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Ligand-modified gene carriers increased uptake in target cells but reduced DNA release and transfection efficiency

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Abstract

DNA delivery to cells can be improved by using particle carriers made from biodegradable polymers such as PLGA. It is speculated that addition of targeting moieties to the particle surface to facilitate uptake can further enhance gene expression in specific cells or tissues. Taking advantage of well-known receptor/ligand interactions in intestinal and renal epithelial cells, we formulated PLGA particles with high density of surface-bound BSA (~768 molecules/particle). BSA-coated particles exhibited significantly higher uptake by cells expressing the albumin receptor, megalin, and resisted degradation in low pH. However, gene expression from BSA-coated particles was 3 to 10-fold lower than unmodified particles; this reduction in transfection efficiency was probably due to the slower DNA release rate from modified particles. In this setting, addition of a targeting feature to particles reduced their effectiveness. Our study highlights the importance of the interplay between cell uptake and payload release in the design of polymer drug carriers.

Keywords

gene delivery; polymer particle; megalin; controlled release; luciferase

Background

Polymeric particles are widely explored as carriers of plasmid DNA for gene therapy or DNA vaccination. Encapsulation of plasmid DNA in polymer particles helps prevent premature degradation and allows for controlled, sustained release of bioactive DNA over time. Biocompatible and degradable polymers such as poly(lactic-co-glycolic)acid (PLGA) are particularly desirable and have been extensively investigated for this purpose[1]. DNA transfection using PLGA carriers have yielded promising results *in vitro* and *in vivo*. In cell culture, PLGA particles loaded with plasmid DNA can elicit higher gene expression compared to naked DNA (i.e., unencapsulated DNA), but lower than cationic polymer or lipid agents at <4 days after transfection[2,3]. At longer incubation times, however, cells treated with particles retain or exhibit increased gene expression level over even those transfected by cationic lipids,

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an effect likely attributed to the latter's high cytotoxicity. Gene expression from DNA encapsulated in PLGA particles, which impart low or no cytotoxicity, generally peaks after 2 days and can be sustained in cell culture up to 10 days[2,4]. *In vivo*, the gene expression from DNA-loaded PLGA particles are sustained longer: chloramphenicol transferase (3 wk)[4], alkaline phosphatase (4 wk)[3], VEGF expression leading to angiogenesis (4 wk)[5] and luciferase expression (10 wk)[6].

Changes to the polymer composition, or “smart” features added to the particle surface, have been explored to increase delivery of particles to target sites. In general, particle targeting schemes fall into one of two main categories. Passive targeting strategies rely on known physiological characteristics of the target site, such as degree of vascularization or pH environment, to increase drug availability at the desired location but not necessarily to enhance active uptake in target cells. Carriers formulated for passive targeting have been made with surface modifications that prolong their systemic circulation time, which lead to higher accumulation in vascularized organs and tumors; produce a charged surface to enhance adhesion (non-specifically) to mucosal surfaces; or confer pH-sensitivity to facilitate payload release in specific pH environments[7-10]. In contrast, active targeting relies on the presence of ligands that interact with specificity and high affinity to receptors/features on some specific cell types. The incorporation of ligands onto particles may be accomplished by surface adsorption or conjugation chemistry[11-14]. Particles decorated with active targeting moieties such as aptamers, folic acid, peptides and proteins have been used to demonstrate the capability of targeted particle delivery. Table 1 provides a representative, partial list of studies that explore targeted delivery for PLGA or other biocompatible particulate carriers; in general, there is a 2 to 4-fold increase in binding or uptake of particles with active targeting.

Most previous studies utilized fluorescent tracers or cytotoxic drugs (eg. Paclitaxel, Docetaxel) as indicators of delivery, but a few have looked at DNA (see ref [20,30] in Table 1). A number of factors must be considered in the design of a polymer particle for DNA delivery: 1. the amount of DNA encapsulated in the particle (i.e., encapsulation efficiency); 2. the bioactivity of the encapsulated DNA; and 3. the rate of DNA release from the particles. The efficacy of the particle formulation is commonly measured by a phenotypical response by cells, such as gene expression for DNA-loaded, and cell death for cytotoxic drug-loaded particles. The magnitude and rate of the cellular response to both types of particles are dependent on their payload encapsulation and release rate. However, we note that since each system requires the participation of different and complex cellular mechanisms, their measure for efficacy (i.e., magnitude of gene expression vs. cell death) may not be directly comparable.

In the present study, we incorporated BSA onto PLGA particles surface for active targeting moiety to enhance uptake in specific epithelial cells. Application of BSA as a carrier of various drugs and ligands has been previously documented[15-18]. Here, we propose the use of BSA as a ligand to target megalin, an endocytic, transmembrane receptor protein (MW 600 kDa) present on the epithelium of the intestine, liver, and kidney. Megalin's role in active sequestration of vitamin-binding proteins, plasma proteins – particularly albumin, lipoproteins, hormones, and small biomolecules has been well-characterized[19]. Interaction of surface-modified particles carrying fluorescent tracer dye, compared to unmodified PLGA particle formulations, was evaluated in an epithelial cell line that expresses the megalin receptor. To confirm the role of megalin in facilitating endocytosis of targeted particles in cells, receptor associated protein (RAP)[20], a ligand with high affinity to megalin, was used as a competitive inhibitor against BSA-coated particles. To explore the capability of modified PLGA particles as gene carriers, we loaded plasmid DNA carrying the luciferase gene into the particles. The DNA conformation, controlled release, and resulting gene expression following transfection of particles to cells were measured and used to evaluate the overall particle efficacy.

Methods

Conjugation of BSA-palmitic acid

Bovine Serum Albumin or BSA (Sigma) and Palmitic acid N-hydroxysuccinimide ester (Sigma) were combined at 1:15 molar ratio in 2% Sodium Deoxycholate in 1× Dubelco's Phosphate Buffered Saline (pH 7, Gibco). The reaction was conducted for 16 hrs at 37 °C, and dialyzed against 0.15% Sodium Deoxycholate in 1× PBS, using Snakeskin dialysis tubing (Pierce, MWCO 3.5 kDa) for 24 hrs.

Formulation of fluorescent dye loaded PLGA particles

Coumarin-6 (Acros Organics) or Rhodamine B (Sigma-Aldrich) was dissolved at 0.5 mg/ml in Methylene Chloride. A 200 µl volume of dye (1 mg/ml) was combined with 200 mg PLGA (50/50, LACTEL) also in 2 ml Methylene Chloride. 200 µl 1× PBS was added drop-wise to the polymer-dye solution while vigorously vortexing. The emulsion was transferred to an ice bath and sonicated at 38% amplitude for 10 s with a probe sonicator (Tekmar). The solution was then added drop-wise to 4 ml 0.5% w/v poly(vinyl)alcohol (PVA, Sigma-Aldrich), or 0.5% PVA with 20 mg BSA-palmitic acid conjugate. The entire mixture was emulsified for a second time, and poured immediately into 0.3% PVA solution with 10% w/v sucrose for 3 hrs to allow for complete evaporation of Methylene Chloride. The hardened particles were washed 3 times in DI water. Briefly, the particles were pelleted by centrifugation at 4 °C, supernatant was discarded and the particles re-suspended in DI water by sonication in a water bath and vortexing. After the last wash, particles were suspended in DI water and lyophilized for 72 hrs. All particles were stored dessicated at -20 °C until use.

Formulation of DNA loaded PLGA particles

DNA encapsulated PLGA particles were made by a double emulsion method. 200 mg PLGA was dissolved in 2 ml Methylene Chloride (Fisher Chemicals). Plasmid DNA (1.4 mg) carrying the coding sequence for luciferase under the control of the CMV promoter, pEGFP/luc (Clontech), suspended in 200 µl 1× TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5) was gradually added to the polymer solution while vortexing. The mixture was transferred to an ice bath and sonicated to create the first emulsion, which was added gradually to 4 ml of either 0.5% PVA, 0.5% PVA and 20 mg unconjugated BSA, 0.5% PVA and 10 or 20 mg BSA-palmitic acid conjugate, or 0.5% PVA and 1mg DSPE-PEG2K-biotin (Avanti Polar Lipids). The second emulsion was performed by sonication, followed by solvent evaporation for 3 hrs in 0.3% PVA with 10% w/v sucrose. Particles were washed, lyophilized and stored as previously described.

Electron microscopy

A small sample of particles was deposited on black carbon tape mounted on a metal stud and sputter-coated with Au for 30 s (Cressington). Samples were observed under a scanning electron microscope with a 5 kV electron beam.

Particle sizing and zeta-potential

Particle sizes were determined by dynamic light scattering using a ZetaPlus Particle Analyzer (Brookhaven Instruments Corporation). Distribution of particle diameters was obtained from a total of 10 sampling runs per particle batch. Here, intensity fluctuations of light scattering from random Brownian motion of particles in solution are analyzed by autocorrelation. The mean diameters were reported from the multimodal size distribution (MSD) that was obtained using the ZetaPlus Particle Sizing software v2.27 (Brookhaven Instruments Corp). Measurement of particle zeta potential was carried out on the Zeta-potential analyzer with ZetaPALS software v3.13 (Brookhaven Instruments Corp). Data was fitted to a Smoluchowski-

based model. Final particle surface charge is reported as a combination of results from 10 runs successfully fitted to the model with residual error ≤ 0.025 .

Evaluation and confirmation of ligand attachment of particle surface

TMB assay—Particles with and without DSPE-PEG(2K)-biotin were incubated with avidin-HRP conjugate (Fisher Scientific). Following 3 washes, particles were combined with TMB reagent (BD Biosciences) in a 96-well plate. HRP signal (absorbance at 450 nm) was obtained after 15 min incubation at room temperature after addition of 2 M H₂SO₄. The amount of HRP/mg PLGA was calculated using a standard of known avidin-HRP concentration.

Immunofluorescence—Particles (2 - 3 mg, without dye) were incubated with an anti-BSA mouse monoclonal antibody (Sigma) to probe for BSA presence on particle surface. Following 3 washes, a second incubation with an antibody directed against mouse IgG conjugated to FITC (Sigma) was carried out for another 3 hrs at room temperature. Particles were washed 3×, and fluorescence was read (490 nm/525 nm) in a SpectraMax microplate reader (Molecular Devices). Unmodified PLGA particles was used as negative control, and particles without prior antibody incubation used as background.

MicroBCA assay—Particles were suspended in 1× PBS at 5 mg/ml, and 150 µl of the particle suspension was used for each assay, against the free BSA standard provided by the microBCA assay kit (Pierce). The assay was carried out as specified by manufacturer. All samples were analyzed in triplicates (n = 3).

Calculation of BSA presentation on particle surface

Calculation for number of BSA molecule per particle surface was performed similar to a previously published method.[21] Briefly, the number of BSA per mg particle (#BSA/mg, or *Value1*) was obtained from the results of a microBCA assay (µg BSA/mg), the molecular weight of BSA (66 kDa) and Avogadro's number). Next, the mass of an average particle (diameter ~ 334 nm) was calculated using an estimated PLGA density of 1.2 g/cm³ and was then used to calculate the number of individual particles in 1 mg (*Value2*). Finally, the number of BSA molecules per individual particle (*Value3*) was obtained by dividing *Value1* by *Value2*.

$$Value1 = \#BSA/mg = \mu g \text{ BSA/mg particle} \div MW \times \text{Avogadro's number}$$

$$Value2 = \#particle/mg = 1 \text{ mg particle} \div [\text{volume particle} \times \text{PLGA density}]$$

$$Value3 = Value1 / Value2 = \#BSA/particle$$

Sample preparation for confocal microscopy

Opossum kidney (OK) cells were cultured on circular glass coverslips in 12-well plates. Coumarin-6 loaded particles or BSA-FITC (Sigma) was delivered through incubation for 6 hrs prior to fixation. Next, coverslips were washed twice with 1× PBS and fixed with 4% Paraformaldehyde (Fisher Scientific). The cells were incubated in permeation buffer (1% BSA, 1% Triton-X in PBS), followed by incubation in 1× Goat Serum Dilution Buffer (17% v/v Goat Serum (Gibco), 20 mM Sodium Phosphate, 450 mM NaCl, 0.3% v/v Triton-X in DI water). Cell membranes were labeled with a primary antibody directed against megalin for (monoclonal, C-tail specific, kindly provided by Dr. Daniel Biesmederfer, Yale University). After 2× wash in 1× PBS, the cells were incubated with a rhodamine-conjugated goat anti-rabbit IgG secondary antibody, washed in permeation buffer and 1× PBS, consecutively, and mounted on microscope slides with VectaShield (Vector Laboratories) mounting medium. Overlay confocal images of cell layer and fluorescent substrates (FITC labeled BSA and

Coumarin-6 encapsulated particles) were analyzed with Imaris v5.5.1 imaging software (Bitplane).

Cell lysate analysis

Cells were grown on 12-well plates to confluency. A fixed dose of rhodamine-loaded particles were delivered to cells (125 $\mu\text{g}/\text{well}$) and incubated at 37 °C and 4 °C (pre-chilled 15 min prior to delivery) for a defined time period: 1, 6 and 12 hrs in either cell growth medium (sterile filtered DMEM +L-glutamine, 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin, all reagents from Invitrogen) or with K1 medium (DMEM/F12 (Invitrogen), 1 $\times$ ITS, 10 ng/ml Epidermal growth factor (BD Biosciences), 50 nM hydrocortisone (Stem Cell Technologies, Inc), 25 ng/ml prostaglandin (Biomol International), 1% Penicillin-streptomycin (Invitrogen)). Each well were washed 3 $\times$ with sterile, ice-cold 1 $\times$ PBS to remove unbound particles. Cell lysate, obtained by adding 200 $\mu\text{l}/\text{well}$ 1 $\times$ lysis buffer (Promega), was read for fluorescence at 540 nm/625 nm. The signal was converted to μg Rhodamine/ μg protein using a standard with known Rhodamine concentration in blank cell lysate, and protein content per well obtained by BCA assay of cell lysate, n = 3.

Flow cytometry

Cells were grown on 12-well plates to confluency. Prior to particle delivery, cells were incubated with 1 μM RAP for 2 hrs. Coumarin-6 loaded particles were added to the cells at 125 - 16 $\mu\text{g}/\text{well}$. After 6 hrs, each well was washed twice with ice-cold 1 $\times$ PBS. Cells were suspended, transferred to 1 $\times$ FACS buffer (1% Formaldehyde, 1% Fetal Bovine Serum in PBS) and analyzed in a FACSCalibur flow cytometer (BD Biosciences). Cell-associated Coumarin-6 fluorescence was detected using a 488 nm excitation laser and green band pass filter. A total of 10,000 events were collected per sample.

Evaluation of DNA loading

DNA was extracted from PLGA particles with and without BSA surface modification and measured by Quant-iTTM PicoGreen assay (Molecular Probes). Approximately 5 mg particles were suspended in 0.1N NaOH and 1% SDS and dissolved for 16 hrs under gentle horizontal agitation, n=3. At the end of the incubation period, the solution is clear and lacks any visible particles. 1N HCl was added to neutralize the pH of sample. The resulting solution was diluted 5-fold in 1 $\times$ TE buffer, and 10 μl was used in the PicoGreen DNA assay. PLGA particles without DNA were used as a negative control. The assay was carried out as specified by the manufacturer, with a known concentration of the original plasmid DNA suspended in a similar buffer as a standard.

In vitro controlled release

2 - 5 mg particles were suspended in 1 ml sterile 1 $\times$ PBS at pH 7 or 5. The particles were kept under gentle horizontal agitation at 37 °C. At specific time points, samples were pelleted by microcentrifugation, 100 μl of supernatant was removed and replaced with an equivalent volume of fresh buffer. The particles were re-suspended by brief water sonication and vortexing. The DNA concentration at each time point was determined by the Picogreen assay, n = 3. The standards for each controlled release set were the original plasmid DNA suspended in 1 $\times$ PBS at pH 7 and pH 5, respectively.

Gel electrophoresis

DNA extracted from particles was analyzed by gel electrophoresis. Approximately 150 - 300 ng of DNA sample, along with 10 kb supercoiled DNA ladder (Invitrogen) were loaded per lane on a 1% w/v agarose gel (American Bioanalytical) spiked with 0.01% v/v SYBRsafe DNA

dye (Invitrogen) in 1× Tris Borate EDTA running buffer (Invitrogen). The DNA samples were separated at 90 V for 1.5 hr and the gel visualized in a UV box.

Transfection of extracted DNA samples

Opossum kidney (OK) cells were seeded at 100,000 cells per well in a 12-well tissue culture plate, total volume = 2 ml. After 24 hrs, cells were transfected with 300 - 400 ng DNA per well of pEGFP/luc, and extracted plasmids from luc-P, luc-P-BSA, using lipofectamine transfection reagent (Invitrogen). Alternately, cell growth medium was replaced with K1 medium for 24 hrs, and transfection of DNA to cells was performed as previously described. Luciferase activity was recorded after 24 hrs using Luciferase Assay System (Fisher Scientific). Briefly, the cells were washed 2 times with 1× PBS and lysed with 0.25 ml 1× Lysis buffer (Promega). 20 µl cell lysate was added to 100 µl luciferase reporter buffer (Promega) and immediately read in a luminometer (Bio-Rad) at 10 s signal iteration time. Gene expression signals were reported as relative luminescence units (RLU) normalized to total protein contents as measured by the microBCA assay (Pierce), $n = 6$.

In vitro gene expression

OK cells were seeded at 100,000 cells per well, and grown in serum-containing medium, followed by 24 hr incubation in K1 medium. DNA loaded particles (luc-P, luc-P-BSA) were suspended in media and added at 62.5 - 500 µg particles per well, $n = 6$. Samples were analyzed for luciferase luminescence after 3 days as described above.

Statistical analysis

Student t-tests were conducted on data samples. Criteria for determination of statistical significance were set at $p < 0.05$.

Results

PLGA particles encapsulating fluorescent dye or DNA payloads were made by the double emulsion (water-in-oil-in-water) and solvent evaporation process. BSA was incorporated onto particle surfaces by first conjugating the protein to palmitic acid to form an amphiphilic molecule (Figure 1 A). Addition of free BSA does not produce a stable protein presentation, as shown in Figure 1 B. Furthermore, the amounts of BSA presentation can be controlled by initial conjugate input to the second emulsion. A two-fold increase of BSA-palmitate conjugate in the second emulsion results in twice the amount of BSA detected on particles. This method was also reported to introduce a dense and stable coating of avidin protein on PLGA particles [11].

We then formulated particles encapsulating plasmid DNA to be used for gene delivery without (luc-P) with BSA on the surface (luc-P-BSA) (Figure 1 C). Analysis by dynamic light scattering indicated that the mean diameter for luc-P was 322 nm with a polydispersity index or p.d.i. = 0.18 and mean diameter of luc-P-BSA was 334 nm, p.d.i. = 0.14. The surface-associated zeta potential for each respective formulation was -37 ± 0.7 mV and -27 ± 1.3 mV. The presence of BSA on particle surfaces was confirmed by two methods. Immunofluorescence staining of particle surface by anti-BSA primary and FITC-labeled secondary antibodies showed a 35-fold higher fluorescent signal in BSA-coated particles than unmodified particles. To calculate the number of BSA molecules on a single particle surface, total protein content for a known mass of particles was measured using a microBCA assay. We detected ~ 3.6 µg BSA/mg particles for luc-P-BSA. Considering the mean diameter of spherical particles to be 334 nm and an estimated PLGA density of 1.2 g/cm^3 , our calculations yielded ~ 768 BSA molecules per PLGA particle (see Methods section).

Expression of megalin on OK cells was visualized using an anti-megalin monoclonal antibody and a rhodamine-conjugated secondary antibody (Figure 2 A). In addition, cell monolayers stained for megalin receptors were also observed in the presence of FITC-labeled BSA or fluorescent particles. Incubation of OK cells with FITC-labeled BSA produced a strong green fluorescence signal after 6 hrs (Figure 2 B). Co-localization of green (BSA-FITC) and red (megalin) signals in intracellular punctuate structures, indicated by yellow regions on the image, was observed. This co-localization likely indicates BSA-FITC internalization in megalin-containing vesicles. Our observation of BSA-FITC endocytosis in association with megalin is in line with previous reports[22, 23]. PLGA particles containing the tracer dye Coumarin-6 with BSA on the surface bind to the surface and were internalized by OK cells (Figure 2 C).

Normalized fluorescent signal ($\mu\text{g fluor}/\mu\text{g protein}$) from OK cells incubated with particles (fluor-P and fluor-P-BSA, loaded with Rhodamine B) was markedly higher when cell monolayers were incubated with BSA-coated particles. In serum-containing media, particle uptake was on average 2-fold higher for fluor-P-BSA at 37 °C and up to 3-fold for longer time points at 4 °C (Figure 3 A, B). In serum-free conditions, fluor-P-BSA also displayed higher uptake at both temperatures, at 2 to 4-fold (Figure 3 C, D). As energy-dependent endocytosis is halted at 4 °C, the higher fluorescence signals are indicative of strong surface binding of BSA-bound particles to receptors on cell membrane. This increase in uptake of fluor-P-BSA over fluor-P is statistically significant with $p < 0.05$ across all time points and at both temperatures.

Quantitative measurement of the particle association with cells made using flow cytometry produced similar results (Figure 3 E). Fluorescent particles loaded with Coumarin-6 were delivered to cell monolayers maintained at 37 °C at a range of concentrations (125 - 16 μg). The fluorescence level (RFU) per cell was measured and the mean signal of 10,000 events (cells) per sample was used to assess the differences in particle uptake. After 6 hrs, cells incubated with fluor-P-BSA displayed on average 2-fold greater fluorescence per cell at the higher doses: 125 and 63 $\mu\text{g}/\text{well}$. The difference was not seen at lower doses, since the overall cell fluorescence was near background level for both particle types. Preincubation with RAP, a ligand with higher affinity to megalin than BSA, and followed by particle delivery resulted in cell populations with no difference in mean fluorescence. This suggests role of megalin in active sequestration of BSA-coated particles, and the reversibility of this receptor's contribution to particle uptake by addition of a competitive ligand of higher affinity.

Both types of particles were found to encapsulate similar amounts of DNA (2.1 - 2.7 $\mu\text{g DNA}/\text{mg PLGA}$). Gel electrophoresis was performed on DNA extracted from particles to assess the integrity of plasmid conformation after encapsulation and extraction (Figure 4). Whereas the original DNA sample (input, lane 2) was mostly supercoiled, extracted and released DNA's (from *in vitro* controlled release) revealed a shift towards relaxed forms. To rule out the possibility that the DNA extraction process damages the plasmid DNA molecule, we mixed DNA plasmids with blank PLGA particles and subjected this mixture to the same extraction procedure as was applied to the encapsulated DNA. Since this DNA sample (lane 3) did not show any conformation change, we speculate that the resulting structure of extracted and released DNA's (lanes 4-9) is an artifact of the particle making process, specifically vortexing and sonication during the first emulsion step. Bioactivity of extracted DNA was confirmed by transfection with Lipofectamine reagent. Though less of the DNA was in the supercoiled form, bioactivity was still maintained. We measured significantly higher luciferase expression (40,000 - 60,000 RLU/ $\mu\text{g protein}$) than untransfected cells (80 RLU/ $\mu\text{g protein}$). This bioactivity is about half that of the positive control, which consists of only supercoiled plasmid DNA's. Naked DNA was also delivered to OK cells, and produced luciferase expression similar to background.

Controlled release of DNA-loaded particles showed consistently lower DNA released from BSA-coated particles (luc-P-BSA) in both pH 7 and 5 buffers as compared to unmodified particles (luc-P). Plasmid DNA released as fraction of total encapsulated DNA is 25% and 47% for luc-P at pH 7 and pH 5, respectively, and 21% and 19% for luc-P-BSA at pH 7 and pH 5, respectively. Furthermore, DNA release from unmodified particles did not plateau after 5 days in buffer at lower pH, but continued to rise over the entire incubation period of 20 days (Figure 5 A). DNA-loaded particles were delivered to OK cells in cultures at a range of concentrations. Luciferase expression for cells incubated with particles after 3 days increased with amount of particle input in serum-containing and serum-free conditions (Figure 5 B, C). Overall, gene expression level was lower in serum-containing media, but both displayed a dose-dependent response in terms of gene expression to the amount of particles delivered. Interestingly, luc-P elicited higher luciferase expression from cells than luc-P-BSA across all delivered doses. Analysis of the dose-dependent behavior was performed by plotting gene expression (RLU/ μ g protein) against the corresponding amount of DNA released from particles (μ g DNA/mg PLGA) after 3 days. While the correlation of DNA dose to gene expression was linear for both types of particles, the degree of dose-sensitivity—indicated by the slope—was 2.5-fold higher in unmodified luc-P particles (Figure 5 D).

The rate of encapsulated DNA release, in this case, seems to be influenced by the presence of proteins and/or fatty acids present on the particle surface. To explore this relationship, we observed DNA release from particles modified with only DSPE-PEG(2K)biotin (no protein) and varied amounts of BSA-palmitate conjugate. Probing of DSPE-PEG(2K)-biotin on particle surface with avidin-HRP yielded a high concentration of $\sim 6.4 \mu$ g avidin-HRP/mg particles, which is comparable in molar concentration to our BSA-P formulations. Controlled release of DNA from a different batch of particles coated with DSPE-PEG(2K)-biotin was similarly delayed at both pH's suggesting that the hydrophobic contribution by DSPE to particle surface is responsible, to some extent, for the delayed DNA release from particles (Figure 6 A). Surface modification with variable amounts of BSA-palmitate conjugates produced similar rates of DNA release: a 50% reduction in the BSA-palmitate surface concentration (luc-P-0.5BSA) produced nearly the same DNA release rate as luc-P-BSA. This suggests that BSA-palmitate surface modification produces an effect on DNA release, which is non-linear with BSA-palmitate concentration (Figure 6 B, C).

The surface modification of particles also seems to convey a protective effect from pH-induced degradation. SEM images of particles at 5 days and 20 days time points in different pH buffers showed considerable changes in particle shape and morphology. At 5 days, the time point at which DNA release from particles are similar, there is little difference between surface morphology of both particle types (data not shown). However, unmodified particles exhibited markedly higher porosity, indicated by arrows, after 20 days, and especially at the lower pH. In contrast, BSA-coated particles maintain a smooth and non-porous surface after prolonged incubation in either pH environments (Figure 7). This protective effect may also be responsible for the slower, more sustained release of the encapsulated DNA from the particles.

Discussion

Design of an efficient drug carrier must take the host's physiology into consideration. Taking advantage of a known ligand-receptor relationship, we formulated PLGA particles with BSA on the surface to facilitate uptake in megalin-expressing epithelial cells. Our results indicate that addition of a BSA coating to the surfaces of PLGA particles does not change their morphology and size distribution. Though BSA readily binds to fatty acid in the body, our previous experience shows that covalent linkage between BSA and palmitate is necessary to obtain a stable protein coating. Addition of BSA reduced the particle surface zeta-potential from -37 to -27 mV. It is possible that the reduced surface negative potential facilitates

interaction of particles with negatively charged cell membrane, but is not able to induce higher uptake when a competitive ligand is present (Figure 3 E).

The cell line used to study particle uptake and gene expression was confirmed to express megalin, and could successfully take up free BSA, or particles associated with BSA, a ligand to megalin (Figure 2). The enhanced uptake of BSA-coated particles by OK cells expressing megalin could be quantitatively measured. Higher fluorescent signal at 37 °C was likely a contribution of particles within cells (endocytosed) and bound on the cell surface. At 4 °C where the cell metabolism rate and membrane fluidity is reduced, the higher fluorescent signal (6 - 12 hrs) on OK cell layers suggests that BSA-coated particles were able to bind at a higher affinity to receptors on the cell surfaces than unmodified. Interestingly, BSA-modified particles showed higher association to cells in serum-supplemented media which contains free BSA that could potentially act as competitive inhibitors (Figure 3 A, B). Introduction of the high-affinity competitive ligand, RAP, inhibited megalin binding to BSA on modified particles and effectively reduced the uptake rate to the level of unmodified particles (Figure 3E). This result leads us to conclude that the receptor megalin plays a significant role in enhancing uptake of BSA-coated particles. The increase in uptake seen here is comparable to other studies for PLGA particles, modified with lectin or peptides as targeting moieties, as shown in Table 1.

The clearance of particles due to cellular processing by OK cells and has been previously confirmed.[24] Here, PLGA particles were shown to localize within the Golgi apparatus within the first 2 hrs after delivery, and are not found in the cell after 24 hrs. In addition, there is evidence that BSA is taken up by clathrin-mediated endocytosis in endothelial cells, which subsequently is transported to Golgi apparatus. [25] We thus speculate that both types of particles are first taken up in the endosomes and do not expect the fates of modified particles to be significantly different.

Payload release from a polymer particle occurs by a combination of diffusion of molecules to the particle surface through channels formed within the polymer matrix, and erosion of the polymer particle which releases molecules deeply trapped within the particle core. DNA release from coated PLGA particles was lower than unmodified particles (Figure 5, 6) and the coated particles undergo less degradation in acidic buffers (Figure 7). This effect is most likely due to the presence of amphiphilic conjugates on the particle surface. It is possible that the presence of hydrophobic fatty acids on the particle surface prevents hydration of the particle interior and is a barrier to DNA release. Also, the presence of BSA protein may sterically hinder DNA diffusion from the particle into the surrounding environment. We tested the effectiveness of hydrophobic fatty acids alone by coating PLGA particles with an amphiphilic molecule, DSPE-PEG(2K)-biotin, which mimics the effect of the palmitate-BSA conjugate on particles but without the protein (Figure 6 A). The slower DNA release found from these particles suggests that the addition of lipids to particle surface significantly hinders release of hydrophilic payload such as DNA, likely by the mechanisms proposed above. We note, however, that this does not preclude the effect of BSA on DNA release. The rate of DNA release is also not directly correlated to the amounts of BSA-palmitate on the surface (Figure 6 B, C)

The slower release of DNA from BSA-coated particles has a negative effect on gene expression in OK cells *in vitro*. This effect was not reversed by increasing the dosage of administered particles. In other words, it is not possible to produce an equivalent level of gene expression by adding more particles to the cells. Considering the uptake rate and gene expression of a single particle dose, 125 µg, there is 2 to 3-fold higher initial uptake of BSA-coated (Figure 3), but 3 to 5-fold lower gene expression level compared to unmodified particles in cells (Figure 5 B, C). These results emphasize both the significant impact of DNA release rate on gene expression and the non-linear relationship between them.

Gene expression by transfection of naked DNA or cationic lipid/DNA complex was transient compared to DNA delivery via particles. While a protective coat and sustained release may be advantageous, the relationship between DNA release rate and gene expression is still not well-understood. For example, fast releasing particles made from poly(ortho)ester may release 3-fold DNA payload at 5 - 7 days over PLGA particles, consequently improving both gene expression in cell culture and tumor reduction *in vivo* due to expression of cancer therapeutic gene[26]. In a separate study, particles carrying DNA made from slow-releasing PLGA-PBAE polymer blends transfect macrophages at 100-fold greater efficiency than faster-releasing PLGA particles[27]. These differences, as further highlighted in our study, may be due to important design components of the particulate drug carrier, such as size, charge, stability, pH-sensitivity, cell-association and payload release rate, that may prove advantageous for a specific particle-drug-target system but not another.

Understanding the nature and cell biological properties of receptor-ligand interactions in target cells or tissues creates opportunities that can be exploited to enhance the delivery of a drug carrier. Targeted drug delivery systems have the potential to maximize the delivery of therapeutic agent to a disease site without risking systemic side-effects. Moreover, a carrier made from a degradable polymer can protect the therapeutic agent from degradation and mediate controlled release of the agent within target sites over time. In spite of these potential benefits, however, there are numerous challenges to particle design that need to be overcome before such targeting techniques can become widely applicable.

Our study demonstrates a method for particle formulation with a high level of ligand presentation, which results in increased uptake of targeted cells even in serum media (with free BSA). In addition, the presence of the target protein receptor, megalin, in the GI tract and the enhanced stability of particles at high pH suggests a promising application in oral gene delivery. However, our efforts to use BSA as ligand to enhance gene carrier binding to epithelial cells indicates that modifications designed to improve one function, such as surface binding, can produce unexpected effects on other properties, such as payload release. Our results indicate that the higher cell-association but slower DNA release, as exhibited in our targeted particle formulation, does not lead to better gene expression. Thus, design of efficient targeted gene carriers needs to extend beyond the modular incorporation of desirable features. Each addition to the “smart” gene carrier must be weighted on its effect on all key parameters pertaining to the system, namely payload encapsulation, release, carrier interaction with cell surface and intracellular processing.

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Figure 1.

Particles were formulated by double emulsion and solvent evaporation method. Payload diluted in aqueous buffer was added to molten PLGA organic solvent to form the first emulsion, which was then added to a second volume of aqueous buffer containing the amphiphilic conjugate intended for surface display (variable amounts of palmitate-BSA or DSPE-PEG(2K)-biotin). Following solvent evaporation, the final particle formulation encapsulates the payload of interest and displays the ligand on the surface (A). Conjugation of BSA to palmitic acid is necessary to achieve a stable coating of the protein on particle surface (B). No BSA associated to particles was detected when free (unconjugated) protein was used. However, BSA was present following addition of BSA-palmitate conjugates, which increased in a controlled manner with amounts of conjugated used (luc-P-0.5BSA indicates the amount of conjugates used in the second emulsion was 50% of that used in making luc-P-BSA). Scanning electron micrograph of particles without and with BSA modification loaded with plasmid DNA encoding for the luciferase gene (luc-P and luc-P-BSA) indicates a spherical and smooth surface morphology (C). Analysis using dynamic light scattering indicates the diameters of particles averaged at 322 nm (polydispersity index = 0.14) for luc-P and 334 nm (polydispersity index = 0.18) for luc-P-BSA formulations. Scale bar = 1 µm.

Figure 2.

Confocal image of OK cells labeled with anti-megalin TRITC (red fluorescence) confirm the presence of the receptor protein (A). Binding and internalization by vesicle of BSA-FITC (B) and Coumarin-6 nanoparticles (C) by OK cells at 37 °C are indicated by green and yellow fluorescence signal (arrows), respectively.

Figure 3.

Analysis of particles (fluor-P and fluor-P-BSA) association to cells over 24 hrs at 37 and 4 °C with serum (A, B) and without serum (C, D) suggested preferential association of BSA-coated particles to cells over unmodified particles. The increase in fluorescence signal per cell was high across all time points (0.5 – 12 hrs), and statistically significant with $p < 0.05$, particle dose = 125 μg/well. Flow cytometry analysis of particle internalization by OK cell at 37°C after 6 hrs was repeated with similar results (E). The mean relative fluorescence unit (mean RFU) fluor-P-BSA per cell event was 2× higher than that of fluor-P. With preincubation of the

megalin inhibitor RAP, however, the increase in uptake conferred by the BSA coating was nullified.

Figure 4.

Gel electrophoresis of DNA extracted and released from luc-P and luc-P-BSA. Lane 1- Supercoiled 10 kb DNA ladder; lane 2- luc; lane 3- luc spiked in PLGA+MCH solution; lanes 4-5- luc extracted from luc-P and luc-P-BSA; lanes 6-7- released from particles in 1×PBS pH7 and pH 5 (lanes 8-9) for luc-P and luc-P-BSA. While the stock plasmid DNA in lanes 2 and 3 appear to be mostly in supercoiled form, DNA's extracted from particles revealed some population of plasmids in relaxed or linear conformations.

Figure 5.

Analysis of supernatant of particles incubated in buffered saline showed higher release from luc-P over luc-P-BSA at pH 7 (A) and pH 5 (B). DNA release from luc-P was overall higher than that from luc-P-BSA particles for both pH's. The percent of DNA released (of total encapsulated) at the end of the incubation period was 25% (0.64 μg DNA) and 47% (1.2 μg DNA) for luc-P at pH 7 and pH 5, and 21% (0.43 μg DNA) and 19% (0.40 μg DNA) for luc-P-BSA at pH 7 and pH 5, respectively. Luciferase gene expression (relative luminescence units normalized to total cellular protein or RLU/μg protein) as a function of amount of particle added after 3 days incubation exhibit a dose-response, and is up to 10-fold higher for luc-P at the highest particle dose 500 μg in serum-containing (B) and serum-free media (C). For the latter, the luciferase expression to DNA released from particles at respective delivered dose at pH 5, both after 3 days, is linear (D). Luc-P exhibit 2.5-fold higher response than luc-P-BSA (slope luc-P=4489 and luc-P-BSA = 1800).

Figure 6.

Coating particles with an amphiphilic molecule, DSPE-PEG-biotin, produced a similar delayed release of DNA, indicating that both the presence of a hydrophobic coat and additional protein on particle surface are responsible in delaying DNA release from particles (A). Plasmid DNA released from BSA-palmitate modified particles was overall slower than unmodified luc-P. In addition, DNA release does not seem to increase linearly with reduction in concentration of conjugates on the particle surface at both pH 5 (B) and pH 7 (C).

Figure 7. Particles with (+) and without (-) BSA coating after 20 days incubation in pH 5 and pH 7 buffers at 37 °C. Where uncoated particles are degraded, depicted by porous surface morphology (arrows), BSA-coated particles remain largely smooth and nonporous. Scale bar = 1 μ m

Table 1

Summary of studies with targeted polymer particles

Polymer	Surface modification and target	Agent	Notes	Reference
PLGA (320-360 nm)	mAb specific to MCF-10DneoT	BSA	2× increase in uptake with mAb	Kocbek <i>et al.</i> [28]
PLGA-PEMA (813 nm)	BSA, streptavidin, IgG, UEA1	BSA-FITC	2× increased in specific uptake	Keegan <i>et al.</i> [12]
PLGA/ PBAE (1-10 µm)	PBAE	plasmid DNA (luciferase)	Gene expression 100× higher in modified	Little <i>et al.</i> [27]
PLGA (120 nm)	SM5-1 Ab	Paclitaxel	Particle uptake is higher in targeted cells. Particles carrying paclitaxel with Ab on the surface 3× more potent.	Kou <i>et al.</i> [29]
PLGA/MMT (294-312 nm)	Trastuzumab mAb	Paclitaxel, Coumarin-6	Ab-modified particles show higher uptake (1.24× in SK-BR-3 and 1.1× in Caco-2 cells)	Sun <i>et al.</i> [30]
PLGA (230-330 nm)	WGA	Paclitaxel	WGA-modified particles carrying paclitaxel increase cell toxicity 2-10×.	Mo <i>et al.</i> [31]
PLGA (150-240 nm)	WGA	thymopentin	1.4-3.1× increased retention in GI tract, especially small intestine	Yin <i>et al.</i> [32]
PLGA (4.8 µm)	WGA	Bet v1	2× higher allergen-specific IgG for WGA-modified particles after oral delivery	Weissenboeck <i>et al.</i> [33]
PLGA (570-1020 nm)	WGA	FITC-conjugated PLGA (tracer)	Increased binding on Caco-2 cells of WGA-modified NP over PLGA or BSA modified NP's. Results indicate 2× higher than unmodified and 1.5× than BSA-conjugate particles	Weissenboeck <i>et al.</i> [34]
PLGA (110 nm)	PLL-peg-folate	none	Higher uptake in cells expressing folate receptor	Kim <i>et al.</i> [35]
PLGA (175 nm)	cLABL peptide	Peptide	2× higher binding	Zhang <i>et al.</i> [36]
PLGA (165 nm)	NLS peptide (PKKRKV)	plasmid DNA (luciferase)	NLS increase luciferase expression by 3-4×.	Jeon <i>et al.</i> [37]
PLGA-PEG (160-290 nm)	RNA aptamer	Docetaxel, 3H-PLGA (tracer)	8-16× higher binding <i>in vitro</i> , 15× higher	Gu <i>et al.</i> [38]

Polymer	Surface modification and target	Agent	Notes	Reference
			accumulation <i>in vivo</i> .	