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A Tunable, Chemoselective, and Moldable Biodegradable Polyester for Cell Scaffolds

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A tremendous amount of research has gone into generating many different types of biodegradable materials for applications ranging from tissue engineering[1] and drug delivery[2] to green chemistry plastics.[3] For biological applications, one of the most studied classes of biodegradable materials is polyesters, due to their versatility in synthetic design and their low cost. Although there have been a few successful applications, the majority of potential polymer candidates are unable to function predictably in a complex and evolving biological environment. If both the material and biological criteria of a polymer are taken into consideration during the design phase, the probability of achieving a particular biological application will be enhanced. For more sophisticated biological applications, there still remain several challenges in using biodegradable polyesters. We believe that, in order to generate a more flexible biodegradable material for a diverse set of cell biological and tissue engineering applications, the materials must be designed to possess the following criteria: the polymer should 1) be easily functionalized, 2) not elicit a cytotoxic response, 3) be moldable to generate a variety of 3D structures and features, and 4) offer the potential to tune the degradation rates.

An important characteristic of polymers for in vivo studies is the inherent cytotoxicity of the material and its degradation byproducts. In synthesizing the material, two approaches relating to cytotoxicity exist. The first and more common approach is to design a material with the anticipation that it will not affect cells negatively. This strategy usually requires little or no prior knowledge of the material's behavior in a biological environment or of the intricate biological processes of cells and tissues. A second, less common strategy involves using natural products as monomers.[4] This reduces the potential cytotoxicity of some of the degradation products as they are naturally occurring and are involved in normal cellular metabolic functions and pathways.

Another challenge in polymer design has been the development of the capability to functionalize biodegradable materials easily, mildly, and specifically. Related to the concept of facile functionalization is the possibility of temporal control of ligand conjugation. Some methods are able to introduce functionality during the polymerization process;[5] this precludes opportunities for introducing chemical groups as a function of time and also for functionalizing selected regions while others remain unmodified. To overcome this limitation, alternate strategies have incorporated functional handles capable of post-polymerization, chemoselective modifications.[6] While these examples are chemoselective, they usually require extra reagents that are often necessary to enable the coupling chemistry. These extra reactions and compounds could lead to the degradation of polymer chains or could introduce compounds that may be cytotoxic and not amenable to cell culture conditions.

One of the most successful subclasses of biodegradable materials has been based on modified poly(ε-caprolactone) systems, which exhibit several advantageous properties. However, some difficulties concerning processing and degradation arise, due to the inherent crystallinity of the polymers.[6a–d] In addition, when macromolecules are designed on the basis of modified lactones, multiple challenges present themselves: 1) a new monomer is required for each new functional group that is introduced, 2) the synthesis of modified rings can be challenging, and 3) post-polymerization deprotection is often required, which can degrade the polymer backbone.

An important feature that would add to the flexibility of biodegradable materials, and therefore increase the scope of applications, would be the ability to mold an amorphous polymer chemically.[7] Cross-linking of a polymer by the introduction of heat or light would allow thermosets to be designed in conjunction with a range of fabrication techniques, which can lead to materials with a spectrum of feature sizes and geometries. This capability would increase the versatility of a polymer and allow the scaling of the material from the millimeter regime, to support cells, to the micro- and nanoscales, for delivery agents of therapeutics to target specific cells and tissues.

The ideal polymer for biological applications would therefore be degradable, noncytotoxic, amorphous, and easily functionalizable with a wide variety of ligands. Also, through the use of condensation polymerization, thermal, mechanical, and solubility properties can be controlled and tuned easily.[8] In this manner, polymers could be designed to investigate many different research areas in biology: geometry effects, size effects, ligand density studies, surface interactions, and cell permeability requirements. A moldable and chemoselective biodegradable polymer would potentially be able to address a wide range of issues from tissue engineering to microparticles for drug delivery in biotechnology.

Here we report the synthesis of a poly(ester ether) that serves as a flexible and straightforward material for biological applications. Our design is based on the chemoselective coupling reaction between ketones and oxyamines.[9] Through the condensation polymerization of tetra(ethylene glycol) and α-ketoglutaric acid, a natural product, a biodegradable polymer containing a ketone in each repeat unit was synthesized (Scheme 1). Upsetting the stoichiometry allowed the polyester

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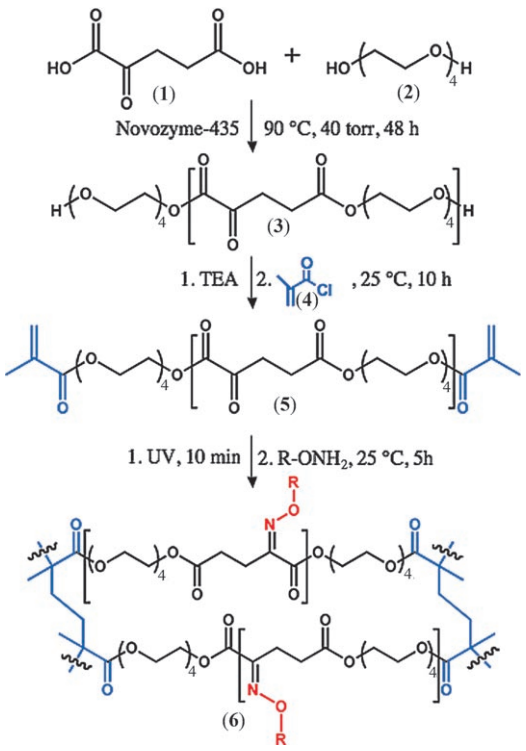

Scheme 1. Preparation of a moldable and chemoselective biodegradable polyester. α-Ketoglutaric acid (**1**) and tetra(ethylene glycol) (**2**) were combined to form prepolymer **3**. End-capping with methacryloyl chloride (**4**) formed **5**, a dimethacrylate macromonomer capable of photoinitiated cross-linking. After irradiation with UV light for 10 min, the polymer was cured, with the ketone groups still capable of chemoselective ligand immobilization.

chains to terminate in alcohol groups, which were capped with methacryloyl chloride to provide cross-linking sites.

To demonstrate that the chemoselective ability of the ketones was not altered or lost during polymerization, the immobilization reaction was carried out with a control molecule, *O*-allylhydroxylamine, to test the oxime-forming reaction (Figures S1–S3). *O*-Allylhydroxylamine was chosen because ^{1}H NMR can easily monitor the extent of reaction. The polymer and the hydroxylamine were mixed in a 1:1 ratio and stirred for 10 h at room temperature. From NMR analysis, the reaction proceeded efficiently and to completion.

The polyester was designed to be 1200 g mol^{-1}, terminating in hydroxy groups to allow for the addition of cross-linking sites; molecular weights were confirmed by gel permeation chromatography. Through the introduction of methacrylate groups onto the chain ends, the polymer could be set through thermal and UV-initiated cross-linking. Because of the flexibility in setting the polyester, as well as the polymer being amorphous, many fabrication methods can be used for molding and shaping **5** into bio-elastomeric devices.[7b,10] To demonstrate the ease with which this material can be molded, micropatterned surfaces were generated by standard imprint lithography. By inversion of an elastomeric stamp, fabricated by soft lithography,[11] into the amorphous polymer, followed by irradiation with UV light (365 nm), various geometries were patterned, molded, and characterized by scanning electron microscopy (SEM; Figure 1). The patterns have feature sizes of 160 μm and depths of approximately 40 μm. Although we have used imprint lithography to show the versatility of the polymer, there are many other methods available for more complex molding and patterning.

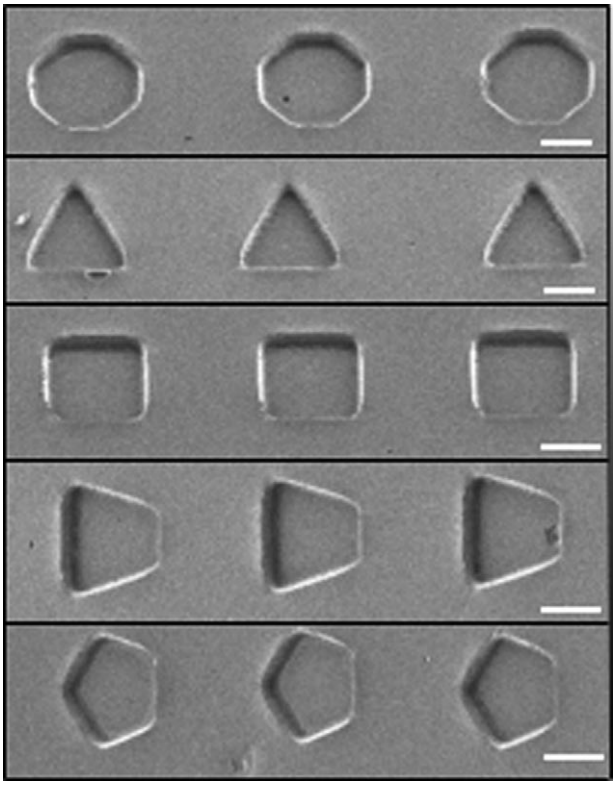

Figure 1. SEM images of micropatterned polymer films. The amorphous polymer **5** was patterned with a PDMS stamp fabricated by soft lithography. After UV cross-linking, the stamp was removed and the pattern was transferred to the newly formed polyester film, creating molded microarrays. Each scale bar represents 100 μm.

To examine the ability of this polymer to be compatible with and to perform in a biological environment, we studied the cytotoxicity of the degradation products and examined the potential for tuning the degradation rates. Degradation studies were carried out in phosphate-buffered saline (PBS) at 37 °C by monitoring the percentage of mass lost as a function of time (Figure 2). After cross-linking of **5** into small discs of approximately 75 mg, samples were placed into PBS for degradation. The nonfunctionalized films degraded completely in 11 days. To determine whether treatment of the ketone groups with oxyamine groups to form oximes affected the degradation rates, we treated the films with *O*-allylhydroxylamine or aminooxyacetic acid in anhydrous methanol. Degradation studies were then performed exactly as with the nonfunctionalized films. We found that oxime formation and the nature of the oxyamine ligands on the cross-linked polyester had significant effects on the rates of degradation. Films functionalized with *O*-allylhydroxylamine degraded in 14 days, while those func-

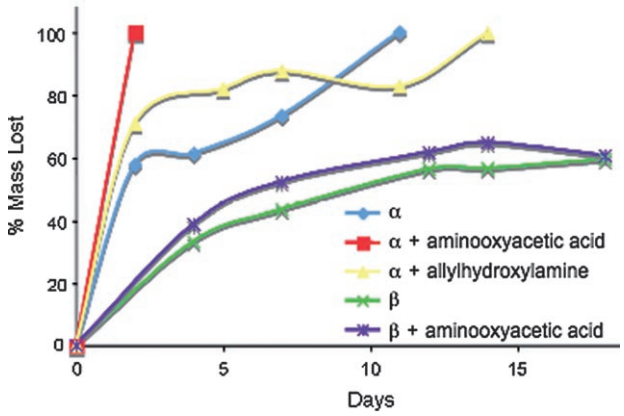

Figure 2. Degradation rates altered by ligand immobilization. Polymer **5** was cross-linked and then functionalized with aminooxyacetic acid, *O*-allylhydroxylamine, or no ligand. A similar polyester, based on β-ketoglutaric acid (Supporting Information), was cross-linked and functionalized with aminooxyacetic acid or no ligand. Films were soaked in PBS at 37 °C and allowed to degrade.

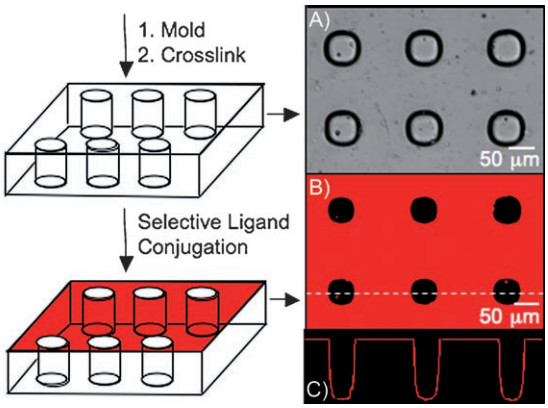

Figure 3. Region-selective ligand conjugation to a molded, micropatterned biodegradable polymer. Geometric patterns were transferred to a polymer film by cross-linking **5** in the presence of a PDMS stamp. Subsequently, a rhodamine analogue, modified to include an oxyamine, was added only to the surface of the film. After 30 min, the film was rinsed with ethanol. A) A phase contrast micrograph shows the square features in the film. B) The dye reacted with the ketones only on the polymer surface. The areas representing the recessed square features show no signs of dye conjugation. C) An intensity profile demonstrates the relative difference in fluorescence between the surface of the film and the microwells after ligand conjugation.

tionalized with aminooxyacetic acid degraded completely in 2 days.

To determine whether the degradation rates can be influenced by the proximity of the ketone groups to the ester groups in the polymer backbone, we prepared a similar polyester based on β-ketoglutaric acid (βKG; Figure S4). The only difference is the location of the ketone groups relative to the ester bonds, as molecular weights were kept constant. In PBS solution, nonfunctionalized films based on βKG required 28 days for 69.3% degradation, while films with immobilized aminooxyacetic acid reached 70.8% degradation in 28 days. These two polyester films degraded at, essentially, the same rate. Comparison of these results with the previously described degradation rates of the polymers derived from **1** shows that the location of the functional handle for polymer modification is important. Through a combination of steric and inductive effects, the macromolecular properties, such as degradation rates, can be altered and tuned simply by the proximity of the immobilization site to the ester bonds.

To demonstrate further how the chemoselective immobilization strategy can be used to control the regions of reactions with a three-dimensional polymer, we first generated a molded, micropatterned polymer and treated only the top face with a fluorescent rhodamine–oxyamine conjugate. Figure 3 shows a schematic and micrographs of the molded pattern—only the top face reacted with the fluorescent dye, with no reaction within the wells of the mold. This result shows the straightforwardness of allowing only select regions to react with ligands on a biodegradable micropatterned mold and might allow for the straightforward immobilization of diverse ligands on different regions of the polymer.

Finally, to demonstrate the utility of poly(αKG-EG_4) as a biomaterial, films were generated and tested as potential cell scaffolds for tissue engineering applications. The amorphous polymer was placed on a glass slide and flattened with a PDMS stamp. The material was then cross-linked by irradiation with UV light for 10 min, after which the PDMS was removed. The exposed ketone groups on the surface of the polymer film were then chemoselectively functionalized by treatment with ~100 μL of a 10 mM solution of a peptide, RGD–ONH_2, for 5 h in PBS. The RGD peptide is a well known and studied ligand that promotes cell adhesion through cell surface integrin receptors.[12] After the films had been rinsed in PBS, 3T3 Swiss Albino mouse fibroblasts were seeded onto the RGD-presenting films and incubated at 37 °C in 5% CO_2. Cells attached to the films and adopted spread morphologies (Figure 4). Over several days the cells migrated and divided, eventually forming a lawn on the polymer film. The cells were able to function normally and to proliferate on **6** for approximately 10 weeks (at which point the material had completely degraded). The

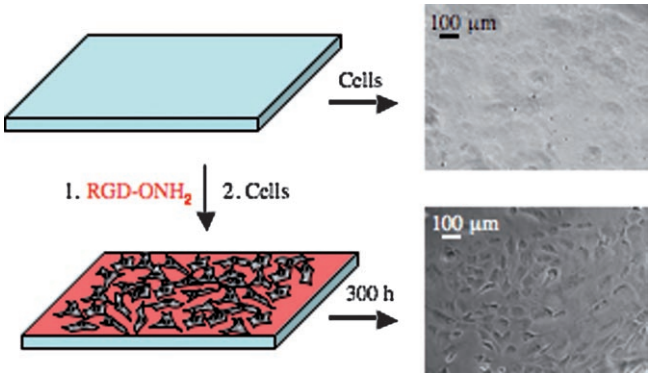

Figure 4. Cell scaffolds based on **6**. After UV cross-linking, the surfaces of films were functionalized with RGD, providing ligands for cell surface receptors. Cells were then seeded and allowed to grow on the polyester surfaces. After 10 days, cells had attached and spread to form a lawn on the film. Cells were also seeded on a nonfunctionalized control film, and no cells had attached after several days, demonstrating the inherent inertness of the polymer without cell adhesive immobilized ligands.

polymer material and its degradation products were found to be noncytotoxic, as no deleterious effects on normal cell function were observed. To show that the interaction was biospecific and mediated only by the immobilized RGD ligands, we seeded cells on films that had not been treated with RGD–ONH_2 and found almost no cell attachment.

In addition, the scrambled peptide RDG–ONH_2 was also immobilized on a film; no cells attached, indicating the exquisite biospecific selectivity of the integrin receptors for bound RGD ligands. Both the nonfunctionalized films and the RDG–ONH_2-functionalized films were also found to be noncytotoxic.

In conclusion, we have demonstrated the design of a versatile biodegradable material capable of mild and chemoselective functionalization. Standard condensation polymerization of α-ketoglutaric acid (**1**) and tetra(ethylene glycol) (**2**) led to the synthesis of **3**. Polymerization with α-ketoglutaric acid accomplished two things: 1) as it is a natural product, cytotoxicity is minimized, and 2) the presence of a ketone in the repeat unit represents a functional handle capable of immobilizing a variety of ligands tethered with oxyamine groups. Photoinitiated cross-linking of **5** allowed for the patterning of various geometries, as well as the design of cell scaffolds. RGD–ONH_2 was coupled to the surfaces of films to present ligands capable of biospecific interactions with cell surface receptors. Cells were able to attach to and proliferate on these films for up to ten weeks, at which time the polymer film had completely degraded. Cellular processes, such as migration and division, were not negatively affected while functioning on the poly(αKG-EG_4) films.

As a number of degradation rates were obtained with the same polymer backbone, several observations were noted: 1) the location of a functional handle for post-polymerization modification can affect macromolecular properties, 2) because of the ease with which ligands can be added, these ketone-containing polyesters have the potential for tunable degradation rates, and 3) the observed degradation rates in PBS were very different from the degradation rates in the presence of cells. A new standard for in vitro degradation studies may be needed in order to gain relevant information concerning how best to translate how the polymer will perform in future in vivo studies. The facile functionalization of poly(αKG-EG_4) allows for the oxyamine-containing ligand to define the function of the polymer through biospecific interactions with cells, as the nonfunctionalized films are biologically inert. For example, by changing the peptide ligand, cell-specific biodegradable materials can be designed. This method of polymer design and ligand immobilization offers the potential for a single polymer backbone to be used in many diverse applications by straightforward variation of the properties of the ligand side-chains. The poly(αKG-EG_4) described is a versatile biomaterial and possesses the necessary features for a range of tissue engineering and drug delivery applications.

Experimental Section

Polyester synthesis: Compound **1** was purified by recrystallization from ethyl acetate, and **2** was used without further purification. Monomers **1** and **2** were combined at 110 °C and stirred until a homogeneous melt had been formed. The temperature was then lowered to 90 °C, followed by the addition of Novozyme-435. The polymerization was allowed to continue for 2 h, followed by 46 h at a reduced pressure of 40 torr. The reaction mixture was then diluted with methylene chloride to allow for removal of the enzyme by filtration. After concentration in vacuo, prepolymer **3** was precipitated in −78 °C methanol and dried under vacuum.

A second polyester was synthesized from diethyl acetone-1,3-dicarboxylate (**7**) and **2**. After distillation, **7** was used instead of the corresponding diacid because of complications caused by decarboxylation upon melting. Compounds **7** and **2** were combined with tin(II) 2-ethylhexanoate (0.01 mol%) at 130 °C. After the mixture had been stirred for 2 h under argon, the pressure was reduced to 20 torr and the reaction was allowed to continue for 22 h. The reaction mixture was then diluted with methylene chloride to reduce the viscosity, followed by precipitation of prepolymer **8** in −78 °C methanol. The polymer was dried under vacuum.

To create thermosets, prepolymers (**3** or **8**) were diluted with tetrahydrofuran. Triethylamine was stirred into the polymer solution, followed by the addition of methacryloyl chloride (**4**) at 0 °C. The reaction was allowed to continue for 2 h, after which the mixture was allowed to warm slowly to room temperature and to react for an additional 8 h. The reaction solution was filtered, combined with a small amount of 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) to prevent cross-linking, concentrated in vacuo, and redissolved in methylene chloride. The workup for macromonomer **5** consisted of six extractions: two with hydrochloric acid (1 M), two with sodium bicarbonate (1 M), and two with a saturated sodium chloride solution. The solution was then dried over Na_2SO_4, filtered, concentrated in vacuo, and precipitated in −78 °C methanol. After drying for two days under vacuum, polymers **5** and **9** were obtained.

Chemoselective ligand immobilization: To test the initial chemoselective coupling to oxyamine-containing ligands, prepolymer **3** and *O*-allylhydroxylamine were dissolved in anhydrous methanol in a 1:1 ratio. The reaction was allowed to proceed for 10 h at room temperature. The solution was then concentrated in vacuo and redissolved in methylene chloride, followed by filtration. The reaction mixture was concentrated in vacuo, after which the polymer was precipitated in methanol at −78 °C and dried for two days under vacuum.

Characterization: ^{1}H NMR spectra were acquired in deuterated chloroform or deuterated acetone on a Bruker 400 MHz AVANCE spectrometer. Molecular weights were measured and compared to polystyrene standards by use of a Waters GPC system with a Wyatt Optilab DSP interferometric refractometer and a Wyatt Dawn EOS as the detector. The measurements were taken at 40 °C with tetrahydrofuran as the mobile phase on three columns in series (Waters Styragel HR2, HR4, and HR5). All polymerizations were designed to achieve molecular weights of approximately 1200 g mol^{-1}.

UV cross-linking: In order to facilitate cross-linking, a photoinitiator—α,α-diethoxyacetophenone—was added to polymer **5**. The mixture was applied to a glass coverslip and patterned with a PDMS stamp, fabricated by soft lithography. To set the material, it was irradiated with 365 nm light for 10 min in an Electro-Cure-500 UV Curing Chamber (Electro-Lite Coporation, Bethel, CT). The PDMS was then removed, resulting in a patterned polymer surface. To cure the polymer for use in a degradation study, the same procedure was followed without the use of PDMS, resulting in discs. To set the polymer for use as a cell scaffold, the same procedure

was followed, except that a flat piece of PDMS was used instead of a patterned stamp.

Degradation studies: To obtain standard degradation rates, discs were weighed (approximately ~75 mg) and placed in scintillation vials. To functionalize the materials, films were submerged in an oxyamine tethered ligand solution in methanol (10 mM, approximately 2 mL) for 5 h. The methanol solution was then discarded, and the vials were filled with Dulbecco's PBS buffer (Sigma). All samples were then stored in an incubator at 37 °C. At predetermined intervals, samples were removed from the incubator, rinsed thoroughly, dried for 2 days, and weighed again. To prevent saturation, PBS was replaced every 7 days. Each data point was repeated in triplicate, and the results were reported as the average percentages of the original mass lost.

Peptide immobilization, cell culture, and microscopy: To functionalize the surface of the thermoset, a solution of RGD–ONH_2 (10 mM, ~100 μL) was added directly to the top of the film and allowed to react for 5 h in PBS, resulting in **6**. After the films had been rinsed in PBS, 3T3 Swiss Albino mouse fibroblasts were seeded onto the RGD-presenting thermosets, and incubation was carried out in Dulbecco's modified Eagle's medium (Sigma) with bovine calf serum (10%) and penicillin/streptomycin (1%) at 37 °C under CO_2 (5%). The cells were added at a low density of 10000 cells mL^{-1} to facilitate in determining how well cells were able to function on **6**. The medium was changed every 3 days, with cells proliferating and functioning normally for approximately 10 weeks, at which time the films had completely degraded. As a control, cells were added onto nonfunctionalized films at a density of 100000 cells per mL. After 24 h, the film was visualized to demonstrate that no cells had attached without the aid of immobilized RGD-mediated interactions. Phase-contrast images were taken with a Nikon Eclipse TE2000-E inverted microscope (Nikon USA, Inc., Melville, NY).

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