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Surface modification of biodegradable polyesters with fatty acid conjugates for improved drug targeting

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Abstract

We describe a general method for incorporating target ligands into the surface of biocompatible polyester poly(lactic-*co*-glycolic acid) (PLGA) 50/50 materials using fatty acids. Avidin-fatty acid conjugates were prepared and efficiently incorporated into PLGA. Avidin was chosen as an adaptor protein to facilitate the attachment of a variety of biotinylated ligands. We show that fatty acid preferentially associates with the hydrophobic PLGA matrix, rather than the external aqueous environment, facilitating a prolonged presentation of avidin over several weeks. We successfully applied this approach in both microspheres encapsulating a model protein, bovine serum albumin, and PLGA scaffolds fabricated by a salt-leaching method. Because of its ease, generality and flexibility, this strategy promises widespread utility in modifying the surface of PLGA-based materials for applications in drug delivery and tissue engineering.

Keywords: Biodegradation; Biocompatibility; Surface modification; Lipid; Drug delivery; Microsphere; Scaffold

1. Introduction

Biodegradable polymers fabricated from poly(lactic-*co*-glycolic acid) (PLGA) have emerged as powerful potential carriers for small and large molecules of therapeutic importance as well as scaffolds for tissue engineering applications. This importance derives from: (1) Physiologic compatibility of PLGA and its homopolymers PGA and PLA, all of which have been established as safe in humans after 30 years in various biomedical applications including drug delivery systems [1–3]. (2) Commercial availability of a variety of PLGA formulations for control over the rate and duration of molecules released for optimal physiological response [4]. (3) Biodegradability of PLGA materials, which provides for sustained release of the encapsulated molecules under physiologic conditions while degrading to nontoxic, low-molecular-weight products that are readily eliminated [4,5]. (4) Control over PLGA manufacturing into nanoscale particles (<500 nm) for potential evasion of the immune phagocytic system or fabrication into microparticles on the length scale of cells for targeted delivery of drugs or as antigen-presenting systems [6,7]. This unique combination of properties—coupled with flexibility over fabrication—has led to interest in modifying the PLGA surface for specific attachment to cells or organs in the body [6,8–12], which may be of interest in both drug delivery and tissue engineering applications. With a functionalized PLGA surface, cells can be attached specifically to scaffolds, enabling control over cellular interactions that lead to formation of optimal neotissue, or encapsulated agent can be delivered specifically to a site of interest potentially reducing deleterious drug side effects and enhancing antigen delivery for vaccine applications.

A major difficulty associated with coupling ligands to PLGA particles has been the lack of functional chemical

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groups on the aliphatic polyester backbone for linking to target ligands. This severely hinders the application of traditional conjugation methods to the PLGA surface. Thus to introduce functionality into PLGA surfaces several approaches have been studied. These include, synthesis of PLGA copolymers with amine [13,14] or acid [14] end groups which can be subsequently fabricated into particles. Another approach involves the blending or adsorption of functional polymers such as polylysine [15,16] or poly(ethylene-alt-maleic acid) (PEMA) [17] or Polyethylene glycol (PEG) [18] into PLGA and forming particles and matrices from these blends [16,17,19–21]. Plasma treatment of the PLGA matrix has also been proposed for the purpose of modifying its surface properties and introducing hydrophilic functional groups into the polymer [22,23]. While these approaches have had good success in their specific applications, their general use is hindered by drawbacks such as difficulty associated with preparing the needed copolymers, limited density of functional groups at the surface of the bulk material, and targeting effects that decrease with time due to desorption or degradation of adsorbed group as the particle or scaffold erodes. It would be most desirable—and perhaps necessary for certain applications—to retain ligand function with control over its density on the surface for prolonged periods of time.

In this work, we engineered PLGA microparticles and scaffolds with improved functionality by incorporating a fatty acid (palmitic acid) conjugated to avidin into particles and previously fabricated scaffolds. We reasoned that this conjugate would naturally position itself at the material surface: the palmitic acid would preferentially partition into the hydrophobic PLGA matrix, whereas the hydrophilic avidin head group would be on display in the hydrophilic external environment, facilitating the attachment of biotinylated ligands to the surface of the microspheres or scaffolds. Here we show that functionalizing PLGA with avidin–palmitic acid imparts the following useful properties to modified matrices: (1) High density of avidin incorporation into the surface. (2) Prolonged display of the avidin over the course of several weeks. (3) Enhancement of protein encapsulation efficiency and particle yield. (4) Control over regional modification of PLGA scaffolds. These improved properties should provide for wide spread utility in basic and clinical applications of targeted drug delivery and tissue engineering.

2. Materials and methods

2.1. Materials

PLGA with an inherent viscosity of 0.59 dL/g, lot D02022 was supplied from Birmingham Polymers Inc. polyvinyl alcohol (M_w average 30–70 kDa), palmitic acid-*N*-hydroxysuccinimide ester (NHS–palmitate), avidin (affinity purified) from egg white and biotin-B-phycoerythrin, biotin immobilized on agarose were all obtained from Sigma Chemical Co. Methylene chloride and trifluoroethanol were of chromatography grade and supplied by Fischer Chemicals. All other reagents were of reagent grade and used as received.

2.2. Preparation of avidin–palmitic acid conjugates

Avidin at 10 mg/mL was reacted with 10-fold excess of NHS–palmitic acid in PBS containing 2% deoxycholate buffer. The mixture was sonicated briefly and gently mixed at 37 °C for 12 h. To remove excess fatty acid and hydrolyzed ester, reactants were dialyzed against PBS containing 0.15% deoxycholate. The resultant avidin–palmitate conjugate was verified by reverse-phase HPLC on a Prevail® C18 column with a linear methanol gradient in PBS as the mobile phase and UV detection at 280 nm.

2.3. Surface modification and characterization

We used a modified water-in-oil-in-water (W/O/W) emulsion method for preparation of fatty acid PLGA particles. In the first emulsion, fluorescent bovine serum albumin (BSA-FITC) in 100 μL of PBS was added dropwise to a vortexing PLGA solution (5 mL) dissolved in methylene chloride and trifluoroethanol (4:1) (v/v). This first emulsion (w/O) was rapidly added to 200 mL of 5% PVA containing the various concentrations of avidin–palmitic acid investigated. This external phase underwent vigorous stirring for 4 h at constant room temperature (RT) to evaporate methylene chloride and trifluoroethanol. The resultant emulsion was then purified by centrifugation at 12,000*g* for 15 min then washed 3 × with DI water. No subsequent filtration or classification of particles took place in this study. The particles were freeze-dried then stored at −20 °C.

Samples were characterized by scanning electron microscopy (SEM). Samples were sputter-coated with gold under vacuum in an argon atmosphere using a sputter current of 40 mA (Dynavac Mini Coater, Dynavac, USA). SEM analysis was carried out with a Philips XL30 SEM using a LaB electron gun with an accelerating voltage of 5–10 kV.

2.4. Surface density and functional specificity

A colorimetric assay with 2-hydroxyazobenzene-4′-carboxylic acid (HABA) was used to quantitate the density of surface avidin groups on PLGA particles. HABA binds to avidin to produce a yellow-orange colored complex which absorbs at 500 nm [24]. First, a linear relationship between avidin in solution and

HABA absorbance was obtained by measuring the absorbance at 500 nm. This standardized relationship was then used to quantitate the density of surface avidin groups. In this assay, 3 mg aliquots of dried particles were suspended in 1 mL of 10 mM HABA (24.2 mg HABA in 10 mM NaOH).

In addition, biotin-phycoerythrin (biotin-PE), a biotin conjugate of the red fluorescent protein (PE) (240 kDa), was used to monitor surface functionality. On a rotary shaker the indicated amounts of biotin-PE in PBS were added to 10 mg of plain and surface modified particles. These solutions were incubated for 15 min then centrifuged (10 min/11,000*g*) and washed 3× in DI water. Particle fluorescence was measured by flow cytometry.

2.5. Affinity to target under dynamic conditions

Biotinylated agarose beads (2 mL of 4% crosslinked agarose) were poured into a fritted glass column and allowed to settle prior to addition of plain or modified particles. The bed was briefly sonicated to eliminate trapped air bubbles. PLGA particles suspended in PBS were gently added to the top of the packing and allowed to settle into the packed bed prior to elution with PBS. The volume of particles added to the bed did not exceed a tenth of the volume of the packed bed. The column was then carefully filled with buffer and a constant flow of buffer at 0.2 mL/min was maintained by a Jasco pump. Fractions were collected every 0.5 mL into polystyrene UV cuevettes and sample turbidity was analyzed by UV spectrophotometry at 600 nm. Turbidity of the mixture was an indicator of particle elution of the column. For modified particles, when turbidity subsided, a 6 M guanidine hydrochloride was added to the column and fractions were collected as described.

2.6. Surface stability and kinetics of BSA release

Release of encapsulated BSA-FITC and surface-bound biotin-PE were carried out in phosphate buffer saline at 37 °C. At the indicated time points samples were centrifuged for 10 min at 11,000*g* and 1 mL supernatant from the samples was removed and replaced with fresh buffer preincubated at 37 °C. The FITC and PE content was measured by fluorescence (($\lambda_{excitation} = 480$, $\lambda_{emission} = 520$) for BSA-FITC and ($\lambda_{excitation} = 529$, $\lambda_{emission} = 576$) for biotin-PE). The fraction of protein released was calculated by dividing the amount of BSA-FITC or biotin-PE at the indicated time points by the total content of both proteins in 10 mg of the same stock of particles. Total BSA-FITC content was measured by dissolving 10 mg of particles in 1 N NaOH overnight. A standard was prepared by titrating BSA-FITC in 1 N NaOH. Since biotin-PE was localized to the surface of the particles, red fluorescence of an aliquot of (5 mg) particles was measured directly without need for dissolution.

2.7. Surface modification of PLGA scaffolds

PLGA 50/50 scaffolds were prepared by a salt-leaching method [25]. PLGA was dissolved in methylene chloride (10 mg in 500 μL). Sodium chloride particles (100 mg sieved to obtain a range of diameters, d, $100 < d < 250$) were sprinkled into a round PVDF containers (Cole Parmer #H-08936-00) followed by addition of PLGA solution. After solvent evaporation (24 h at RT), scaffolds were washed thoroughly in DI water for 3 days. Scaffolds were freeze-dried and stored at −20 °C for later use.

Avidin–palmitic acid incorporation was achieved by a simple deposition procedure. A 100 μL drop was regionally placed on top of dried scaffolds and allowed to soak in for 15 min at RT, followed by washing 5× in 1× PBS + 1% BSA. For surface staining, the entire scaffold was incubated in a biotin-PE solution for 10 min at RT followed by a second wash in DI water.

3. Results and discussion

3.1. Palmitoylation of avidin

The overall scheme to modify a protein with palmitic acid is shown in Fig. 1. In the scheme depicted (Fig. 1), NHS–palmitic acid is added to avidin at 10× molar excess and reacted in the presence of 2% deoxycholate detergent. The NHS ester reacts with avidin amine groups producing a stable amide linkage and rendering the protein hydrophobic. Both reaction and purification steps were performed in the presence of detergent to prevent palmitate vesicle formation [26]. Compared to free avidin, which eluted as a single uniform peak with buffered water alone, avidin–palmitic acid exhibited some aggregation and eluted with methanol in the mobile phase (Fig. 2). This reflects the enhanced hydrophobicity of the conjugate. At higher methanol concentrations in the mobile phase we observed several elution peaks indicating different degrees of conjugate association with the column. A possible explanation is that NHS–palmitic acid targets individual lysine residues as well as the amino terminus of the protein for conjugation; a process that can yield heterogeneous populations of palmitoylated avidin that associate differently with the hydrophobic stationary phase. Since homogeneity of the avidin conjugate was not essential for the present work, this preparation was used without further purification.

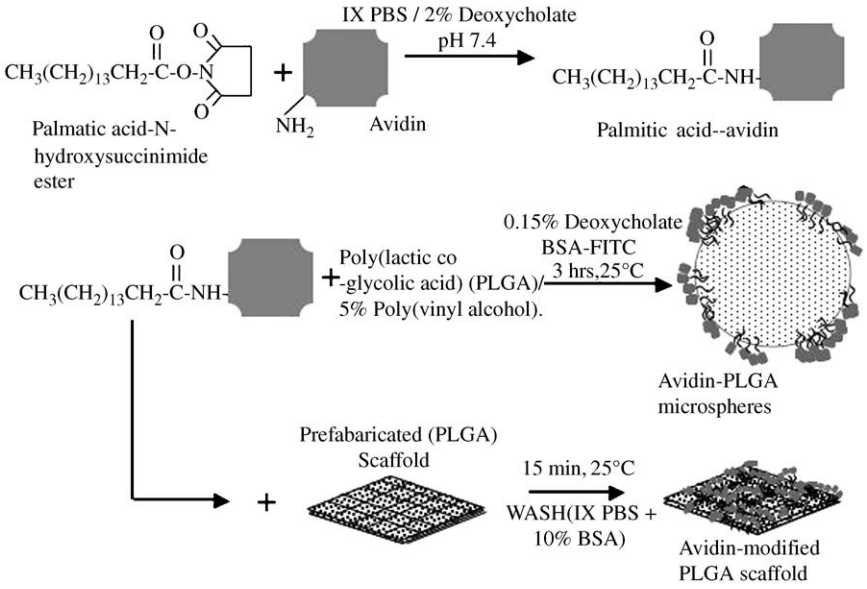

Fig. 1. Overall scheme depicting the modification of PLGA particles and scaffolds with palmitoylated avidin. (A) Conjugation of palmitic acid to avidin and (B) incorporation of avidin–palmitate on the surface of PLGA particles using the double emulsion-solvent evaporation method. Surface modification of scaffolds was more direct with avidin–palmitate added directly to the PLGA matrix followed by a washing step in blocking buffer.

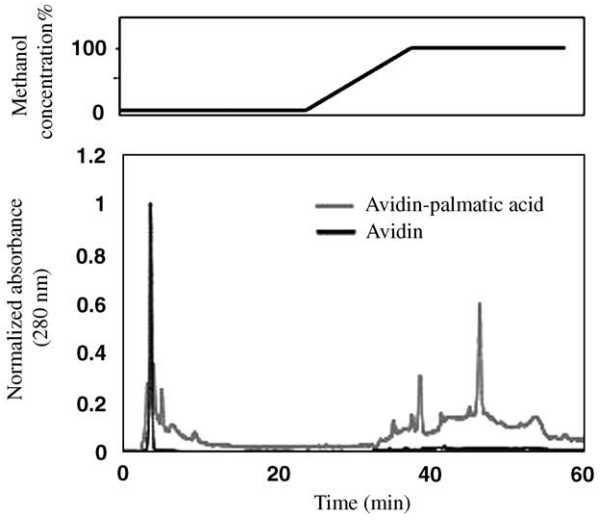

Fig. 2. Palmitoylated avidin verified by reverse-phase HPLC (C18 column) with a methanol gradient in the mobile phase. Palmitoylated avidin was eluted of the column with a linear gradient of methanol in 1× PBS.

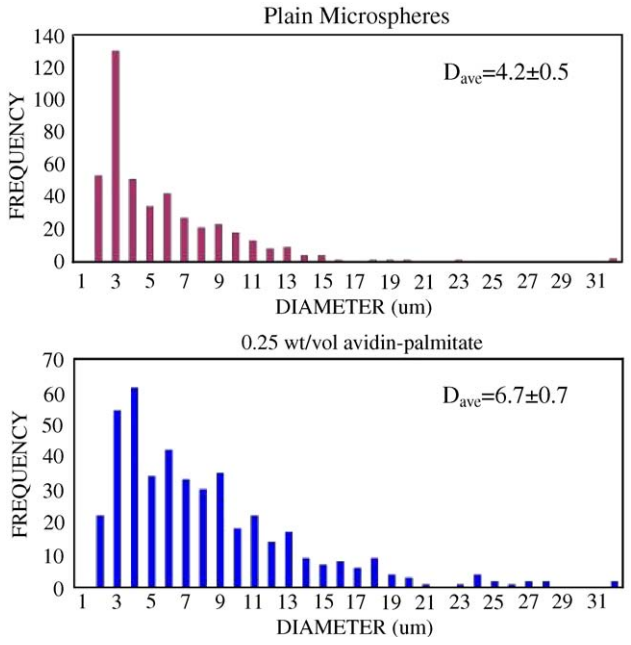

Fig. 3. Particle size distribution analysis of (A) plain and (B) modified PLGA particles. Particle size distributions were based on SEM images and performed with NIH SCION® image analysis software.

3.2. Effect of surface modification on particle morphology

Both plain and palmitoylated avidin particles displayed heterogeneous size distributions (Fig. 3). The average diameter of plain and surface modified particles ranged from 4–7 μm. Therefore, the presence of avidin–palmitate in the emulsion, at the concentrations used in this study, did not significantly impact the size distribution of the particles. Strikingly, microspheres prepared with conjugate in the emulsion showed a characteristic texture and surface roughness by SEM. This characteristic varied with the concentration of avidin–palmitic acid in the emulsion (Fig. 4). Our interpretation of these images is that palmitic acid in the form of vesicles or lamellae spread onto the surface of the PLGA during formation of the particles. Surface

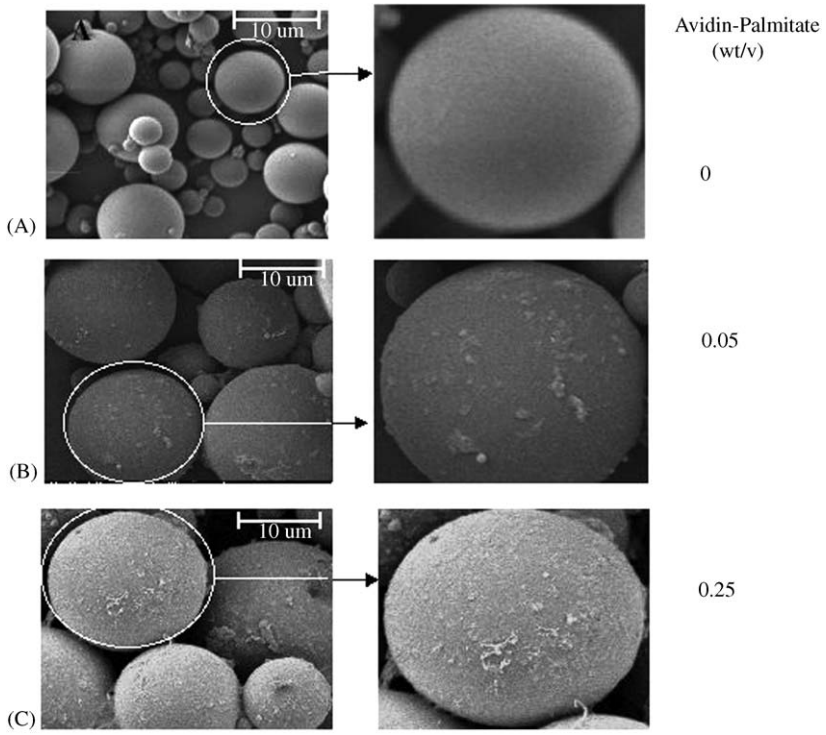

Fig. 4. Surface microtopography correlates with increasing concentrations of avidin–palmitate in the emulsion. Representative examples of three batches with (A) 0, (B) 0.05, and (C) 0.25 w/v avidin–palmitate in a 5% PVA emulsion.

spreading would be facilitated by mechanical dispersion or the presence of solvent (methylene chloride and trifluoroethanol during the solvent evaporation step) or the presence of low concentrations of detergent (0.15% deoxycholate) in the final emulsion and during formation of the particles.

The observed characteristic changes in the surface morphology of PLGA upon the addition of lipid or other amphiphilic co-stabilizers has been observed previously in similar systems. For example, when 1,2-dipalmitoylphospatidylcholine (DPPC) was used to stabilize PLGA emulsions, significant changes in the surface chemistry were observed by X-ray photoelectron spectroscopy [27]. Our study is consistent with this observation and supports the fact that the low surface energy of lipid (DPPC) or palmitic acid, in contrast with the high surface energy of PVA, dominates the surface chemistry of PLGA contributing to the observed morphological changes. Our study, however, highlights that these changes may also facilitate the presentation of surface functional groups for coupling to proteins.

3.3. Surface density and functionality of avidin–palmitic acid on PLGA particles

An increase in the absorbance of HABA at 500 nm correlates with the presence of avidin in solution. This relationship was used to verify and quantitate the density of surface avidin groups on PLGA particles (Table 1). We observed an apparent maximum in surface density with 0.25 mg of the conjugate per mg of PLGA in emulsion. The efficiency of avidin–palmitate incorporation into particles ranged between 14% and 24% with higher efficiencies of incorporation observed at lower concentrations of the avidin–palmitate in the emulsion. The presence of an apparent maximum may therefore reflect the natural tendency of the fatty acid to aggregate at higher concentrations; aggregation would limit fatty acid partitioning into the forming PLGA phase.

To ascertain the functionality and specificity of incorporated avidin to target biotinylated ligand, we compared the fluorescence of plain and modified particles treated with biotin-PE by flow cytometry. The mean channel fluorescence (MCF) of surface modified particles was approximately three orders of magnitude greater than control microspheres (Fig. 5E and F). This functional specificity was also qualitatively confirmed by fluorescence microscopy (Fig. 5C and D). Fluorescence images showed regions of brighter fluorescence indicating local high density binding regions on the particles where conjugate was localized.

To determine the degree of molecular crowding on the surface of treated particles, we titrated biotin-PE onto microspheres prepared with various concentrations of avidin–palmitic acid (Fig. 6). Surfaces modified with

Table 1
Avidin–palmitate and PVA in the emulsion are on a w/v basis (mg avidin–palmitate or PVA per mL of emulsion)

Avidin–palmitate (w/v)	PVA (w/v)	Particle yield (%)	% Encapsulation ((mg BSA/mg polymer)$_{final}$/(mg BSA/mg polymer)$_{initial}$)	Avidin density (μg/mg polymer)	Maximal biotin-PE binding (μg/mg polymer)
0	2.5	40±5	18.3±2	N/A	N/A
0.025	2.5	57±5	30.7±2	6±1	1
0.05	2.5	56±7	38.1±4	9.5±2	1.25
0.15	2.5	92±6	46.0±3	30±2	2.0
0.25	2.5	98±10	77.8±5	35±3	2.5

Note: Particle yield is the final dry weight of prepared particles normalized to the initial weight of polymer used in the preparation.

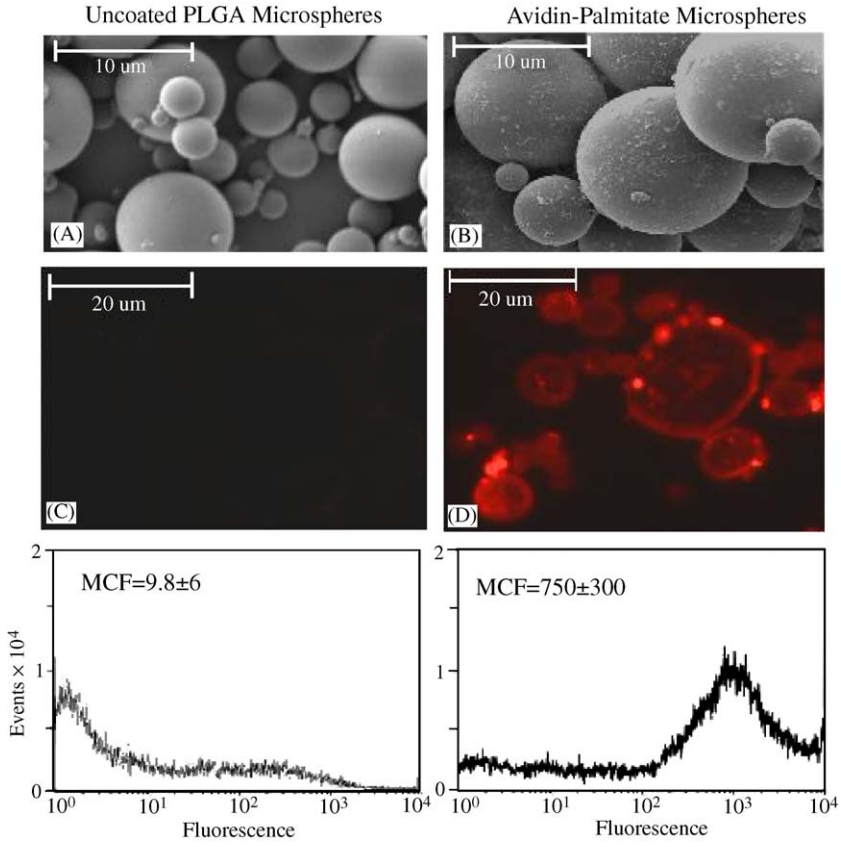

Fig. 5. Comparison of surface staining with biotin-PE for both plain (A, C, E) and modified particles (B, D, F). Fluorescence imaging of particles (C, D) and flow cytometry (ungated) of modified and plain particles (E, F). MCF is the mean channel fluorescence of FL2 (red channel).

increasing amounts of the conjugate bound more of the biotinylated fluorophore, as reflected by the higher MCF. Interestingly, we observed a self-quenching of PE with higher concentrations of biotin-PE added to the particles. Self-quenching, which results in a slight decrease in MCF with increasing concentration of fluorophore, occurs with the 'crowding' of fluorophores in localized regions in the proximity of 50–100 Å [28]; an indication of the molecular crowding and high density of biotin-PE at the surface of the particles.

3.4. Functional avidity of surface modified microspheres under dynamic conditions

In physiological settings injected particles rarely remain static but undergo a shearing due to flow and encounters with cells and tissue. Critical to the function of surface active particles in these settings is their ability to bind and remain with their target in the presence of flow [29]. To assess functional avidity under dynamic conditions, plain and surface modified microspheres

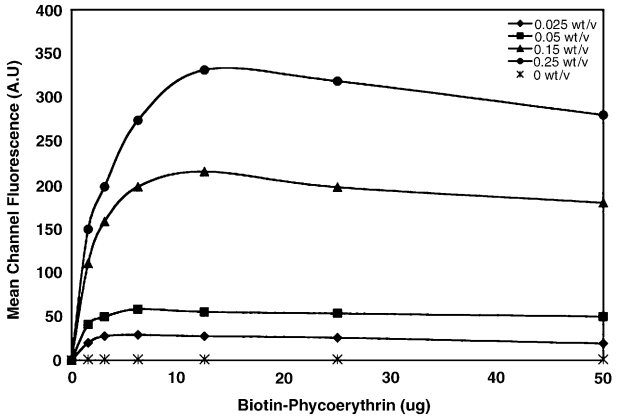

Fig. 6. Biotin-PE titration on particles modified with different concentrations of avidin–palmitate. MCF reflects the FL2 readout channel with no gate.

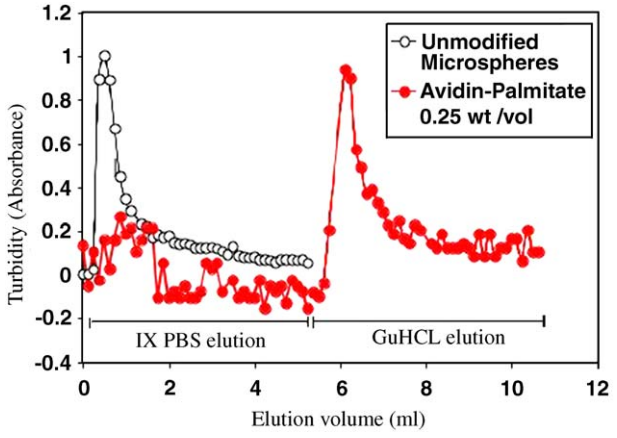

Fig. 7. Analysis of particle affinity to a biotinylated target under dynamic conditions.

were injected into a column packed with biotinylated agarose beads followed by elution with buffered saline. Plain microspheres eluted quickly from the column with PBS; modified microspheres, however, visibly adhered to the packing and did not elute even with high saline flow rates that physically disrupted the packing (Fig. 7). Elution of the modified particles required the addition of 6 M guandium hydrochloride (GuHCl); a strong protein denaturant known to disrupt the biotin–avidin linkage. A mass balance showed that while 1–3 wt% plain microspheres adhered nonspecifically to the column packing after buffer elution, 80–90% of surface modified particles remained associated with the column prior to GuHCl elution.

3.5. The effect of surface modification on the encapsulation efficiency of BSA

Because our strategy involved the simultaneous encapsulation and surface modification of particles at the emulsion stage, we examined how the addition of avidin–palmitic acid might affect the encapsulation efficiency of BSA. We measured the amount of encapsulated BSA in PLGA particles modified with various concentrations of avidin–palmitate in the emulsion (Table 1). Our results indicated that palmitoylation of microspheres enhanced BSA encapsulation in a concentration dependent manner. The encapsulation efficiency of particles modified with 0.25 (w/v) avidin–-palmitate was four-fold greater than unmodified particles. We also noted an increase in the yield of particles with higher concentrations of avidin–palmitate in the emulsion (Table 1). Others have found similar effects on the encapsulation efficiency and particle yields with the addition of pegylated Vitamin-E or the lipid DPPC to a PLGA emulsion [30,31]. A possible mechanism for this general effect might involve the increased hydrophobic stabilization due to the presence of co-stabilizing amphipathic molecules such as fatty acids or lipids, facilitating enhancements in PLGA particle formation and encapsulation efficiency [32].

3.6. Kinetics of BSA release and stability of the avidin–palmitate layer

Fig. 8A shows the release profiles of plain and surface modified microspheres over the duration of a controlled release experiment at 37 °C for 25 days. Both plain and modified particles had very similar BSA release kinetics, with an initial burst release during the first 24 h followed by a gradual release and a bulk erosion step (12 days) taking place nearly at the same time for surface modified and unmodified particles. PE fluorescence was almost negligible in the supernatant. Visually, centrifuged particles appeared bright red (picture not shown) during the entire time course of the experiment. A cumulative loss of less than 10% of the total PE fluorescence was detected over this period of time indicating stable surface functionality over the time course of the experiment.

Using SEM we examined the morphology of the both plain and modified particles after 21 days. Surprisingly, we found that while plain microspheres showed substantial morphological changes at the endpoint, modified particles were relatively spherical in shape (Fig. 8B and C). In addition to showing less drastic morphologic changes by SEM, a distinct capping layer was observed in most microspheres examined; a representative image is shown in Fig. 8C. Because of the distinct surface topology associated with surface modification (Fig. 4), coupled with persistent binding avidity over the time course of the experiment, we hypothesize that the additional surface layer observed in eroded modified microspheres might be due to surface rearrangement of the avidin–palmitic acid groups and reorganization during sphere degradation.

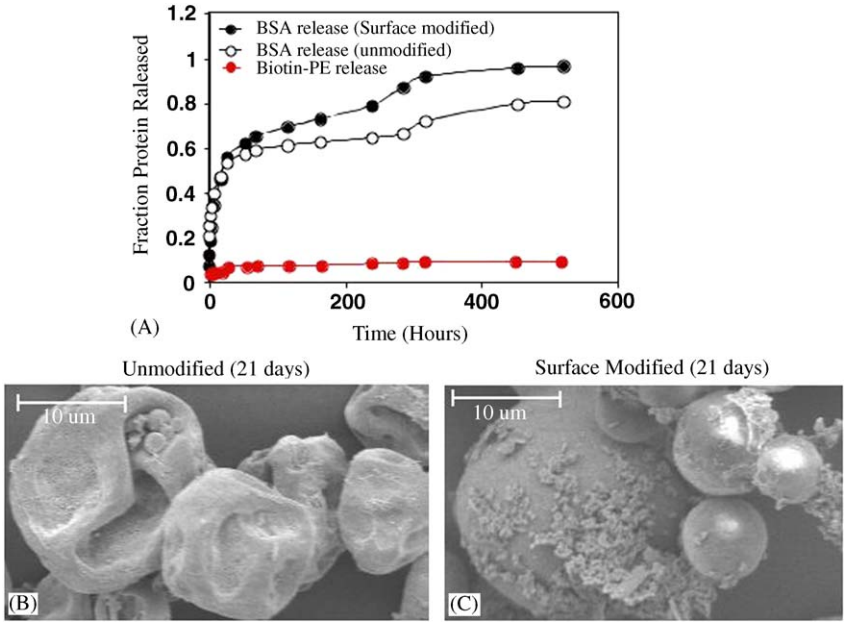

Fig. 8. Controlled release of BSA and surface biotin-PE from modified microspheres and comparison to release of BSA from plain microspheres. (A) Controlled release experiment in 1 × PBS and 37 °C for 25 days. (B, C) SEM images of degraded plain and modified microspheres (21 days).

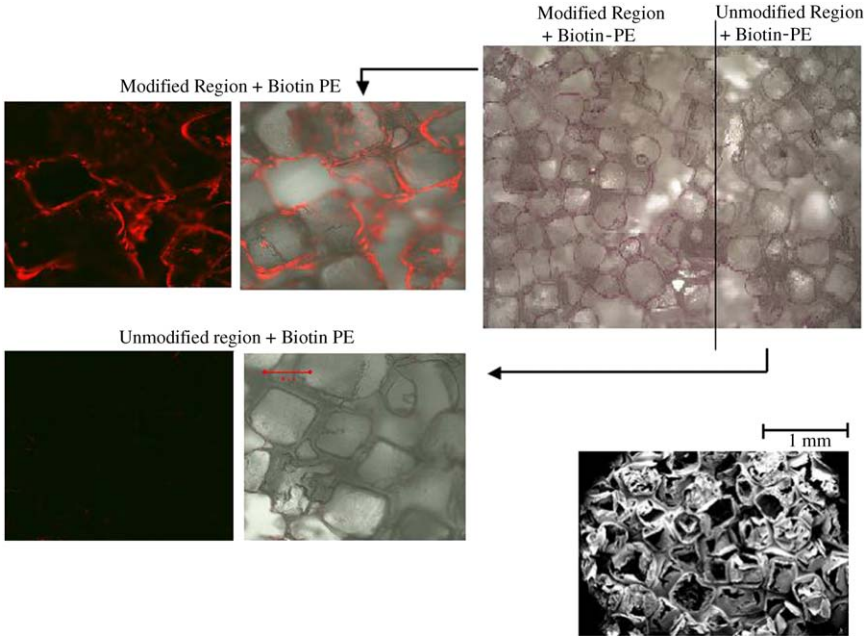

Fig. 9. Regional surface modification of PLGA scaffolds with avidin–palmitate followed by biotin-PE staining. Fluorescence and phase contrast comparison of modified and unmodified regions on the same scaffold.

The fact that surface activity (>90%) was persistent for several weeks, coupled with greatly reduced changes in morphology and a possible reorganization of targeting groups during controlled release, suggests a significant robustness and resiliency of the palmitoylated avidin surface.

3.7. Surface modification of PLGA scaffolds

Our approach to surface modification of PLGA particles was translated to an effective strategy for modifying synthetic matrices for tissue engineering applications. Scaffolds regionally treated with avidin–

palmitic acid displayed bright red fluorescence, when incubated with biotin-PE, indicative of surface functionality only in those treated regions (Fig. 9). Moreover, these scaffolds still maintained their red color after 3 weeks in PBS and 37 °C (data not shown). This surface modification approach is simple and facilitates three important properties for tissue engineering applications: (1) The ability of the matrix to be reliably and easily functionalized for selective cell attachment; (2) versatility in terms of attaching a variety of ligands; and (3) sustained presentation of ligands for long-term proliferation and differentiation of attached cells on the matrix.

4. Conclusion

In this study, we developed a strategy for surface modification of PLGA by introducing a functionally active amphipathic fatty acid, palmitic acid coupled to the ligand of interest (avidin), during the emulsion preparation of PLGA particles. This strategy was also translated to regional modification of PLGA scaffolds for tissue engineering applications. Because of the generality of this system and its flexibility, different ligands may be attached to palmitic acid facilitating surface modification of particles with a variety of ligands; we expect these particles to have improved in vivo targeting or clearance characteristics. For example, combinations of palmitoylated PEG and palmitoylated-avidin incorporated on the same particle may serve as ideal vehicles that combine high circulation lifetime with prolonged targeted drug delivery for in vivo applications. In addition, with respect to PLGA scaffolds, regional modification and ease of adjusting the density and type of ligand combine to produce a powerful strategy for adjusting scaffold affinity for different applications such as co-culture and growth of functional tissue composed of several cell types [33].

Acknowledgements

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