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Published on Web 12/14/2006

High-Yield Activation of Scaffold Polymer Surfaces To Attach Cell Adhesion Molecules

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Abstract: Zirconium tetra(*tert*-butoxide) reacts with surface amide groups of polyamide nylon 6/6 to give (η^2-amidate)zirconium complexes in high yield. These surface complexes react to bond the cell-adhesive peptide arginine-glycine-aspartic acid (RGD) to the polymer surface. A surface loading of 0.18 nmol/cm^2 of RGD is achieved, which is 20−1000 times higher than previously reported attainable on natural or synthetic polymers by other strategies. Approximately 40% of the nylon surface is covered by the RGD, which gives a surface that is both stable to hydrolysis and highly active for cell adhesion and spreading in vitro.

Introduction

Engineered bioactive polymeric scaffolds[1] are of increasing importance for use in tissue regeneration in a variety of clinical applications, and interest in them continues to grow because they display significant versatility with wide-ranging physical properties, including biodegradability, compared to metals.[2] For these reasons, standard metallic implant technologies may someday be replaced by new, polymer-based ones. Yet, though many polymers do show much promise as biomaterials, the lack of an appropriate interface between the polymer and bodily tissue remains a substantial problem.[2] Due in part to their wetting properties,[3] polymer surfaces are often prone to nonspecific protein adsorption which can lead to nonspecific cell-type adhesion and fibrous encapsulation.[2] Successful strategies to create biocompatible polymeric implant surfaces that support desired cell growth would provide the means to improve device biointegration and would thus significantly impact the biomaterials field.

Metallic implant materials surface-derivatized with high yields of the cell attractive peptide Arg-Gly-Asp (RGD) can foster substantial cell adhesion and growth in vitro.[4] But comparable results have not yet been achieved using therapeutic polymeric devices, as the polymers most often used as biomaterials are not amenable to surface treatments that give high-yield surface coverage.[2] Though polymer scaffold materials with improved bioactivity have been prepared by blending,[5−8] copolymerization,[9−12] or physical treatment,[13,14] these methods can alter the bulk properties of the polymer[14] and yield only low peptide surface coverages[15,16] that do not approach that achieved on metallic substrates.[4]

We now report a unique, high-yielding strategy for surface activation of pre-cast polymers that we illustrate for nylon polyamides, which are currently used in burn and chronic wound treatment applications.[17,18] In our method, a simple zirconium alkoxide complex is allowed to react with surface amide groups of the nylon to give a Zr complex-activated surface that is then easily functionalized with peptides. Fluorescence spectroscopic methods show that approximately 40% of the nylon surface is activated with the RGD. This is the highest yield reported to date for peptide surface attachment by derivatization of a preformed biopolymer, and in vitro studies demonstrate substantially increased fibroblast cell binding and spreading on surfaces functionalized with RGD compared with the untreated polymer. Our activation strategy should be suitable for a range of scaffold materials with acidic N−H moieties to attach any biomolecule containing functionality that is reactive either directly with Zr alkoxides or with their simple derivatives.

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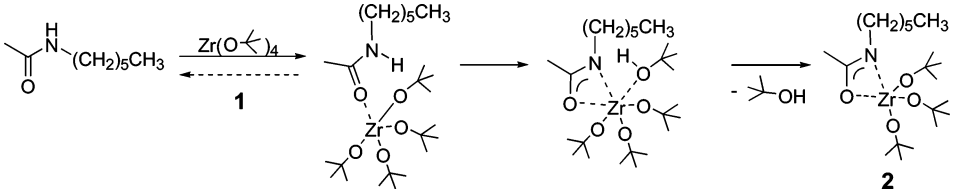
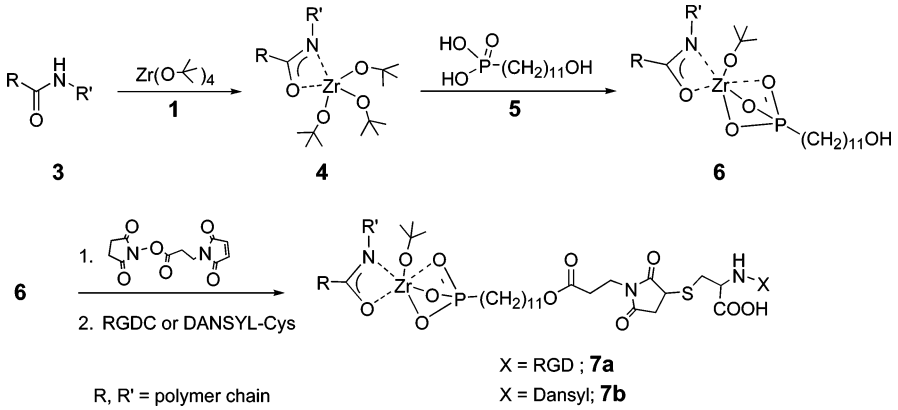
Scheme 1. Reaction of *N*-Hexylacetamide and Zirconium Tetra(*tert*-butoxide)



Scheme 2. Reaction of Nylon-Zr-amide Complex with a Phosphonic Acid and RGDC or DANSYL-Cys Coupling



Results and Discussion

Our novel approach to surface modification enables high surface density derivatization of a preformed polyamide device with RGD under ambient conditions. We hypothesized that since the surface of nylon 6/6 exposes backbone amide functionality containing acidic N−H bonds, most of these groups could serve as sites for chemical derivatization if appropriately activated. Coordination of the carbonyl group to an appropriate metallic center would further acidify these N−H bonds and facilitate such activation. Zirconium tetra(*tert*-butoxide) (**1**) is an excellent activation reagent because of the high oxyphilicity of Zr and because alkoxide groups remaining in its coordination sphere following reaction with the amide are readily replaceable ligands,[19] which accomplishes the desired derivatization of the polymer. This hypothesis was substantiated first in a small-molecule amide model system: *N*-hexylacetamide was treated with **1** to yield reactive complex **2** (>95% by ^{1}H NMR [$CDCl_3$]: δ 0.8 [t, 3H]; 1.3 [m, 35H]; 1.9 [s, 3H]; 3.2 [quartet, 2H]; Scheme 1). The η^2-coordination to Zr for the amidate moiety in **2** is indicated by the 8 ppm downfield shift of the acyl carbon vs the free amide (^{13}C NMR [$CDCl_3$]: δ 170.1 for *N*-hexylacetamide; δ 178.1 for **2**); a similar shift was observed on formation of η^2-zirconium carboxylates from carboxylic acids[20] while η^1-Pd(II) amidates show a smaller downfield shift.[21] Zirconium amidate hydroamination catalysts[22] show a crystallographic preference for η^2-coordination.

Surface derivatization of solid nylon 6/6 (**3**) proceeded according to our model system. Films of **3** (R = $(CH_2)_4CO$; R′ = $(CH_2)_6NH$) were cast from formic acid solution on glass microscope slides and were treated with vapor of **1**. The IR spectrum of polymer surface-bound Zr complex (**4**) showed ν_{C-H} = 2976 cm^{-1}, indicative of *tert*-butoxide groups. The **4**-coated slide was treated with phosphonoundecanol (**5**) to yield surface complex **6**, which is active for bonding RGDC peptides (Scheme 2). IR analysis of **6** showed peaks in the aliphatic region ($\nu_{CH2,asym}$ = 2922 cm^{-1}; $\nu_{CH2,sym}$ = 2851 cm^{-1}) characteristic of disordered alkyl chains.[23] Surface **6** was activated for RGD-Cys (RGDC) derivatization by reaction with the *N*-hydroxysuccinimide ester of 3-maleimidopropionic acid.

We have also found that an active surface for RGD binding can be prepared by directly reacting surface **4** with the *N*-hydroxysuccinimide ester of 3-maleimidopropionic acid to produce surface **8**. This reaction may proceed by transetherification (Scheme 3); this would give *N*-(*tert*-butoxy)succinimide, which was observed as a byproduct (confirmed by LC-MS). It is also possible that adventitious hydrolysis of the succinimidyl ester generates 3-maleimidopropionic acid in situ, which reacts with **4** via ligand exchange. Indeed, treating **4** with 3-maleimidopropionic acid (instead of the ester) gave an activated surface that performed identically to **8**. IR analysis of **8** showed a characteristic maleimide stretch (ν_{CO} = 1705 cm^{-1}). RGDC-modified nylon **7a** or **9a** was prepared by immersion of **6** or **8** in an aqueous solution of RGDC. A substantial change in surface hydrophilicity was confirmed by a decrease in water contact angle (75° for **3** compared to 50° for **9a**).

Hydrolytic stability and surface content of both derivatized nylons **7** and **9** were measured by a fluorescence spectroscopy-based experiment using a DANSYL analogue:[24] DANSYL-Cys was added at the reactive termini instead of RGDC (Schemes 2 and 3). Samples of **7b** and **9b** were immersed in water (adjusted to pH 7.5 with NaOH) for 7 days. Release of DANSYL groups was measured by fluorescence intensities of supernatants from treated **7b** and **9b** that were compared to the control sample (**3**) over this 7-day period. Unreacted DANSYL-ating reagent desorbed from the nylon surfaces in about 3 h.

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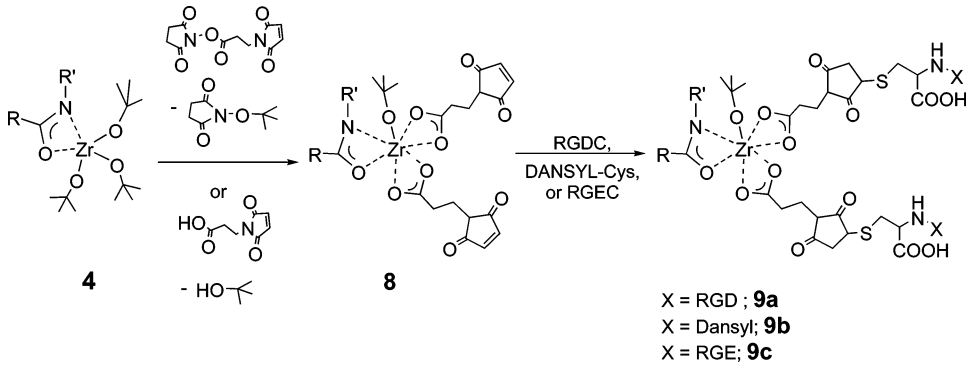
Scheme 3. Reaction of Nylon-Zr-amide Complex **4** with 3-Maleimidopropionic Acid or Its *N*-Hydroxysuccinimidyl Ester and Subsequent Coupling with RGDC, RGEC, or DANSYL-Cys



No release of surface-bound DANSYL material on **7b** or **9b** occurred over the next 7 days (see Supporting Information). Thus, zirconium-amidate surface-bound complexes are stable to hydrolysis under these conditions.

The usefulness of our approach for polymer surface modification is enhanced by the high surface coverage that it can attain: it has been shown[25] that cell adhesion and motility both increase as a function of RGD surface density. Surface complex DANSYL contents of **7b** and **9b** were quantified by immersion in water at pH 12 for 3 h, which cleaves the Zr complexes from the surface, precipitates ZrO_2, and releases fluorophore from **7b** and **9b** into solution. The amount of DANSYL surface-bound through Zr complexes **7b** and **9b** was measured to be 0.10 and 0.18 nmol/cm^2, respectively. These amounts are consistent with the DANSYL:Zr stoichiometries of 1:1 and 2:1 indicated for **7b** and **9b**, respectively (Schemes 2 and 3). Coverage of the nylon surface by RGD is 20−1000 times higher than has been reported by copolymerization or by purely organic chemical surface modification routes[5−7,9−11,13,15,16,26] and is indeed comparable to that obtained on titanium or other metallic surfaces.[4]

Nylon 6/6 activated by our procedure and terminated with RGDC peptides is highly active for supporting cell adhesion. NIH3T3 cells attached and spread on the RGD-modified surface **9a**, forming membrane extensions that stained with anti-vinculin antibodies (Figure 1A,C) and actin filaments that stained with fluorescent phalloidin (Figure 1C). Significantly more cells attached to the RGDC-modified surface **9a** than to untreated nylon (**3**) (Figure 1D). Similar numbers of cells attached to **9a** prepared through treatment of **4** with 3-maleimidopropionic acid compared to treatment with the ester. A one-way ANOVA test showed a statistical significance at both 3 and 6 h time points (3 h, $p = 1.8 \times 10^{-5}$; at 6 h, $p = 7.9 \times 10^{-6}$). To determine if the enhanced cell attachment observed for **9a** was specific for RGD, we also treated **8** with the non-cell-adhesive peptide (Arg-Gly-Glu-Cys) RGEC, which gives **9c** (water wetting contact angle = 50°). Cell adhesion on **9c** was less efficient than that on **9a** (Figure 1B,D); cells on **9c** also spread less, tending to remain round without forming vinculin-positive cellular extensions (Figure 1B,E). One-way ANOVA tests showed statistically significant differences in cell counts ($p = 1.9 \times 10^{-2}$) and cell spreading ($p = 9.0 \times 10^{-4}$) on surfaces **9a** and **9c**. Thus, while cell adhesion on nylon may be somewhat affected by changes in surface composition in general, cell spreading on treated nylon is specifically enhanced by the attachment of RGD.

Conclusions

We have shown that surface-bound Zr-amidate complexes, which are readily synthesized on the surface of nylon polymer, are effective for activation of that surface for further organic chemical transformation. Our derivatization of nylon 6/6 with RGD peptides, measured to be about 0.1−0.2 nm/cm^2 (corresponding to 20−40% spatial surface coverage for **7a** and **9a**, respectively) is far higher than has been attained on synthetic and natural polymers by copolymerization or by purely organic chemical surface modification routes,[5−7,9−11,13,15,16,26,27] and our RGD-derivatized surfaces are highly cell-attractive. Since our activation process involves simple reaction of amide N−H groups, it should be broadly applicable to other therapeutically important synthetic and natural polymers that contain this functionality such as polyester-*co*-polyamides,[28] polyurethanes,[29] polyureas,[29] polyimides,[30] or even silk.[27,31]

Experimental Section

General. All reagents were obtained from Aldrich and used as received unless otherwise noted. Tetrahydrofuran was dried over KOH and acetonitrile was dried over CaH_2 overnight; both were distilled prior to use. *N*-Hexylacetamide was synthesized by reaction of acetyl chloride (1.9 g, 24 mmol), hexylamine (2.0 g, 20 mmol), and 0.1 mL of triethylamine in CH_2Cl_2 at 0 °C for 3 h. The crude product was washed successively with Millipore water until the pH of the aqueous layer was greater than 6. The CH_2Cl_2 fraction was dried over Na_2SO_4, filtered, and evacuated to yield *N*-hexylacetamide (^{1}H NMR [$CDCl_3$]: δ 0.7 (t, 3H); 1.3 (m, 6H); 1.5 (quintet, 2H); 1.9 (s, 3H); 3.2 (quartet, 2H); 5.5 (s, 1H)). Phosphonoundecanol was synthesized as previously described.[24] Surface-modified samples were analyzed using a Midac M2510C Interferometer equipped with a surface optics SOC4000 SH specular reflectance head attachment. Fluorimetry experiments used a Photon Technology International Fluorescence Spectrometer.

(η^2-[*N*-Hexyl]amidate)zirconium Tri(*tert*-butoxide), 2. *N*-Hexylacetamide (0.15 g, 1.0 mmol) was treated with zirconium tetra(*tert*-butoxide) (Strem), **1** (0.40 g, 1.0 mmol), in dry CH_2Cl_2 for 1 h under

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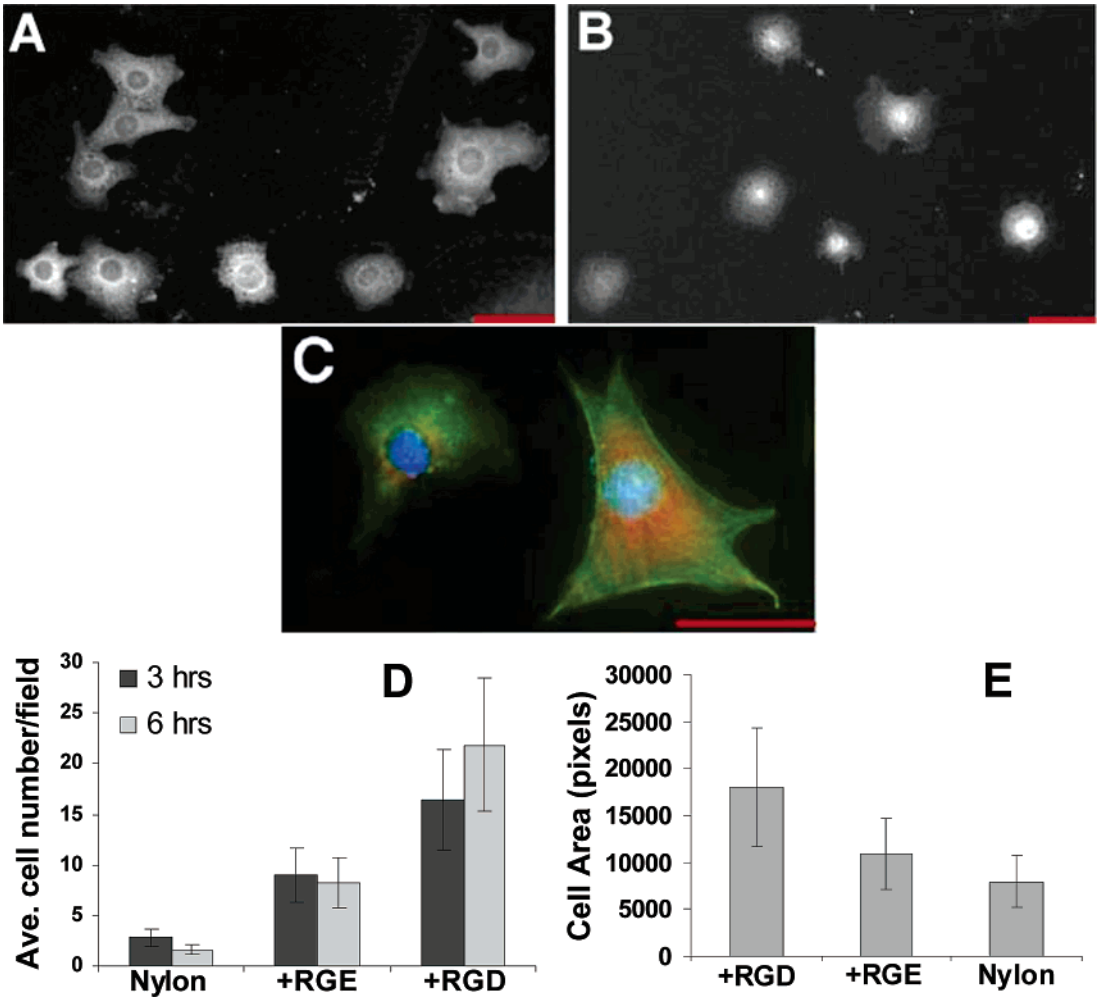

Figure 1. NIH3T3 cell attachment on derivatized nylon. Cells on RGD-modified nylon 6/6 (**9a**) (A) or RGE-modified nylon 6/6 (**9c**) (B) surfaces after 3 h, fixed and stained with anti-vinculin antibodies and rhodamine-conjugated secondary antibodies. (C) Cells on RGD-nylon 6/6 after 3 h, triple-stained with anti-vinculin antibodies (red), FITC-phalloidin (green), and DAPI (blue). Scale bars are 50 μm. (D) Number of cells per 10× microscope field counted for untreated nylon 6/6, RGD-derivatized, and RGE-derivatized nylon 6/6. (E) Average cell area determined for nylon 6/6, RGD-derivatized, and RGE-derivatized nylon 6/6 after 3 h per 20× microscope field with IPLab software. For (D) and (E), average values for at least three fields are shown with error bars representing ±1 standard deviation.

nitrogen. Solvent and reaction byproducts were removed in vacuo to yield **2** (^{1}H NMR [$CDCl_3$]: δ 0.8 (t, 3H); 1.3 (m, 35H); 1.9 (s, 3H); 3.2 (quartet, 2H)).

Surface Reaction of Nylon 6/6 with 1. Films of nylon 6/6 (**3**) were cast from 0.1 mM formic acid solution on glass microscope slides that were then rinsed copiously in Millipore water and evacuated at 10^{-2} Torr for 3 h. The coated slides were then placed in a deposition chamber that was equipped with two stopcocks for exposure either to vacuum or to vapor of **1**. The chamber was evacuated to 10^{-3} Torr for 30 min, and slides of **3** were exposed to vapor of **1** (with external evacuation) for 30 s followed by 5 min exposure without external evacuation. This cycle was repeated twice and was then followed by an additional 10 min of exposure without external evacuation. The chamber was then evacuated for 16 h at 10^{-3} Torr to ensure removal of excess **1** to give activated nylon **4**.

RGD-Modified Nylon 6/6 7a. A slide coated with **4** was immersed in a 0.1 mM solution of phosphonoundecanol (**5**) in dry THF for 15 min to yield Zr phosphonate complex **6.** Treatment of **6** in a 0.1 mM solution of 3-maleimidopropionic acid *N*-hydroxysuccinimide ester for 24 h under dry N_2 followed by copious rinsing successively in acetonitrile and Millipore water, drying in vacuo, and immersion in a 0.1 mM aqueous solution of RGDC at pH 6.5 for 24 h produced **7a**.

RGD-Modified Nylon 6/6, 9a. RGD-derivatized surface **9a** was prepared by immersing a slide coated with **4** in a 0.1 mM solution of 3-maleimidopropionic acid *N*-hydroxysuccinimide ester in dry acetonitrile for 16 h to produce **8**. Immersion of **8** in a 0.1 mM aqueous solution of RGDC at pH 6.5 for 24 h gave **9a**.

RGE-Modified Nylon 6/6, 9c. Immersion of **8** in a 0.1 mM aqueous solution of RGEC (Canadian Peptide) at pH 6.5 for 24 h gave RGE-derivatized surface **9c**.

Determination of Nylon 6/6 Surface Loading Using Fluorescent Molecule-Labeled Analogues 7b and 9b. These adducts were prepared as described for **7a** and **9a**, but a 0.1 mM aqueous solution of *N*-(5-(dimethylamino)-1-naphthylsulfonyl)-cysteine (DANSYL-Cys) was used instead of RGDC (Schemes 2 and 3). To address the issue of solvent-induced polymer swelling, control films of **3** were prepared by soaking in 0.1 mM DANSYL-cys solution for 24 h. A calibration curve of fluorescence intensity versus concentration was measured for DANSYL-Cys solutions from 0.16 to 21 μM at pH 7.5 and pH 12. Nylon films (2 cm^2) derivatized as **7b** and **9b** and control films of **3** were immersed in water at pH 7.5 for 7 days at room temperature, and the supernatants were analyzed by fluorescence spectroscopy. The samples were then removed from solution, dried, and immersed in water at pH 12 for 3 h, after which the supernatants were again analyzed by fluorescence spectroscopy. The spatial surface coverage of **9a** by RGD was calculated from its measured surface loading, 0.2 nmol/cm^2; assuming an RGD "footprint" of 40 Å^2 (determined using Chem 3D), this corresponds to coverage of about 0.4 cm^2/cm^2 of surface, or 40%.

Fibroblast Adhesion and Spreading on RGD-Derivatized Nylon Surfaces. Cell response to surfaces **3**, **9a**, and **9c** were evaluated in vitro. NIH 3T3 cells maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% calf serum (Hyclone) were prepared for cell adhesion experiments as previously described.[24] Cells (2.65×10^4 cells/cm^2 in serum-free DMEM) were added to tissue culture wells containing untreated or derivatized nylon surfaces which had been preblocked for 1 h in 1% bovine serum albumin in PBS. After 90 min, medium with nonadherent cells was removed and replaced with fresh, serum-free DMEM. At 3 and 6 h cells were fixed, permeabilized, and stained with anti-vinculin antibody (Sigma) followed by rhodamine-IgG secondary antibody (for focal adhesions). In some cases, cells were also stained with FITC-phalloidin (for actin filaments) and DAPI (for DNA). Images were obtained as described previously.[32] Brightness and contrast of color levels in Figure 1C were adjusted for the merged image using IPLab software. Cell adhesion was quantified by counting the number of attached cells in at least three microscope fields (10× magnification).

Acknowledgment. The authors thank the National Science Foundation and the National Institutes of Health for financial support of this research.

Supporting Information Available: Fluorescence intensity vs time traces showing hydrolytic stability and surface loading of **7b** and **9b** are available via the Internet.

JA065217T

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