue can be visualized directly to provide a more direct method to study the mechanism of endocytosis. Intestinal absorption of free biotin has been studied *in vivo* in rats, and these studies have demonstrated the involvement of a specialized Na⁺-dependent receptor system localized in the apical domain of both the proximal and distal small intestine.³¹

Time-dependent fluorescent images show the typical feature of clathrin-mediated endocytosis (CME).^{15,16,17} Nanoparticle fluorescence was visible outside the tissue after 5 min (Figure 3A). A transient microtubular reticulum appeared at 10–12 min (Figure 3B) and featured the formation of early endosomes. This observation is consistent with the notion that in absorptive epithelial cell cultures, the early endosomal microtubular network has the appearance of a tubular reticulum.¹⁶ Here we identified ~50 µm endocytic network reticular

units. The reticulum was doubly stained with LysoTracker Red and coumarin 6-labeled nanoparticles to show the processing of nanoparticles within endolysosomal structures. We noticed numerous vesicular structures $\sim 0.5\text{--}1.0\text{ }\mu\text{m}$ on the surface of the microtubular reticulum (Figure 3B). The size of these structures corresponds to that of late endosomes and lysosomes, which have been reported to reach 0.5 and 1.2 μm , respectively, in the most mature stages of development.¹⁷ The presence of late endosomes and lysosomes was confirmed by the fluorescent colocalization of LysoTracker Red and coumarin 6-labeled nanoparticles.

The CME pathway comprises primarily four types of organelles: early, recycling, and late endosomes, and lysosomes. These four classes of endocytic organelles are not preexisting, stable structures but rather are dynamic and difficult to recognize based on their morphology or position in the cytoplasm alone. Early endosomes represent a dynamic network of tubules and vesicles dispersed throughout the cytoplasm.^{15,16} Late endosomes are defined as vesicular structures that accumulate and concentrate internalized cargo intended for degradation.^{16,17} Late endosomes degrade their contents progressively, thus providing for the recycling of surviving receptors, and ultimately increase in density as digestible membrane and content are processed, catabolic products are released, and the remaining undigestible material (lysosomal hydrolases, and certain membrane and lipid components) is concentrated. At this stage, they become resting lysosomes, which can be activated again upon fusion with late endosomes.^{16,17}

At the 15–20 min time point, we noted larger vesicle–cell clusters that resembled the remains of microtubular elements (Figure 3C). That the number of such large vesicular structures resembling tubular elements was much higher after 15–20 min probably reflects tubular elements that pinch off or serve as sites for budding and forming recycling vesicles and larger vesicular elements that proceed to late endosomes and lysosomes. In parallel with these tubular vesicles within the size range of $\sim 5\text{--}25\text{ }\mu\text{m}$, we also identified late endosomal and lysosomal vesicles $\sim 0.5\text{--}1.0\text{ }\mu\text{m}$ that stained with both labels.

The presence of late endosomes and lysosomes was confirmed by the fluorescent colocalization of LysoTracker Red and coumarin 6 vesicular structures, which were observed to be the dominant structures after 30 min and 1 h. (Figure 3D,E). After 2 h, no visible vesicular or tubular structures could be detected, and the tissue looked like equivalent tissue treated with the pure fluorescent marker or bare fluorescent nanoparticles (Figure 3F and Supplementary Figures 2 and 3). Significantly smaller lysosomes and a low level of label colocalization indicated that the lysosomes were converted to the rest mode.

To confirm further the nanoparticle intestinal uptake mechanism, we used endocytosis inhibitors (Figure 4). The fact that the nanoparticle intestinal absorption reflects uptake through CME was supported by the observation that endocytosis was unaffected by filipin (Figure 4B) but was inhibited by chlorpromazine (Figure 4A) and cholesterol-dependent uptake inhibitors (Figure 4C).

Quantitative analysis of nanoparticle endocytic uptake is expressed as arbitrary units of an average fluorescence intensity fluctuation (Figure 5) meaning that the level of relative

endocytic uptake reflects the formation of early endosomal network. Interestingly, the development of DSPE-PEG2000-SS-Biotin endocytic network was shown to be a function of the nanoparticle concentration (Figure 5A). At the concentration of 1 ng/ml, dramatic, sharp and transient increase in endocytic uptake (~0.2 to ~18) is notable. At higher nanoparticle concentrations of 500 and 1000 ng/ml endocytic network subsequently evolves after 10 min. and reaches maximum after 15 and 20 min., respectively. An average network life-span of 2-3, 5-6 and 10-12 min. varies with nanoparticle concentrations of 1, 500 and 1000 ng/ml, respectively. It is difficult to explain the observed phenomenon since the molecular basis of endocytosis is yet to be fully elucidated. The phenomenon appears to involve concentration dependent receptor-ligand events at the level of cell membrane. It has been found that different endocytic receptors require different adaptor proteins for endocytosis.^{15,17} However, the significance of the diversity of adaptor proteins and how these adaptors might direct endocytic network formation remains unclear.^{15,17} The presence of CME inhibitors led to an attenuation of endocytosis confirming the uptake mechanism (Figure 5B).

Biotin-conjugated dendrimer nanoparticles have been reported to exhibit receptor-mediated endocytosis at lower concentrations (0.1 μ M) and macropinocytosis at higher concentrations in ovarian cancer cell cultures.³² It is believed that vesicles larger than 0.2 μ m, and often reported to be ~1–5 μ m, are a reliable sign of macropinocytosis.²⁰ Macropinosome-like structures were not detected at lower or higher nanoparticle concentrations, indicating that in contrast to cancer cells, healthy intestinal cells do not engage in macropinocytosis. Taken together, these data indicate that CME is an attribute of biotin-conjugated nanoparticle intestinal absorption.

Recently, there has been a renewed interest in nanoparticle-based therapeutics and the critical role that nanoparticle cellular uptake plays in conferring growth and advantages of nanoparticle-based therapeutics. Here, we provide evidence that the mechanism of endocytosis-mediated internalization of vitamin-conjugated nanoparticles can be visualized directly in healthy rat intestinal tissue. Our findings raise the question of how endocytosis in healthy intestinal tissues can be controlled and used for noninvasive nanoparticle-based therapies.

In summary, we have developed 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. We have demonstrated an approach to visualize directly the mechanisms of nanoparticle processing within intestinal tissues through time-dependent fluorescence imaging of intestinal cross sections after incubation with model fluorescent nanoparticles. Crucially, this methodology allows us to determine with a high level of confidence the mechanism by which healthy intestinal cells process nanoparticles, and points to the possible use of this approach in the design of noninvasive nanoparticle-based therapies. A major advantage of our methodology compared with the methodology of mass transport through Caco-2 cells or everted rat intestinal sac models is the possibility of directly visualizing nanoparticle transport events across healthy intestinal tissues. In conventional mass transfer studies, there is always the possibility of nanoparticle retention by cellular structures.^{33,34} In addition, cancer cells, including Caco-2 cells, have specific

metabolic needs, and consequently, exhibit nutritional phenomena that are not typical for healthy cells, such as the overexpression of nutritional ligands and the uptake of nutrients from the extracellular space through macropinocytosis.²⁰ The methodology of visualized endocytosis is generally applicable to any type of fluorescent nanoparticle, and allows us to understand the mechanism of intracellular processing of nanoparticle therapeutics. Our findings indicate the high level of correlation between *in vivo* bioavailability and visualized endocytic uptake. Consequently, the developed methodology for visualizing endocytic uptake has the potential to become an important tool in the development of nanoparticle-based therapeutics. However, further investigations are required before proposing our methodology as a predictor of the oral efficacy of a nanomedicine. To achieve a full mechanistic understanding of nanoparticle crossing of intestinal barriers, live cell imaging investigations using techniques such as spinning disk confocal microscopy and total reflection fluorescence microscopy need to be used in conjunction with our methodology, because these techniques enable the visualization of nanoparticle interactions with cellular structures under dynamic conditions, e.g., time-dependent retention of nanoparticles in lysosomes.^{33,34} Furthermore, a methodology to visualize the endocytic uptake presented here has a potential to be employed to investigate the endocytic uptake of various nanoparticles by numerous healthy and cancer tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported by the Cancer Council SA Beat Cancer Fellowship provided by the South Australian Health and Research Institute.

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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-NH₂); 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-NH₂ 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) ¹H NMR spectra of model nanoparticles. Blue: ¹H NMR of DSPE-PEG2000-NH₂: δ 0.87 (t, 6H, (-CH₃)₂), 1.25 (s, 60H, H from the hydrophobic chain), 1.58 (s, water from the solvent), 1.9 (s, blunt peak from heteroatoms NH₂), 2.2 (m, 4H, CH₂CO-), 3.2 (m, 2H, CH₂NHCO-), 3.3 (m, 2H, CH₂-NH-), 3.6 (s, 176H, -OCH₂CH₂-), 3.8 (m, 2H, CH₂O-), 3.9 (m, 2H, CH₂OP-), 4.2 (m, 2H, CH₂OPO-), 4.3 (d, 2H, CH₂OCO-), 5.2 (s, 1H, CHCO-), 6.5 (s, CONH-). Red: ¹H NMR of DSPE-PEG2000-biotin: δ 0.87 (t, 6H, (-CH₃)₂), 1.25 (s, 60H, H from the hydrophobic chain), 1.45 (m, 2H, CH₂-biotin chain), 1.6 (s, water from the solvent), 1.7 (m, 4H, CH₂ biotin chain), 2.15 (t, 2H, biotin), 2.2 (m, 4H, -CH₂CO-), 2.7 (d, 1H, CH₂-S- biotin), 2.9 (m, 1H, CH₂S-biotin), 3.1 (m, 1H, biotin), 3.2 (m, 2H, CH₂NHCO-), 3.3 (m, 2H, CH₂-NH-), 3.6 (s, 176H, -OCH₂CH₂-), 3.8 (m, 2H, CH₂O-), 3.9 (m, 2H, CH₂OP-), 4.2 (m, 2H, CH₂OPO-), 4.3 (d, 2H, CH₂OCO-), 4.4 (m, 1H, biotin), 4.5 (m, 1H, biotin), 5.1 (s, 1H, CHCO-), 5.8/6.7 (s, - CONH-). Green: ¹H NMR of DSPE-PEG2000-SS-biotin: δ 0.87 (t, 6H, (-CH₃)₂), 1.25 (s, 60H, H from the hydrophobic chain), 1.45 (m, 2H, CH₂-biotin chain), 1.6 (s, water from the solvent), 1.7 (m, 4H, CH₂ biotin chain), 2.15 (t, 2H, biotin), 2.2 (m, 4H, -CH₂CO-), 2.37 (t, 2H, CO-CH₂-), 2.7 (d, 1H, CH₂-S- biotin), 2.9 (m, 1H, CH₂S-biotin), 3.1 (m, 1H, biotin), 3.15 (t, 2H, -CH₂-S-), 3.2 (m, 2H, CH₂NHCO-), 3.3 (m, 2H, CH₂-NH-), 3.38 (t, 2H, -S-CH₂), 3.39 (t, 2H, -CH₂-NH-) 3.6 (s, 176H, -OCH₂CH₂-), 3.8 (m, 2H, CH₂O-), 3.9 (m, 2H, CH₂OP), 4.2 (m, 2H, CH₂OPO-), 4.3 (d, 2H, CH₂OCO-), 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-NH₂. 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).

Figure 2.

A) Example HPLC chromatograms of fluorescently labeled nanoparticles; the peak area for the encapsulated fluorescent molecule is directly proportional to the size of the hydrophilic corona DSPE-PEG2000-SS-biotin > DSPE-PEG2000-biotin > DSPE-PEG2000-NH₂. B) Plasma concentration-time profiles of nanoparticles in male Sprague Dawley rats following a single oral administration of fluorescently labeled nanoparticles equivalent to 1.42 mg/kg: DSPE-PEG2000-NH₂ (◇); DSPE-PEG2000-biotin (□), and DSPE-PEG2000-SS-biotin (▲) (mean ± SD, n = 6). C) Pharmacokinetic parameters of nanoparticles following a single

dose based on noncompartmental calculation (mean $\pm$ SD, n = 6). D) Different behavior of intestinally processed and unprocessed DSPE-PEG2000-SS- biotin nanoparticles: 1, in the presence of 0.5 M glutathione, the processed nanoparticles are not released from the complex with avidin-FITC; 2, unprocessed nanoparticles are released from the complex with avidin-FITC in the presence of glutathione.

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Figure 3.

Time-dependent visualization of clathrin-mediated endocytosis (CME) through histological cross-sectioning of healthy rat ileum. A) An endocytic uptake assay using 1 ng/ml of coumarin 6-fluorescently labeled DSPE-PEG2000-SS-biotin nanoparticles (green) and 1 ng/ml of the marker for lysosomes LysoTracker Red. After 5 min, the green fluorescence is visible outside the tissue (1), the red fluorescence is distributed equally inside the tissue (2), and the overlay image shows nanoparticles located on the surface and outside the tissue. B) After 10–12 min, the tissue resembles a reticulum and features early endosome formation. The reticulum stained dually with both red (2,4) and green nanoparticle (1) labels, as shown in the overlay image (3). Vesicular dually stained structures measuring 0.5–1 μm on the surface of the microtubular reticulum correspond to late endosomes and lysosomes, consistent with the dynamic nature of endolysosomal structures. C) At the 15–20 min time point, larger vesicle–cell clusters resembling the remnants of microtubular elements are notable. The vesicles measuring 5–25 μm are doubly stained with green (1,4) and red (2,5), and this pattern is confirmed in the overlay images (3,6). D) At the 30 min time point, the tissue is stained homogenously with both labels (green 1, red 2, overlay 3). The presence of late endosomes and lysosomes is indicated by the fluorescent colocalization of red and green vesicular structures in the size range 0.5–1 μm (green 4, red 5, overlay 6). E) Representative tissue cross-sections after 1 h show the nonreticular appearance and the presence of doubly

stained 0.5–1 μm vesicular structures (1–3) F) At the 2 h time point, vesicular or tubular structures are not visible (1–3), indicating that lysosomes are in the rest mode.

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Figure 4.

Endocytic uptake of 1 ng/ml – 1 µg/ml coumarin 6-labeled DSPE–PEG2000–SS– biotin nanoparticles in the presence of various inhibitors and the competitive absorption agent biotin visualized through histological cross-sectioning of healthy rat ileum tissue.

Lysosomes were labeled with 1 ng/ml LysoTracker Red. A) Representative time-dependent fluorescent images show blocked nanoparticle uptake in the presence of the selective inhibitor of clathrin-mediated endocytosis chlorpromazine (10 µg/ml); the endocytic network and structural features of clathrin-mediated endocytosis were not detected. Left: green fluorescence from nanoparticles; middle: red fluorescence from the LysoTracker Red; right: overlay image of green and red fluorescence. B) Representative time-dependent fluorescent images show typical features of clathrin-mediated endocytic nanoparticle uptake in the presence of the caveolae-mediated endocytosis inhibitor filipin (1 µg/ml). Left: green fluorescence from nanoparticles; middle: red fluorescence from LysoTracker Red; right: overlay image of green and red fluorescence. C) Representative time-dependent fluorescent

images show blocked nanoparticle uptake in the presence of cholesterol inhibitors lovastatin (1 $\mu\text{g/ml}$) and methyl- β -cyclodextrin (0.13 g/ml). Left: green fluorescence from nanoparticles; middle: red fluorescence from LysoTracker Red; right: overlay image of green and red fluorescence. D) Histological cross-sectional fluorescent images of nanoparticle uptake in the presence of 1 $\mu\text{g/ml}$ of the competitive inhibitor biotin. The presence of the endocytic network from the LysoTracker Red (red fluorescence 1,2) and the absence of the nanoparticle network (green fluorescence at nanoparticle concentrations 1 ng/ml (3), 0.01 $\mu\text{g/ml}$ (4), 0.1 $\mu\text{g/ml}$ (5), and 1 $\mu\text{g/ml}$ (6) indicate that biotin inhibited nanoparticle endocytosis.

Figure 5.

Quantification of endocytic uptake is presented as arbitrary units of an average fluorescence intensity amplitude for $n = 3$ independent experiments with at least 25 tissue samples scored per experiment. A) Time dependent endocytic uptake of nanoparticles and control pure fluorescent marker sample; B) Quantification of endocytic uptake of 1 ng/ml DSPE-PEG2000-SS-Biotin in the presence of specific inhibitors. Statistical significance was determined by Student's t-test; $*P < 0.05$. For all graphs, error bars indicate mean $\pm$ S.D.