Biodegradable Microspheres with Enhanced Capacity for Covalently Bound Surface Ligands

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ABSTRACT: A poly(lactic-*co*-glycolic acid) (PLGA) microsphere formulation was developed which incorporates carboxylic acid groups into the microsphere surface. These functional groups are suitable for coupling to a variety of ligands and form linkages that remain stable in aqueous environments for extended periods of time. The ligand binding capacity of these microspheres compares favorably to that of similarly sized carboxylated polystyrene microspheres, which are commonly used as model particles for targeted delivery studies. Targeting microspheres to specific cell types by ligand−cell surface receptor interactions can increase the site specificity of microspheres administered intravenously or mucosally. The morphology and drug release kinetics of this PLGA microsphere formulation are not significantly different from those made with traditional reagents. This formulation allows for covalent surface modification of degradable microspheres with encapsulated payloads, which will enable studies that evaluate the ability of targeted microspheres to increase the effectiveness of payload delivery to sites of interest compared to nontargeted formulations.

Introduction

Degradable microspheres of poly(lactic-*co*-glycolic acid) (PLGA) have been evaluated for use in a wide variety of specialized drug delivery applications, including but not limited to vaccines,[1] tumor treatment,[2] drug delivery to the retina,[3] and control of inflammation.[4] Microspheres are of specific interest in these applications in part because they allow for localized delivery of the encapsulated agent directly to the desired site. Often this site specificity comes from direct physical administration of the microspheres at the site of interest (such as injection into the center or periphery of tumors).[2] Oral or intravenous administration of the microspheres is simpler, but with a loss of site specificity. Investigators are approaching the problem of maintaining site specificity for microspheres delivered by mucosal or intravenous routes by modifying the surface properties of the microspheres to increase their bioadhesiveness.[5] Of particular interest is modification of the microsphere surface with ligands that bind to cell surface receptors. Targeting microspheres to specific cell types has great potential for increasing the efficiency of drug delivery to the desired location, especially for microspheres delivered intravenously or at mucosal surfaces. For example, surface-bound ligands enable microspheres to mimic the rolling adhesion behavior of leukocytes[4] or increase adhesion to intestinal M cells for subsequent presentation to the immune system.[6]

Most degradable microsphere formulations use materials that are relatively inert, aside from the desired degradation reaction. This chemical inertness is desirable for biocompatibility but complicates attempts to covalently link ligands to the microsphere surface. Despite this difficulty, a number of groups have devised techniques for immobilizing ligands to the surface of PLGA microspheres, including biotin−streptavidin complexes,[7] glutaraldehyde cross-linking,[8] and carbodiimide coupling of primary amines on the ligand to microspheres made of PLGA with carboxylic acid end groups (PLGA-COOH).[9,10] Carboxylated polystyrene microspheres have been used as model particles for a number of targeting studies,[6,11−13] taking advantage of a high density of carboxylic acid groups at the microsphere surface for ligand conjugation by a well-established carbodiimide chemistry protocol. Using carbodiimide chemistry to conjugate ligands to the surface of PLGA microspheres, however, is not as straightforward. With carboxylic acid groups only at the end of polymer chains, PLGA-COOH microspheres have a limited number of available conjugation sites compared to the model carboxylated polystyrene microspheres. In addition, common emulsion methods for making PLGA microspheres use surfactants such as poly(vinyl alcohol) (PVA) to stabilize the emulsion. PVA has been shown to remain at the surface of the microspheres, even after repeated washing[14] (Figure 1A), creating a potential barrier for efficient ligand conjugation to the acid end groups of PLGA chains.

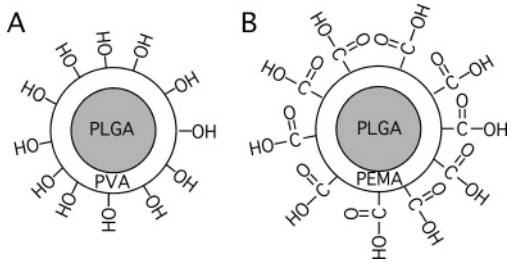

Figure 1. Schematic of microspheres made by emulsion methods using different stabilizers. (A) Stabilizing the emulsion with PVA results in microspheres with surface hydroxyl groups. (B) Using PEMA as the stabilizer produces microspheres with surface carboxylic acide groups.

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To increase the number of carboxylic acid groups at the surface of PLGA microspheres, we have replaced the traditional stabilizer PVA (with hydroxyl side chains) with a stabilizer bearing carboxylic acid side chains, poly(ethylene-*alt*-maleic acid) (PEMA) (Figure 1B). We compared properties of microspheres made with PEMA as the stabilizer (PLGA/PEMA) to those of PLGA/PVA microspheres. In addition, we evaluated the capability of the microspheres to bind ligands by a standard carbodiimide protocol.

Experimental Section

Materials. PLGA with lauryl ester end groups, a 50:50 lactide:glycolide ratio, and inherent viscosity of 0.59 dL/g in hexafluoro-2-propanol was from Birmingham Polymers (Birmingham, AL), as was 50:50 PLGA with carboxylic acid end groups (PLGA-COOH) and inherent viscosity of 0.32 dL/g in chloroform. PEMA and carboxylated polystyrene microspheres (diameter = 0.792 ± 0.023 μm) were from Polysciences (Warrington, PA). 5-(Aminoacetamido)fluorescein and R-phycoerythrin labeled goat-anti-mouse IgG were from Molecular Probes (Eugene, OR). Polystyrene fluorescence standard beads (Quantum 26) were from Flow Cytometry Standards Corp. (San Juan, PR). Dichloromethane was from Fisher Scientific (Fairlawn, NJ). Clone HSA11 mouse IgG specific for human serum albumin (HSA) was from Sigma (St. Louis, MO), and mouse IgG specific for human IgA was from Zymed (South San Francisco, CA). PVA, fluorescein isothiocyanate conjugated to bovine serum albumin (FITC-BSA), ovalbumin (OVA), HSA, human IgA (hIgA), 1-(3-(dimethylamino)propyl)carbodiimide (EDC), and ethanolamine were each from Sigma.

Microsphere Preparation. Microspheres were prepared using a double-emulsion technique. Four hundred milligrams of PLGA or PLGA-COOH was dissolved in 2 mL of dichloromethane in a glass tube. One hundred microliters of a 75 mg/mL solution of FITC-BSA in water was added to the polymer solution while gently vortexing the tube. For microspheres with no encapsulated protein, 100 μL of Milli-Q water was added to the polymer solution. The polymer solution was then sonicated for 15 s at 40% amplitude with a TMX 400 sonic disruptor (Tekmar, Cincinnati, OH) to create the primary emulsion. Four milliliters of an aqueous 1% w/v solution of the desired stabilizer (either PVA or PEMA) was added to the tube, and the sonication step was repeated. Immediately after the second sonication, the emulsion was poured into 100 mL of 0.3% w/v aqueous solution of the same stabilizer used for the second emulsion, under rapid stirring with a magnetic stirrer. The resulting microspheres were stirred in this solution for 3 h in order to evaporate away the dichloromethane. The microspheres were then washed three times with Milli-Q water, resuspended in 4 mL of Milli-Q water, and lyophilized to dryness.

Conjugation of Ligands to Microsphere Surface. The technique used to conjugate ligands to the microsphere surfaces was a slightly modified version of a manufacturer's protocol for protein conjugation to carboxylated polystyrene microspheres (Polysciences Technical Data Sheet 238C). Briefly, microspheres were washed twice with 0.1 M sodium bicarbonate buffer, pH adjusted to 9.0. Microspheres were then washed three times with 0.02 M sodium phosphate buffer, pH = 4.8. Microspheres were then resuspended to 20 mg/mL in phosphate buffer. Microspheres were diluted down to 10 mg/mL with 2% w/v EDC in phosphate buffer. This suspension was incubated for 3 h at 25 °C on an end-to-end shaker to activate carboxylic acid groups. After incubation, microspheres were washed three times in phosphate buffer and resuspended to 10 mg/mL in borate buffer (0.2 M boric acid, pH adjusted to 8.5) with ligand at a concentration of either 80 μg/mL 5-(aminoacetamido)fluorescein or 320 μg/mL of the desired protein ligand (HSA, hIgA, or OVA). Microspheres were incubated with ligand overnight at 25 °C on an end-to-end shaker. Microspheres were then centrifuged at 10000*g* for 5 min, and supernatant was removed. Microspheres were resuspended to 10 mg/mL in borate buffer, and 4 μL of 0.25 M ethanolamine in borate buffer was added for each milligram of microspheres. Microspheres were incubated for 30 min at 25 °C to quench any unreacted sites. Microspheres were then washed twice with Milli-Q water, resuspended in 4 mL of Milli-Q water, and lyophilized to dryness.

Detection of Conjugated Ligand. A model ligand, 5-(aminoacetamido)fluorescein, was coupled to the surface of the microspheres and was detected by flow cytometry (FACScalibur, Becton Dickinson, San Jose, CA). For microspheres with protein conjugated to the surface, aliquots of microspheres were suspended at 5 mg/mL in a 1:500 dilution in PBS of mouse antibody specific for the conjugated protein. Microspheres were incubated for 1 h at room temperature. As a control for nonspecific antibody adsorption, separate aliquots of the microspheres were incubated with primary antibody not specific for the conjugated protein. Microspheres were washed three times with PBS and resuspended to 5 mg/mL in a 1:50 dilution in PBS of R-phycoerythrin labeled secondary antibody specific to the primary mouse antibody. Microspheres were incubated for 1 h at room temperature and then washed three times with PBS before analysis by flow cytometry.

Encapsulated Protein Release Assay. Microspheres with encapsulated FITC-BSA were suspended in PBS at a concentration of 2 mg/mL and incubated at 37 °C on an orbital shaker at 125 rpm. To evaluate the effect of ligand conjugation on encapsulated protein release, microspheres with OVA conjugated to the surface were included in this study. At selected time points the microspheres were centrifuged at 10000*g* for 5 min. Supernatant aliquots were removed from each sample and replaced with an equal volume of fresh PBS. Microspheres were resuspended and returned to the shaker. Supernatant samples were stored at −80 °C until analysis. Sample fluorescence was measured on a Perkin-Elmer LS 55 luminescence spectrometer (Shelton, CT), with excitation and emission wavelengths set at 488 and 520 nm, respectively.

Ligand Release Assay. PLGA/PEMA microspheres conjugated to 5-(aminoacetamido)fluorescein were suspended in PBS at 5 mg/mL and incubated at 37 °C for various lengths of time. After incubation, microspheres were washed twice with fresh PBS and resuspended. Retention of fluorescent ligand was measured by flow cytometry of microspheres. Total surface conjugated ligand was estimated by comparison of the fluorescence intensity of microsphere−ligand conjugates to fluorescent standard beads.

Microsphere Morphology Characterization. Microspheres were fixed to aluminum sample stubs with double-sided carbon tape and sputter-coated with gold for viewing by scanning electron microscopy. Micrographs were analyzed with Scion Image software (Scion Corp., Frederick, MD) to determine size distributions of different microsphere samples.

Results

Switching from PVA to PEMA as the stabilizer for PLGA microsphere production did not noticeably change microsphere morphology. Both PLGA/PVA and PLGA/PEMA microsphere types exhibited smooth, unbroken surfaces (Figure 2) and nearly identical size distributions (Figure 3), with a mean diameter of 686 nm for PLGA/PVA and 682 nm for PLGA/PEMA. The PLGA-COOH/PVA microspheres also had smooth, unbroken surfaces but were noticeably smaller, with a mean diameter of only 361 nm. During PLGA-COOH/PVA microsphere production, undissolved polymer was found in the tube after the sonication steps. Microsphere yield calculations indicated that the PLGA-COOH had dissolved to a concentration of roughly 150 mg/mL dichloromethane compared to 200 mg/mL used for making both PLGA/PVA and PLGA/PEMA microspheres. With this reduced polymer concentration comes a reduction in

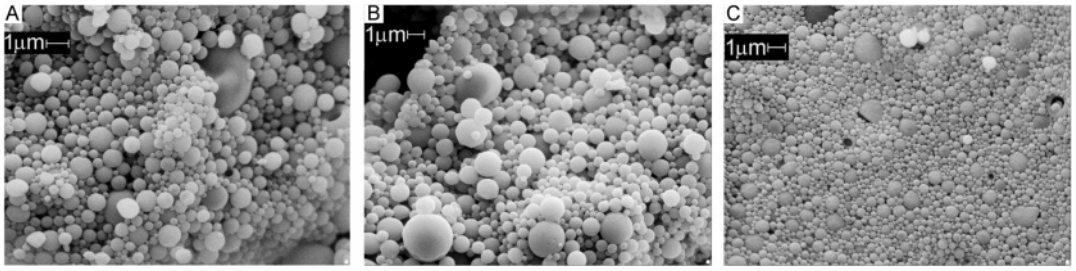

Figure 2. Scanning electron micrographs of (A) PLGA/PVA, (B) PLGA/PEMA, and (C) PLGA-COOH/PVA microspheres.

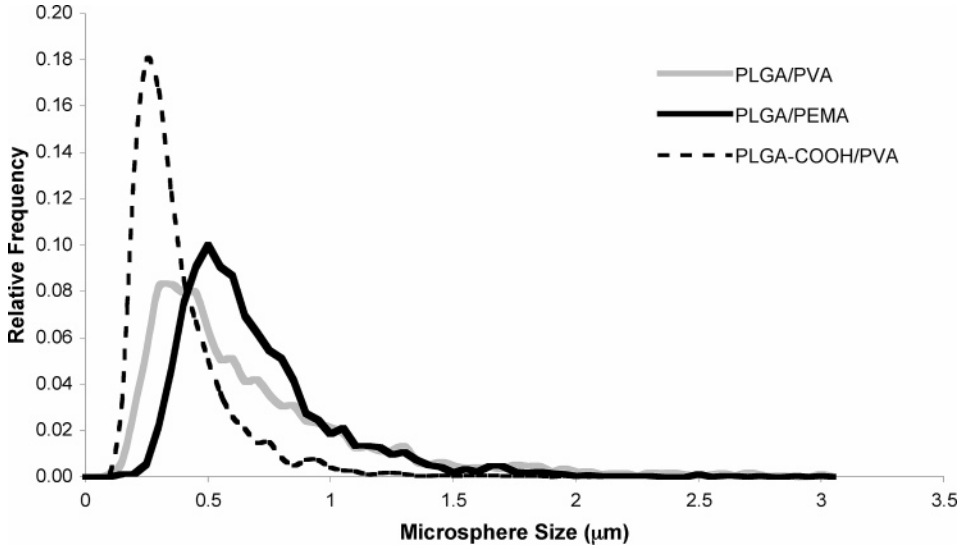

Figure 3. Microsphere size distributions (n = 3246 for PLGA/PVA, 2605 for PLGA/PEMA, 3246 for PLGA-COOH/PVA).

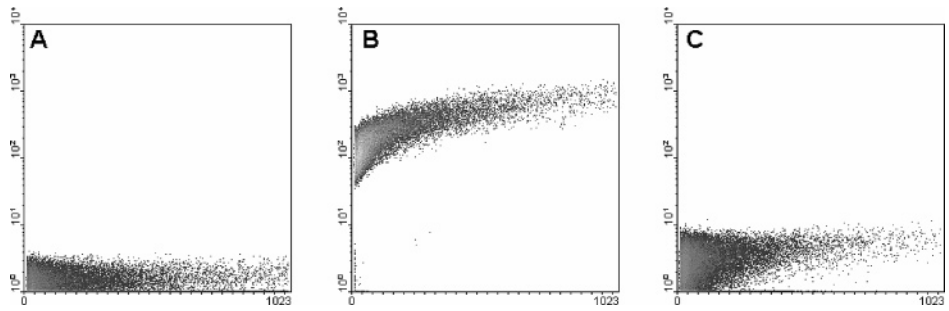

Figure 4. Dot plots of fluorescence intensity vs forward scatter for PLGA/PEMA microspheres: (A) microspheres before conjugation to 5-(aminoacetamido)fluorescein; (B) microspheres after conjugation to ligand; (C) microspheres run through conjugation protocol without EDC (ligand adsorption).

solution viscosity, resulting in more effective droplet dispersion during the sonication steps, and therefore smaller microspheres.[15]

The fluorescein analogue 5-(aminoacetamido)fluorescein was used as a model ligand for evaluation of conjugation to the surface of PLGA/PEMA microspheres; no encapsulated protein was present in these studies. Ligand attachment was verified by flow cytometry, which showed substantial binding (Figure 4B). Ligand binding was due to conjugation to the microsphere surface, not adsorption, as only trace amounts of ligand were detected on microspheres when cross-linking reagents were excluded from the conjugation protocol (Figure 4C). Conjugations of the fluorescent ligand were performed for microspheres of different formulations, which were then compared for ligand binding levels. PLGA/PEMA microspheres were similar in ligand binding capacity to carboxylated polystyrene microspheres of similar size (Figure 5). The presence of encapsulated protein did not adversely affect the ability to bind ligands, as the fluorescent ligand could also be conjugated to PLGA/PEMA microspheres containing encapsulated OVA (not shown). Microspheres made with PVA as the stabilizer showed limited capacity for ligand binding, whether the core polymer was standard PLGA or PLGA-COOH. The carbodiimide coupling protocol used in this study did not result in any visible change in microsphere morphology, as indicated by scanning electron microscopy (Figure 6).

Fluorescence intensity of PLGA/PEMA microspheres with bound fluorescent ligand was converted to ligand density by comparison to a standard curve relating mean fluorescence to molecules of equivalent soluble fluorochrome (MESF) of polystyrene calibration beads. This comparison indicated that 26280 ± 276 (mean ± SD of three measurements) ligand molecules were conjugated per microsphere. During incubation in PBS at 37 °C the microspheres lost approximately 10% of the conjugated ligand within 3 days, at which point release ceased (Figure 7). Microsphere fluorescence

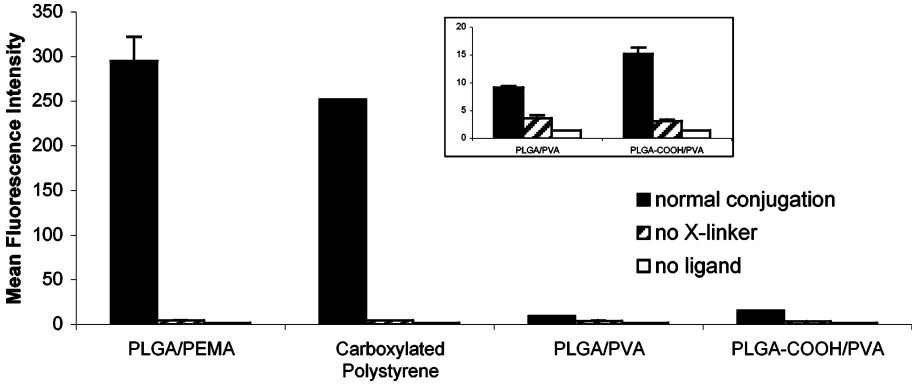

Figure 5. Comparison of capacity for coupling of 5-(aminoacetamido)fluorescein to the surface of various microsphere formulations. Data are the mean fluorescence measurement for microspheres as analyzed by flow cytometry. Inset: fluorescence intensity of PLGA/PVA and PLGA-COOH/PVA microspheres.

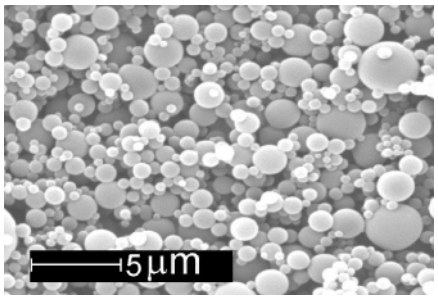

Figure 6. Scanning electron micrograph of PLGA/PEMA microspheres after conjugation to 5-(aminoacetamido)fluorescein.

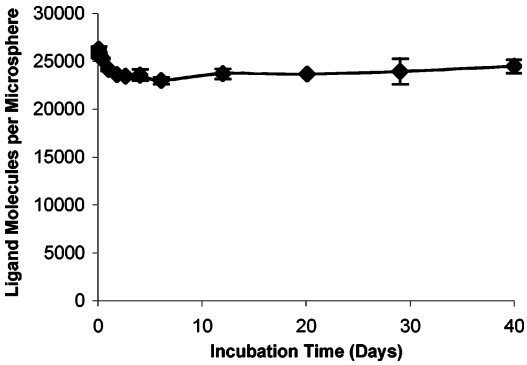

Figure 7. Retention of conjugated 5-(aminoacetamido)fluorescein at the surface of PLGA/PEMA microspheres.

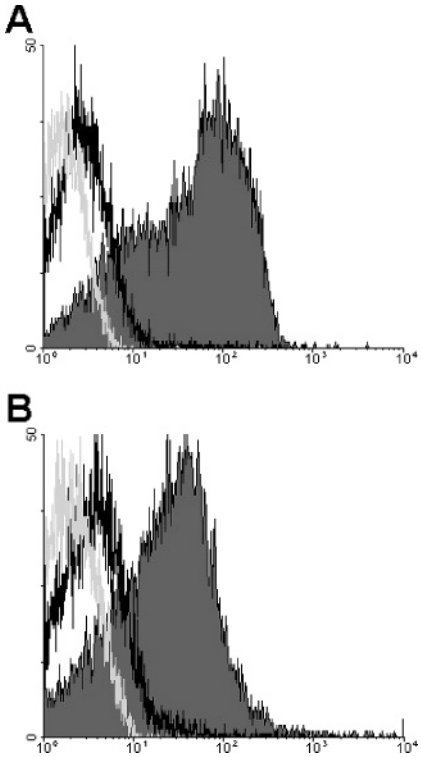

Figure 8. Detection of (A) HSA and (B) hIgA conjugated to PLGA/PEMA microspheres by immunocytometry. Histograms are of fluorescence intensity. Gray line: microspheres before incubation with antibodies. Black line: microspheres incubated with irrelevant 1° antibody (control for nonspecific antibody adsorption). Solid fill: microspheres incubated with 1° antibody specific for conjugated protein.

remained constant from this point to the remainder of the incubation period of 40 days. Supernatants of the incubation buffer were analyzed by fluorometry, with results that were consistent with the release profile of Figure 7 (data not shown).

The PLGA/PEMA microspheres can also be functionalized with macromolecules, as demonstrated by conjugation of the proteins HSA (MW ~ 66000) and hIgA (MW ~ 160000) to the microsphere surface. The shift to the right of fluorescence histograms for microspheres incubated with antibodies specific for the surface-conjugated proteins (Figure 8, solid fill) indicates that both HSA and hIgA can be conjugated to the microspheres. Antibody binding to the microspheres was dependent on the presence of the conjugated protein, as attempts to label the microspheres with antibodies not specific to the conjugated protein (black lines) produced only slight shifts in microsphere fluorescence compared to unlabeled control microspheres (gray lines).

Changing from PVA to PEMA as the stabilizer during microsphere production did not significantly change the encapsulation efficiency of FITC-BSA (21.4% for PLGA/PVA vs 24.9% for PLGA/PEMA). Microspheres produced with each surfactant also displayed similar release profiles for encapsulated protein (Figure 9). Both sets of microspheres had an initial burst release of encapsulated protein lasting for approximately 2 days, followed by an extended period of slow protein release. PLGA/PEMA microspheres conjugated to protein ligands had lower overall levels of FITC-BSA release due to loss of encapsulated protein during the ligand conjugation process. In effect, the initial burst release of encapsulated protein for these microspheres took place during ligand conjugation, resulting in microspheres with lower, but still significant, levels of encapsulated protein.

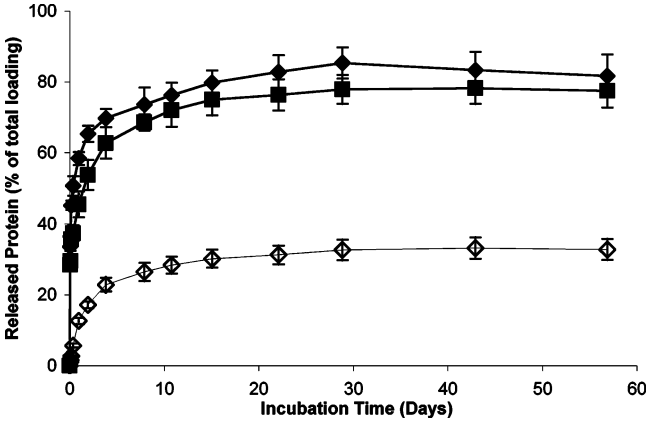

Figure 9. Cumulative release of encapsulated FITC-BSA from PLGA microspheres during incubation in PBS at 37 °C. Data for PLGA/PEMA microspheres after conjugation protocol (open symbols) represented as percent of original (preconjugation) protein loading. PLGA/PVA microspheres (filled squares); PLGA/PEMA microspheres, before conjugation (filled diamonds); PLGA/PEMA microspheres, after conjugation to OVA (open diamonds).

Discussion

Replacing PVA with PEMA as the stabilizer for PLGA microsphere production results in microspheres with carboxylic acid groups at the microsphere surface, which can be used to conjugate primary amine-bearing ligands to the microsphere by carbodiimide chemistry. Among the ligands used in these studies was hIgA, which has previously been shown to increase particle uptake when adsorbed to the surface of polystyrene microspheres,[16] suggesting that the PLGA/PEMA microspheres might be an effective vehicle for targeted delivery of orally administered vaccines. Changing from PVA to PEMA does not significantly change microsphere size distribution, protein encapsulation efficiency, or release kinetics for the encapsulated protein. That these microsphere properties are unaltered by the switch in stabilizer is not surprising, given the similarity in structure of PVA and PEMA. Both surfactants have simple hydrophobic backbones of repeating $-CH_2-$ or $-CH-$ groups, with equal densities of short hydrophilic side chains. PEMA was used in this study to provide the desired carboxylic acid groups at the microsphere surface, and the positive results obtained here suggest it is likely that other stabilizers could be used, such as poly(acrylic acid). The surface could likely be functionalized with other hydrophilic groups by selection of a stabilizer with side chains of the desired functional group, such as poly(allylamine) to introduce primary amines. It is even conceivable that certain ligands, such as short hydrophilic peptides, could be conjugated to a surfactant in advance of microsphere production, resulting in microspheres with the desired ligand at the surface without the need for an additional conjugation step after microspheres are already formed.

Inclusion of the desired functional group as the side chain of the emulsion stabilizer has two important features that lead to a high density of functional groups at the microsphere surface. First, the functional side chains are repeated along the entire length of the stabilizer molecule, providing a high density of functional groups as compared to PLGA molecules that are functionalized only at the ends of the polymer chain. Second, the stabilizer molecules partition at the interface of the organic and aqueous phases of the emulsion that is formed during microsphere production. The surfaces of the microspheres form at this interface during the solvent evaporation step, meaning that the functional groups are localized at the microsphere surface and not buried within the microsphere core. The importance of these features is emphasized by the relative capacity for ligand binding of different microsphere formulations shown in Figure 5. The capacity for ligand binding of PLGA/PEMA microspheres is substantially improved over those made of PLGA with acid end groups and PVA as stabilizer. Ligand binding to PLGA/PEMA microspheres compared favorably to that of commercially available carboxylated polystyrene microspheres. It should be noted that for the PLGA-COOH/PVA microspheres used in this study the PVA may have interfered with ligand conjugation; microspheres of PLGA-COOH made by methods not requiring stabilizers (such as spray drying) may have higher capacity for ligand binding than demonstrated here.[9]

For ligand−receptor mediated targeting of degradable microspheres, it is important for the linkage between ligand and microsphere to remain stable long enough for microspheres to reach the site being targeted. This requirement would seem to make linkage of the ligand to stabilizer side chains instead of directly to molecules in the microsphere bulk (i.e., PLGA) an unfavorable strategy. The results shown in Figure 7, however, show that the linkage between ligand and microsphere is quite durable. The release profile of conjugated ligand from the microsphere surface is similar to that for encapsulated protein. Both release profiles show an initial burst release in the first few days of microsphere incubation in buffer, followed by a rapid reduction in the release rate. Approximately 90% of the conjugated ligand remains fixed at the microsphere surface even after 40 days of continuous incubation in buffered saline, a result also reported for release of ligands conjugated to acid end groups of PLGA in spray-dried microspheres.[9] The durability of the ligand binding can be attributed in part to the relative stability of the amide bond formed during conjugation, but also to the bulk erosion behavior of PLGA. Stabilizer molecules have been shown to remain at the microsphere surface despite repeated washing.[14] These investigators suggest that the hydrophobic backbone of the surfactant may become physically entrapped by PLGA chains at the microsphere surface. Our results are consistent with this view. During the emulsification process, surfactant molecules partition at the interface between organic droplets and the aqueous continuous phase, with the hydrophobic backbone of surfactant molecules within the organic droplets. When the microspheres harden, the microsphere surface is formed at the interface between each organic droplet and the aqueous phase, leaving surfactant molecules entrapped at the surfaces of the microspheres. Since the microspheres remain intact over the course of the bulk erosion process, it would therefore be reasonable to expect that significant amounts of the surfactant (and ligands conjugated to the surfactant) will remain bound to the microsphere surface for extended periods of time. Interestingly, comparison of the surface ligand and encapsulated protein release curves indicates that the conjugated ligand remains at the microsphere surface even after release of encapsulated protein has ceased.

Microspheres released a significant amount of encapsulated protein during ligand conjugation, which occurred in aqueous conditions. The conjugation protocol

exposed the microspheres to a buffered aqueous solution for approximately 20 h, during which time the microspheres released encapsulated protein. The conjugation process did not change the kinetics of payload release for the microspheres, as the difference in release profiles in Figure 9 for microspheres with conjugated ligands vs microspheres not exposed to the conjugation protocol is simply a shift of the release curve forward by 20 h. The net effect of this premature loss of microsphere payload will ultimately depend on the increased efficiency of delivery that can be obtained through the targeting ability generated by ligand conjugation to the microsphere surface. The conjugation protocol used in these studies was originally designed for nondegradable polystyrene microspheres, for which exposure to buffers with acidic or basic pH values and the duration of incubation steps are not important concerns. Optimization of the ligand conjugation protocol for degradable microspheres with encapsulated payloads such as those used in these studies will require the balancing of conditions that favor increased ligand binding with those that limit payload release.

Conclusion

By replacing PVA with a stabilizer that has carboxylic acid side chains, we have generated a PLGA microsphere formulation with the capability for covalent ligand conjugation to the microsphere surface by carbodiimide chemistry. Furthermore, the ligand density is similar to that obtained with the carboxylated polystyrene microspheres that have previously been used as model particles in uptake and targeting studies. The microsphere surface can be functionalized with small molecules as well as biological macromolecules such as antibodies, with linkages that remain stable for several weeks in solution. Aside from the ability to conjugate molecules to the microsphere surface, replacing PVA with PEMA did not significantly alter microsphere properties. The size distribution and surface morphology of the microspheres made with each stabilizer type are similar, as are the release profiles for encapsulated molecules. The new microsphere formulation will facilitate uptake and targeting studies utilizing biodegradable microspheres with encapsulated payloads, instead of nondegradable polystyrene model particles. These studies will be able to investigate not just whether microspheres can be targeted to specific cell types, but also whether this targeting can be used to improve the effectiveness of therapeutic delivery in clinically relevant situations.

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