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Enhancement of surface ligand display on PLGA nanoparticles with amphiphilic ligand conjugates

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ABSTRACT

Biodegradable polymeric nanoparticles are widely recognized as efficacious drug delivery vehicles, yet the rational engineering of nanoparticle surfaces in order to improve biodistribution, reduce clearance, and/or improve targeting remains a significant challenge. We have previously demonstrated that an amphiphilic conjugate of avidin and palmitic acid can be used to modify poly(lactic-co-glycolic acid) (PLGA) particle surfaces to display functional avidin groups, allowing for the facile attachment of biotinylated ligands for targeting or steric stabilization. Here, we hypothesized that the incorporation, density, and stability of surface-presented avidin could be modulated through varying the lipophilicity of its fatty acid conjugate partner. We tested this hypothesis by generating a set of novel conjugates incorporating avidin and common fatty acids. We found that conjugation to linoleic acid resulted in a ~60% increase in the incorporation of avidin on the nanoparticle surface compared to avidin–palmitic acid, which exhibited the highest avidin incorporation in previous studies. Further, the linoleic acid–avidin conjugate yielded nanoparticles with enhanced ability to bind biotinylated ligands compared to the previous method; nanoparticles modified with avidin–linoleic acid bound ~170% more biotin–HRP than those made with avidin–palmitic acid and ~1300% more than particles made without conjugated avidin. Most critically, increased ligand density on anti-CD4-targeted nanoparticles formulated with the linoleic acid–avidin conjugate resulted in a 5% increase in binding of $CD4^+$ T cells. Thus we conclude that the novel avidin–linoleic acid conjugate facilitates enhanced ligand density on PLGA nanoparticles, resulting in functional enhancement of cellular targeting.

1. Introduction

Biodegradable polymeric nanoparticles (NPs) have long been investigated as drug delivery vehicles. These particles can be used to solubilize concentrated drug payloads, improve drug stability and bioavailability, and extend drug effect through sustained delivery [1,2]. Among the most commonly used and extensively investigated biodegradable polymers are poly(lactic-co-glycolic acid) (PLGA) and its constituent polymers, polylactic acid (PLA) and polyglycolic acid (PGA) [1]. These polymers have a long history of safe use in humans and their degradation under physiologic conditions releases lactic and/or glycolic acid monomers that are easily metabolized or eliminated [3]. Copolymers of PLGA are of particular interest for drug delivery as the degradation rate of the polymer and subsequent drug release rate can be modulated by varying the ratio of the PLA to PGA segments; higher ratios of the more hydrophobic PLA decrease the penetration of water and overall degradation rate of the polymer while higher ratios of the more hydrophilic PGA have the opposite effect [1,3].

The biodistribution and pharmacokinetic properties of nanoscale drug delivery vehicles are largely dependent on their size, material composition, and surface properties [4–6]. There has been tremendous interest in the development of biodegradable nanoparticles that display targeting ligands in order to improve the biodistribution, safety, and efficacy of encapsulated agents. Antibody, aptamer and even small molecule-targeted PLGA nanoparticles have been shown to be preferentially bound to or internalized by target cells, compared to cells lacking the targeted receptor or ligand [7–10]. In vivo, targeting has been demonstrated to increase dose accumulation and persistence at sites of disease, such as tumor beds, where the target ligand is either uniquely or highly expressed [11,12]. Interestingly, localization or internalization of nanoparticles can enhance the potency of encapsulated agents, as measured by, for example, lowered IC50 values of chemotherapeutic drugs [7,10,12,13]. However, as PLGA lacks functional chemical groups on the aliphatic polyester backbone, a significant challenge has been the development of methods that enable facile surface modification of nanoparticles made from this

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polymer [1,14]. One popular method has been to utilize co-block polymers, such as those based on PLGA and the hydrophilic polymer polyethylene glycol (PEG), that contain functionalized endgroups which enable covalent conjugation of ligands. These polymers have long been used to fabricate nanoparticles in which the hydrophilic properties of PEG and relatively hydrophobic properties of PLGA determine the formation of core–shell structures [15,16]. Covalent conjugation of ligands to functionalized PEG, either before [10] or after [11] particle manufacture therefore results in their preferential display on the particle surface. However, one downside of this approach is the potential exposure of the drug-loaded NP to hydrolysis and the potential for ligand denaturation. Significant loss of the surface ligand can occur, likely due to hydrolysis of the PLGA [15], and a comparison of covalent conjugation versus adsorption has shown that conjugation can compromise the binding ability of targeting antibodies [7]. Likewise, fine control over the number or density of ligands has not been well discussed in the literature.

We have previously demonstrated that an amphiphilic avidin–palmitic acid conjugate can be utilized to present functional avidin groups on the surface of drug-loaded PLGA scaffolds and microparticles [17]. The avidin molecules are then available to bind biotinylated ligands at any point after particle manufacture and storage. Importantly, this methodology enables titration of targeting ligands and thereby precise control over the surface properties. For example, avidin-coated polystyrene particles, although ineffective as in vivo drug delivery vehicles, have been useful in investigating the effect of NP targeting due to the wide availability of biotinylated ligands [18]. Compared to other surface functionalization techniques, this novel methodology for modifying PLGA NP surfaces spares potentially labile ligands from harsh manufacturing processes and does not require modification of either the encapsulant or the polymer. We have demonstrated the versatility of this linker system by modifying PLGA nanoparticles with polyethylene glycol (PEG) for improved biodistribution of doxorubicin [19] and enhanced transport across mucosal barriers [20], lipoglycans for enhancement of encapsulated vaccine efficacy [21], ligand modification to improve cell uptake [22], and targeting antibodies for enhanced T-cell stimulation [23] and improved cytokine delivery [24].

As the preferential surface presentation of the avidin–fatty acid conjugate is thought to be driven by its amphiphilic nature [17], we hypothesized that varying the fatty acid lipophilicity would influence the density and stability of avidin–lipid incorporation in PLGA nanoparticles. Ligand density is a critical factor in the efficacy of targeted drug delivery systems; higher density is a particularly useful feature for ligands that, in their monomeric form, have a weak affinity for their target receptors [4,6,25–27], such as single-chain variable fragments (SCvF) and peptide/major histocompatibility complexes (peptide/MHCs), which have weak affinity to target T cell receptors [27–29]. Thus, the results of this study suggest new opportunities in the design of a high avidity nanoparticle platform for targeted drug delivery in a number of therapeutic scenarios.

2. Materials and methods

2.1. Preparation of avidin–fatty acid bioconjugates and avidin-functionalized nanoparticles

Stable avidin–lipid conjugates were formed using a zero-length crosslinking agent to create a covalent bond between the lipid carboxyl end groups and free amines on the avidin protein. Lipids (butyric, caprylic, palmitic, stearic, or linoleic acid; all from Sigma) were first reacted in 0.1× PBS with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) and *N*-hydroxylsulfosuccinimide (sulfo-NHS) (Invitrogen) to convert the terminal carboxyl group to an amine-reactive sulfo-NHS ester. Avidin (Sigma) at 5 mg/ml was then reacted with 10-fold molar excess of the NHS-functionalized fatty acid in 0.1× PBS and the solution was gently mixed at 37 °C for 2 h. Reactants were then dialyzed against 1.0× PBS at 37 °C for 24 h to remove excess reactants and/or hydrolyzed esters.

PLGA nanoparticles (NPs) were manufactured using an oil-in-water emulsion method. One hundred (100) mg of PLGA with molecular weight of 92–112 kDa and 50/50 lactide:glycolide ratio (Durect Corporation) were dissolved overnight in 2 ml dichloromethane. To make BSA-FITC loaded NPs, 100 μl of BSA-FITC (Fisher Scientific) (10 mg/ml in 1× PBS) were added to the polymer solution with vortexing. This solution was then added dropwise with vortexing to a 4 ml aqueous solution consisting of 2 ml (5 mg/ml) avidin–lipid bioconjugate and 2 ml 5% PVA (MW 30–70 kDa, Sigma) to make surface-functionalized, "avidin+" NPs. To make "blank" or "unconjugated" NPs controls, 2 ml of 1× PBS or 2 ml of freshly dissolved avidin (5 mg/ml in PBS) were used, respectively, instead of the avidin–lipid conjugate solution. The organic polymer/aqueous surfactant emulsion was then sonicated on ice 3× at 10 s intervals using a 600 watt Misonix 3000 sonicator with a 3/16" microtip. Solvent was removed and particles hardened by magnetic stirring for 3 h in 120 ml of 0.3% PVA aqueous solution. Nanoparticles were collected by centrifugation for 10 min at 10,000 rpm and resuspended/washed in sterile DI water. Particles were washed a total of 3 times to remove excess surfactant, conjugate, and encapsulant prior to lyophilization and storage at −20 °C. Nanoparticles of polylactide (PLA) (100/0 lactide:glycolide ratio, Durect Corporation) were made in identical fashion.

2.2. Characterization of avidin–lipid conjugates

Avidin–lipid conjugates were previously characterized by HPLC [17]. We also examined the biotin-binding ability of conjugates using 4′-hydroxyazobenzene-2-carboxylic acid (HABA) (Sigma). HABA binds to avidin to yield a yellow-orange complex that absorbs at 500 nm. As the dye binds with weaker affinity to avidin ($K_d = 5.8 \times 10^{-6}$ M) than biotin ($K_d = 1 \times 10^{-15}$ M), traditionally the HABA assay is utilized to quantify biotin concentrations as the addition of free biotin displaces the HABA dye with an associated decrease in absorbance [30]. Here, the linear relationship between avidin concentration and absorbance was used to calculate avidin concentration.

2.3. Characterization of nanoparticles

Nanoparticle morphology was characterized by scanning electron microscopy (SEM). Samples were sputter-coated with gold using a Dynavac Mini Coater and imaged with a Philips XL30 SEM using a LaB electron gun with an accelerating voltage of 5–10 kV. Particle size and distribution were determined using ImageJ image analysis software (available from the NIH). Mean particle diameter was calculated by analysis of >250 counts per sample and statistical difference between groups assessed by two-tailed Student's t tests. The hydrodynamic mean effective diameter and polydispersity were measured in 1× PBS using a ZetaPALS particle sizing instrument (Brookhaven Instruments Corporation, Holtsville, NY). Particle counts (number of particles per milligram of sample) were obtained using a Nanosight instrument (NanoSight, Ltd., Wiltshire, UK).

2.4. Quantification of avidin incorporation, stability, and effect on release profile

Avidin incorporation in nanoparticles was quantified using a micro-BCA assay (Fisher Scientific). Unloaded, surface-modified nanoparticles were suspended in dilutions starting at 2 mg/ml in 1× PBS and 150 μl of sample or standard added to a 96 well u-bottom microplate. 150 μl of the micro-BCA working reagent was added to each well and the plate incubated for 2 h at 37 °C. The plate was then centrifuged to collect NPs and 100 μl of supernatant transferred to a new plate. Protein content was measured by absorbance at 562 nm and background measured at 650 nm. Mean avidin incorporation was calculated by performing