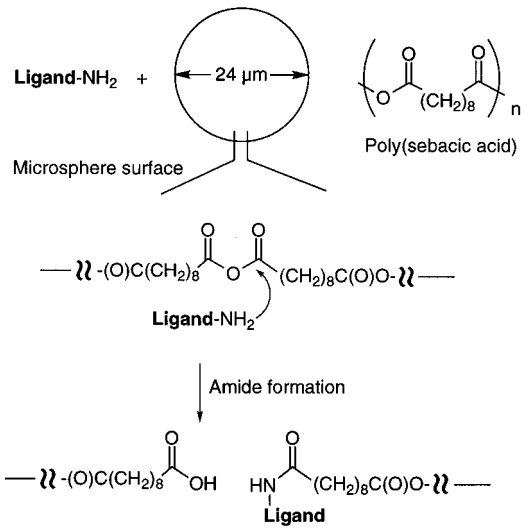
Surface Modification of Polyanhydride Microspheres

JINMING GAO[†,‡], LAURA NIKLASON[†,‡], XIAO-MEI ZHAO[§], AND ROBERT LANGER*[,†]

Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, Department of Anesthesia, Massachusetts General Hospital, Boston, Massachusetts 02114, and Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138

Received July 25, 1997.
Final revised manuscript received October 31, 1997.
Accepted for publication November 6, 1997.

Polyanhydrides are emerging as a new class of biodegradable polymers for drug delivery.[1−3] The degradation of polyanhydride delivery systems is largely through surface erosion, potentially leading to zero-order release of encapsulated drugs.[4,5] Recently, the Food and Drug Administration has approved the use of the polyanhydride poly[sebacic acid-*co*-1,3-bis(*p*-carboxyphenoxy)propane] to deliver drugs for treatment of brain cancer.[6] This is one of the few examples where an implantable synthetic degradable polymer has been approved for human use. The use of polyanhydride polymers in oral delivery of insulin and genes further broadens the scope of their applications in drug delivery.[7] Despite numerous studies on polyanhydrides, the compositions thus far developed do not have the capability to target specific organs or cell types. To achieve this goal, it would be important to have an approach that could enable the chemical functionalization of microspheres to allow the attachment of targeting ligands (e.g. antibodies, etc). The recognition of ligands on the surface of delivery systems by organ-specific or cell-specific receptors may result in organ-targeted drug delivery[8,9] or in improved drug uptake through ligand-induced endocytosis.[10,11] Here we describe a one-step procedure to covalently modify the surface of poly(sebacic acid) (pSA)[12] microspheres with ligands containing amino groups. Amino groups react with the anhydride groups on the surface of microspheres, which results in covalent attachment through an amide bond (Scheme 1).



Scheme 1—Surface modification of pSA microspheres with ligands containing amino groups via amide formation.

Experimental Section—*Materials*—pSA was synthesized from sebacic acid following a published procedure.[12] L-Argininamide dihydrochloride and guanidine hydrochloride were purchased from Sigma. *N*-(5-Aminopentyl)-biotinamide, 6-((biotinoyl)amino)hexanoic acid, and fluorescein-conjugated avidin were purchased from Molecular Probes (Eugene, Oregon). The pSA (M_w = 34 kDa) microspheres (mean diameter = 24 ± 1 μm) were formed by solvent evaporation.[13]

Surface Modification of Microspheres by Argininamide—The pSA microspheres (30 mg for each concentration of argininamide) were suspended in solutions containing different concentrations of argininamide (0, 0.40, 1.2, 4.0, 12, and 40 mM, 2 mL each) in borate buffer (0.20 M, pH 9.0) at room temperature. After 15 min, the surface modification was stopped by acidifying the solution to ~pH 6 by addition of acetate buffer (0.20 M, pH 5.0, 5 mL).[14] The microspheres were collected after centrifugation (10 000 rpm for 10 min) at 4 °C, washed with 2 M aqueous NaCl, distilled water (twice), and ethanol, and dried in vacuo. The surface density of argininamide was analyzed by X-ray photoelectron spectroscopy (XPS), and its bulk concentration was analyzed by ^{1}H NMR (400 MHz) of microspheres dissolved in $CDCl_3$. The size of the microspheres was measured by a Coulter Multisizer (Coulter Electronic Limited, Luton, U.K.).

Guanidine Hydrochloride Experiment—The pSA microspheres (20 mg) were suspended in 30 mM guanidine hydrochloride solution in borate buffer (0.20 M, pH 9.0, 2 mL) at rt. After 15 min, acetate buffer (0.20 M, pH 5.0, 5 mL) was added. The microspheres were collected, washed with 2 M NaCl, distilled water (twice), and ethanol, and dried in vacuo.

Self-Assembled Monolayer (SAM) Experiments—The gold substrates were prepared by e-beam evaporation of 5 nm of titanium and 200 nm of gold to a single-crystal silicon wafer in a deposition chamber. The gold-coated wafers were cut into ca. 1 cm × 2 cm pieces and self-assembled monolayers (SAMs) were formed by immersing the gold substrates in an ethanolic solution of 16-mercaptohexadecanoic acid (2 mM, 10 mL) at room-temperature overnight.[15] One sample was used as an unmodified control. Two other samples were soaked in 10 mM argininamide solution in borate buffer (0.20 M, pH 9.0) at rt. After 15 min, one sample was taken out and blown dry by Dust-Off (Falcon Safety Products, Inc., NJ). The other sample was washed by 2 M aqueous NaCl and distilled water (twice) and finally blown dry. XPS analyses showed nitrogen signals in the unwashed SAM sample but not in the washed SAM sample nor the control sample (data not shown).

The carboxylic acid groups on the SAMs were further transformed to interchain anhydrides using trifluoroacetic anhydride following a literature procedure.[15] The resulting SAM sample was soaked in 10 mM argininamide solution in borate buffer (0.20 M, pH 9.0) at rt for 15 min. The sample was washed by 2 M aqueous NaCl and distilled water (twice) and blown dry. XPS analyses showed the appearance of nitrogen signals.

Avidin Binding to the Surface of Biotin-Attached pSA Microspheres—The pSA microspheres (10 mg) were suspended in a solution of *N*-(5-aminopentyl)biotinamide (10.0

* To whom correspondence should be addressed. Telephone: (617) 253-3107. Fax: (617) 258-8827. Email: rlanger@mit.edu.
† Massachusetts Institute of Technology.
‡ Massachusetts General Hospital.
§ Harvard University.

S0022-3549(97)00284-0 CCC: $15.00
Published on Web 01/07/1998

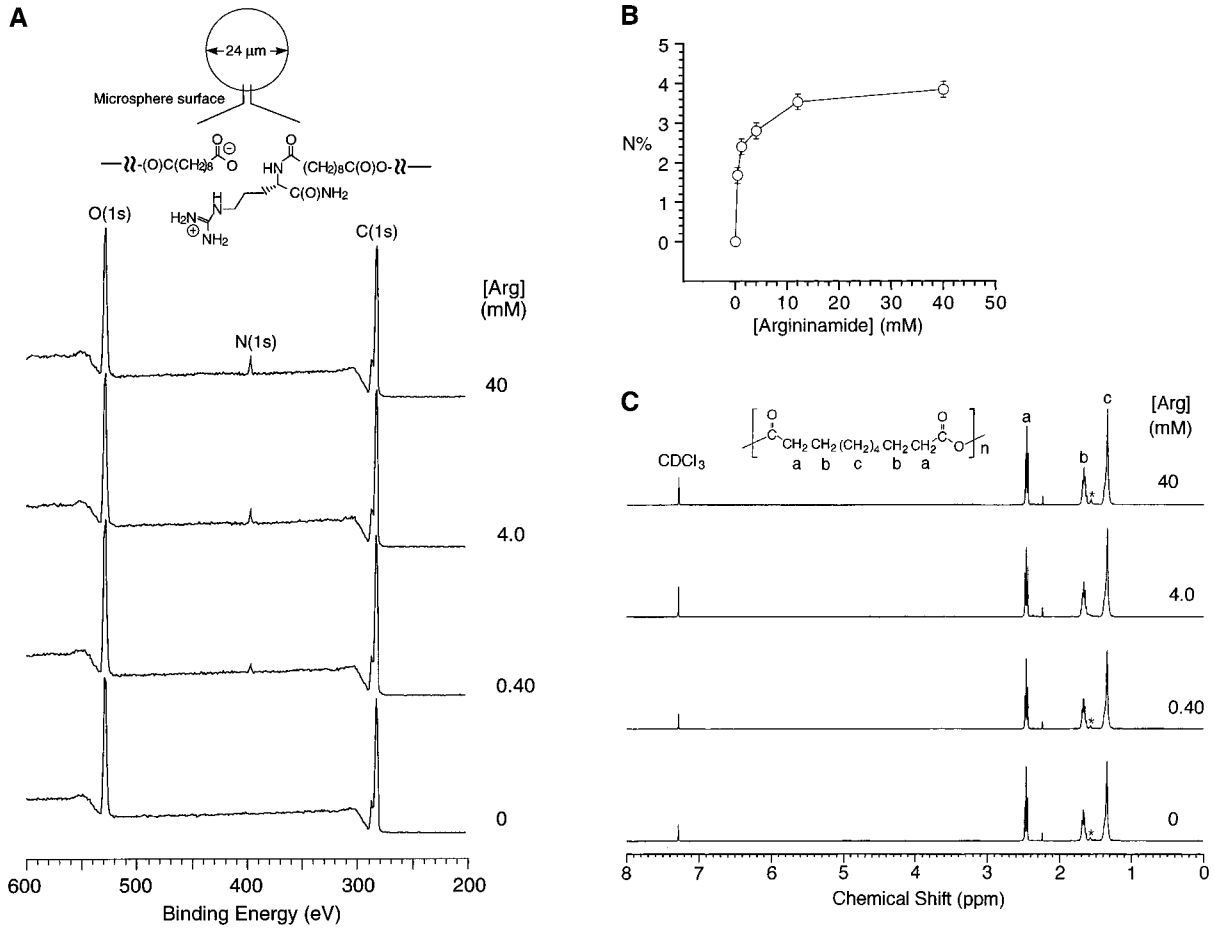


Figure 1—(A) XPS analyses of the surface of pSA microspheres covalently modified by argininamide. XPS detects only the photoelectrons from 1s orbitals of nitrogen, carbon, and oxygen elements between 200 and 600 eV of binding energy. The scale of binding energy at the bottom of 1A applies to all the spectra. (B) Dependence of the value of N% at the microsphere surface on the concentration of argininamide in the reaction solution. The error bars were obtained from duplicate measurements. C) ^{1}H NMR (400 MHz) of surface modified microspheres in $CDCl_3$. The peak due to residual amount of water is indicated by an asterisk (chemical shift ~1.6 ppm).

mM) in borate buffer (0.20 M, pH 9.0, 2 mL) at rt. After 15 min, acetate buffer (0.20 M, pH 5.0, 5 mL) was added. The microspheres were collected, washed with 2 M NaCl, distilled water (twice), and ethanol, and dried in vacuo. Then these microspheres were suspended and mixed in a solution of fluorescein-conjugated avidin (0.5 mg/mL, 2 mL) in phosphate-buffered saline (PBS, pH 7.4) at rt. After 30 min, the microspheres were collected, washed with PBS and distilled water and dried in vacuo. In the control experiment, the identical procedure was applied to 6-((biotinoyl)amino)hexanoic acid (replacing *N*-(5-aminopentyl)-biotinamide). The microspheres were analyzed by fluorescence microscopy (λ_{ex} = 480 nm, λ_{em} = 520 nm).

Results and Discussion—Attachment of argininamide introduced a new element, nitrogen, into the otherwise carbon, oxygen, and hydrogen environment of the pSA surface, thereby providing a basis for determining the extent of surface modification by N analysis. We chose argininamide as the model ligand because it has a free amino group for covalent attachment and its high nitrogen content (5 nitrogen atoms out of 12 total non-hydrogen atoms) enhances its detection on the surface by XPS. XPS determined the surface density of argininamide by analyzing the nitrogen percentage (N%) on the pSA surface (Figure 1A). Atomic sensitivity factors were used to correct the different values of photoelectric cross-sections for different nuclei during the data analysis. The value of N% increased with the concentration of argininamide ([argininamide]) in the reaction solution, and it reached a plateau as [argininamide] approached 12 mM (Figure 1B). The observed maximum value of N% was 3.9%, corresponding to 10 mol % of argininamide based on each repeating unit of pSA (i.e. one out of 10 repeating units of pSA reacted with argininamide).[16]

The surface-modified pSA microspheres were dissolved in $CDCl_3$, and the bulk concentration of argininamide was examined by ^{1}H NMR. Due to the tiny quantities of argininamide in solution, ^{1}H NMR was not able to detect its proton signals (e.g., the proton from α-carbon having a chemical shift of ~4.5 ppm) in any of the microsphere samples (Figure 1C), suggesting that the high density of argininamide detected by XPS only occurred at the microsphere surface. After surface modification, the diameter of the microspheres decreased from 24 to 22 μm as [argininamide] increased from 0 to 40 mM in the reaction solution, while the value of M_w decreased from 34 to 19 kDa, respectively. The decrease in diameter is a result of reaction of argininamide with pSA on the microsphere surface: argininamide broke down the pSA chain to smaller fragments, which became soluble in solution. The decrease in Mw of pSA may reflect the degradation of the polymer at the microsphere surface or the small amount of argininamide that diffused into the microspheres and reacted with the polymer. We did not distinguish between the two possibilities in this study.

To confirm the covalent attachment of argininamide to the microsphere surface, we conducted two control experiments. First, we repeated the surface modification reaction in 30 mM guanidine hydrochloride ($[NH_2{=}C(NH_2)_2]^+Cl^-$) solution at pH 9.0. XPS analysis did not show any nitrogen

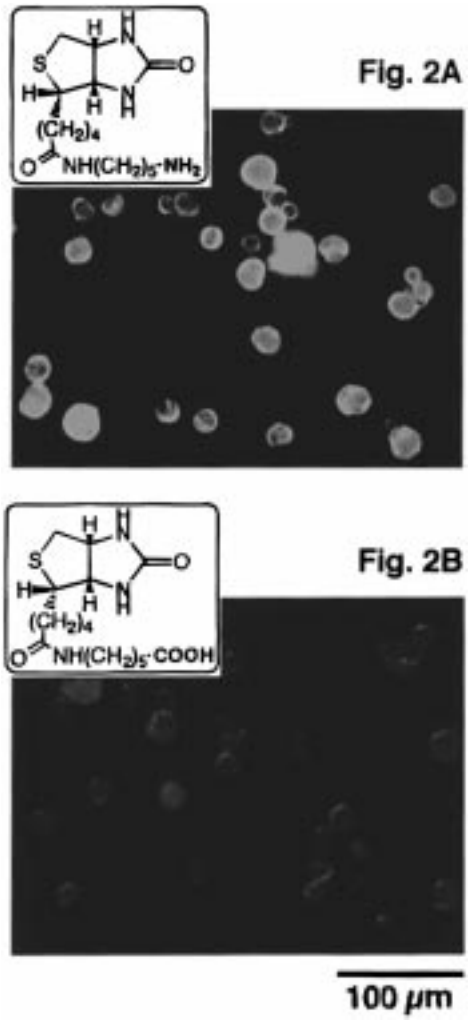


Figure 2—Fluorescence micrographs of microspheres modified by (A) *N*-(5-aminopentyl)biotinamide and (B) 6-((biotinoyl)amino)hexanoic acid, followed by recognition with fluorescein-labeled avidin in solution. The chemical structure of the two ligands is shown in the insert of each figure. Condition for fluorescence microscopy: $\lambda_{ex} = 480$ nm, $\lambda_{em} = 520$ nm. The scale bar on the bottom of Figure 2B applies to both 2A and 2B.

signals on the microsphere surface. Second, we prepared a self-assembled monolayer of $HOOC(CH_2)_{15}SH$ on gold. This SAM surface provides densely packed alkyl chains with negatively charged carboxylate head groups at pH 9.0 without the reactive anhydride groups. Even though the density and structural order of the carboxylate anions on the SAMs are different from those on the microsphere surface, the higher density of negative charges on the SAMs make it more sensitive to detect ionic interactions with positively charged argininamide in solution. After soaking the SAM sample in 10 mM argininamide solution at pH 9.0 for 15 min followed by washing with 2 M aqueous NaCl and water, we did not observe any nitrogen signals by XPS. As a comparison, we formed interchain anhydride groups from carboxylic acids on the SAMs, and these surfaces readily reacted with argininamide in solution, which resulted in covalent attachment of argininamide on the SAMs. These experiments help to exclude the possibility that ionic interactions between the positively charged argininamide and the negatively charged pSA surface caused the attachment of argininamide onto the pSA microsphere surface.

To examine the capability of the ligands attached on the microsphere surface to interact with their receptors in solution, we modified the surface of pSA microspheres with *N*-(5-aminopentyl)biotinamide (see insert in Figure 2A for its structure). Thus, biotin was covalently attached to the surface from the amino group. The microspheres were then suspended in the solution of fluorescein-labeled avidin at pH 7.4 for 30 min, washed with PBS and water, and dried in vacuo. Examination under a fluorescence microscope demonstrates that avidin bound to biotin on the microsphere surface (Figure 2A). In the control experiment, we repeated the surface modification and binding experiments using carboxylic acid terminated biotin, and observed a very weak fluorescence signal (Figure 2B).

This surface modification procedure has the following advantages: it is a one-step procedure, it requires a small amount of ligand to achieve high surface density,[17] the surface density of the ligand can be controlled by its concentration in the reaction solution, the ligands attached on the surface can freely interact with their receptors in solution, and the method should be general to a variety of ligands that contain amino groups. The ease, versatility, and generality of the procedure should open new opportunities in designing novel polyanhydride drug delivery systems to target specific organs or specific cell types within an organ, and in providing useful model systems to study the pharmacokinetics and pharmacodynamics of organ-targeted drug delivery.

References and Notes

1. Langer, R. *Ann. Rev. Biomed. Eng.* **1995**, *23*, 101−111.
2. Langer, R. *Acc. Chem. Res.* **1993**, *26*, 537−542.
3. Tamada, J.; Langer, R. *J. Biomater. Sci. Polymer Ed.* **1992**, *3*, 315−353.
4. Tabata, Y.; Langer, R. *Pharm. Res.* **1993**, *10*, 391−496.
5. Domb, A. J.; Gallardo, C. F.; Langer, R. *Macromolecules* **1989**, *22*, 3200−3204.
6. Brem, H.; Langer, R. *Sci. Med.* **1996**, *3*, 52−61.
7. Mathiowitz, E.; Jacob, J. S.; Jong, Y. S.; Carino, G. P.; Chickering, D. E.; Chaturvedi, P.; Santos, C. A.; Vijayaraghavan, K.; Montgomery, S.; Bassett, M.; Morrell, C. *Nature* **1997**, *386*, 410−414.
8. Takakura, Y.; Hashida, M. *Pharm. Res.* **1996**, *13*, 820−831.
9. Daemen, M. J.; Smits, J. F.; Thijssen, H. H.; Struyker-Boudier, H. A. *Trends Pharm. Sci.* **1988**, *9*, 138−141.
10. Sato, J.; Sugiyama, Y.; Tsuji, A.; Horikoshi, I. *Adv. Drug Delivery Rev.* **1996**, *19*, 445−467.
11. Brown, M. S.; Goldstein, J. L. *Science* **1986**, *232*, 34−47.
12. Leong, K. W.; Simonte, V.; Langer, R. *Macromolecules* **1987**, *20*, 705−712.
13. Mathiowitz, E.; Langer, R. *Microcapsules and Nanoparticles in Medicine and Pharmacy*; Donbrow, M., Ed.; CRC Press: Inc.: Boca Raton, FL, 1992; pp 99−123.
14. At pH 6.0, reaction of pSA microspheres with 10 mM argininamide in acetate buffer (0.20 M) showed less than 1% of nitrogen on the microsphere surface by XPS analysis.
15. Yan, L.; Marzolin, C.; Terfort, A.; Whitesides, G. M. *Langmuir* In press.
16. The attenuation length (the thickness of material required to reduce the flux of photoelectrons to 1/e) in the XPS study is 33 Å for photoelectrons from elemental nitrogen. See: Laibinis, P. E.; Bain, C. D.; Whitesides, G. M. *J. Phys. Chem.* **1991**, *95*, 7017−7021.
17. For example, only 1.9 μg of argininamide is required to achieve a surface density of 4.5 pmol/cm^2 per gram of pSA microspheres (24 μm in diameter).

Acknowledgments

The work at MIT was supported by the NIH grant NCDDG UO1-CA52857. This work made use of MRSEC Shared Facilities supported by the National Science Foundation under Award Number DMR-9400396. Laura Niklason was supported by an Anesthesiology Research Fellowship from the Foundation for Anesthesia Education and Research. We thank Dr. Lin Yan for helpful discussions on the SAM experiments.

JS970284U