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Tunable Elastomers with an Antithrombotic Component for Cardiovascular Applications

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Abstract

Here we report the development of a novel family of biodegradable polyurethanes for use as tissue engineered cardiovascular scaffolds or blood-contacting medical devices. Covalent incorporation of the antiplatelet agent dipyridamole into biodegradable polycaprolactone-based polyurethanes yields biocompatible materials with improved thromboresistance and tunable mechanical strength and elasticity. Altering the ratio of the dipyridamole to the diisocyanate linking unit and the polycaprolactone macromer enables control over both the drug content and the polymer crosslink density. Covalent crosslinking in the materials achieves significant elasticity and a tunable range of elastic moduli similar to that of native cardiovascular tissues. Interestingly, the crosslink density of the polyurethanes is inversely related to the elastic modulus, an effect attributed to decreasing crystallinity in the more crosslinked polymers. *In vitro* characterization shows that the antiplatelet agent is homogeneously distributed in the materials and is released slowly throughout the polymer degradation process. The drug-containing polyurethanes support endothelial cell and vascular smooth muscle cell proliferation, while demonstrating reduced levels of platelet adhesion and activation, supporting their candidacy as promising substrates for cardiovascular tissue engineering.

Keywords

cardiovascular tissue engineering; biodegradable; polyurethane; antithrombotic; elastomer

1. Introduction

Hemocompatible materials have broad application as blood-contacting substrates for medical devices and tissue engineered constructs such as vascularized organs, cardiac valves, and arterial bypass grafts.^[1–7] Synthetic polymers are appealing substrates for off-

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

the-shelf cardiovascular grafts due to their ease of production and reproducible mechanical strength; however, as of yet no single material possesses the entire suite of properties necessary for the long term success of such implants. Current synthetic blood vessel grafts are most commonly fabricated from the non-degradable polymers polyethylene terephthalate (PET)^[7, 8] and polytetrafluoroethylene (PTFE).^[7, 9] These grafts show satisfactory long-term patency when used in arteries with diameters larger than 6 mm, but in small diameter contexts, such as the coronary arteries,^[10] synthetic grafts suffer from high rates of occlusion.^[11, 12] Loss of vessel graft patency is most commonly associated either with acute thrombosis caused by the inherent thrombogenicity of the polymer substrates^[12–14] or with mechanical mismatch between the stiff graft materials and the surrounding native tissues, which often leads to complications such as intimal hyperplasia and atherosclerosis.^[7, 12, 15–17] The grafts of choice for patients requiring bypass surgery of small diameter vessels are autologous veins or arteries harvested from the patient, with the saphenous vein, internal mammary artery, and radial artery being the most common.^[18] However in many cases, suitable autografts are unavailable due to previous harvesting or pre-existing conditions, highlighting the existing need for synthetic alternatives.

The development of new biocompatible materials suitable for blood-contacting devices would similarly benefit the treatment of cardiac valve disease. Current mechanical cardiac valve prostheses are composed of rigid polymers, metals, and pyrolytic carbon,^[19, 20] and are prone to thromboembolic complications due to the thrombogenicity of the graft materials and disruption of native hemodynamics caused by the stiff valve materials and unnatural architectures.^[6, 19, 21–24] The heightened risk of thromboembolism requires patients receiving these mechanical valves to undergo lifelong anticoagulant therapy, which itself increases the incidence of hemorrhage.^[20, 25] Furthermore, implant materials with greater mechanical stiffness than the surrounding tissues risk inducing inflammation that can lead to unwanted fibrotic encapsulation of the foreign material, hindering graft integration into the body.^[26] An ideal substrate for cardiovascular scaffolds would be non-toxic and non-immunogenic; it would present a hemocompatible blood-contacting surface to avoid damaging blood tissue or triggering thrombosis; and it would have appropriate strength and compliance to prevent graft rupture and to approximate the hemodynamics of native tissues. As no current material displays all the above properties, the development of novel materials tailor-made to meet the specific requirements of their target biological tissues would greatly expand the cardiovascular biomedical engineer's toolkit.

Tissue engineering strategies might offer the best long term solution to the current challenges facing synthetic implants. Biodegradable polymer-based synthetic grafts that can provide templates for native tissue regeneration hold great promise for restoring long term tissue function and minimizing risks associated with chronic exposure to foreign materials because cellular invasion and proliferation within degradable implants allows the body to gradually replace the synthetic substrate with living biological tissue^[3, 4, 12, 27, 28] without the need for lifelong anticoagulation or antiplatelet therapy. And regenerated living tissues that can adapt and grow are particularly important for pediatric patients. Furthermore, the inclusion of bioactive molecules within degradable scaffolds can deliver drugs such as antiplatelet agents directly at the site of need to prevent platelet adhesion and activation.^[29, 30]

Previously, we have reported the use of biodegradable polyurethanes for application in cardiovascular tissue engineering.^[31–33] Here we report the development of a novel family of biodegradable polyurethanes with improved mechanical strength, elasticity, biocompatibility, and thromboresistance by incorporation of an antiplatelet agent. Our goal was to design a gradually degrading biocompatible polymer that would present a hemocompatible blood-contacting surface throughout the degradation process, with tunable mechanical properties to better match the range of strength and elasticity found in native cardiovascular tissues compared to current grafting materials. To this end, we designed our novel elastic polymers using three major constituents: polycaprolactone (PCL), dipyridamole (DPY), and hexamethylene diisocyanate (HDI). PCL, an FDA-approved biocompatible polyester, was selected as a flexible and strong macrodiol that gradually degrades under biological conditions. Dipyridamole, a tetrol antiplatelet drug,^[34] was selected as a small molecular chain extender capable of forming crosslinks to tune the toughness and elasticity of the product materials and to augment the polyurethanes' thromboresistance. Previous studies have shown that the drug dipyridamole can improve vascular patency when loaded into a drug-eluting polymer graft^[30] or tethered covalently to a graft surface.^[35] However, diffusion-mediated drug release and surface coatings often exhaust their active component before total degradation of the synthetic polymer scaffold is achieved, which could leave the graft vulnerable to late stage thrombosis. Covalent incorporation of the dipyridamole unit directly into the backbone of our bulk polymer couples the rate of drug loss with scaffold degradation, localizing the antiplatelet agent at the polymer surface throughout the graft lifetime. To link the PCL and dipyridamole components, we selected HDI, an aliphatic isocyanate widely used to produce biocompatible degradable polyurethanes.^[36–38] These building blocks allow for a straightforward catalyst-free one-pot reaction, and the use of a short diisocyanate unit to link varying hydroxyl functionalized materials could easily accommodate the addition of other alcohol or amine moieties with specific properties to further tune the scaffold characteristics for greater applicability in cardiovascular tissue engineering and other areas.

The drug dipyridamole was of particular interest due to its chemical compatibility with polyurethane synthesis and the presence of four equivalent reactive hydroxyl groups on the molecule, which not only allow it to be used as a polyurethane chain extender, but enable its use as a crosslinking site within the product polyurethanes by adjusting the ratio of isocyanate groups to hydroxyl groups in the reaction mixture. We therefore hypothesized that manipulation of the monomer feed ratio could give rise to materials with variable drug concentrations for pharmacological delivery and with tunable elasticity to better match the heterogeneity of Young's moduli found in native cardiovascular tissues.^[39–42] In this study, a range of polyurethanes was synthesized and characterized in terms of mechanical and biological properties to determine the candidacy of our degradable dipyridamole-loaded materials as substrates for blood contacting contexts.

2. Results and Discussion

2.1. Polymer synthesis and characterization: tuning the drug content and crosslink density

The polymers were prepared via a one-pot catalyst-free procedure. As shown in Scheme 1, the PCL macrodiol was first reacted with HDI in a 1:2 ratio at 60 °C for 2 hours in order to form polyester segments end-capped by isocyanate groups. The polyol dipyrindamole was subsequently added to the vessel and the reaction was continued at 70 °C for an additional 18 hours, resulting in extended polyurethane sequences. Polymerization was confirmed by Fourier transform infrared spectroscopy (FTIR) and gel permeation chromatography (GPC). In Figure 1, the FTIR spectra show formation of the Amide II band at 1529 cm⁻¹ associated with the newly forming urethane bonds^[43] and the concurrent disappearance of the peak at 2267 cm⁻¹ characteristic of isocyanate group stretching.^[44] The peak near 1724 cm⁻¹ is attributed to the carbonyl stretching from the ester groups in the PCL component and from newly forming urethane bonds as the reaction proceeds.^[44] Further support of polymerization was given by GPC (Table 1), which demonstrated a 10-fold increase in M_n and a 27-fold increase in M_w from the PCL prepolymer to the uncrosslinked polyurethanes. The presence of isocyanate groups was also monitored to follow the reaction progress. Quenching the reaction with an excess of dibutylamine followed by back titration with hydrochloric acid revealed $54.5 \pm 5.6\%$ of the initial isocyanate groups remained at 2 hours, indicating end-capping of most of the polyester prepolymer segments. At 20 hours – after the addition of dipyrindamole – $0.0 \pm 4.9\%$ of the isocyanate content remained, indicating complete reaction ($n=3$).

The four available hydroxyl groups on the dipyrindamole molecule enable the drug to be used as either a chain extender for the preparation of branched polymers when an average of two hydroxyl groups are reacted per dipyrindamole unit, or as a crosslinking unit for the assembly of polymer networks when more than two of its arms react with the isocyanate. The crosslink density can be altered by adjusting the ratio of the three components with respect to each other. We developed protocols enabling us to tune both the drug content of the material and the crosslink density, as shown in Table 2 – with the added requirement that in cases where the ratio of OH to NCO groups does not equal 1, the material would present free hydroxyl groups rather than NCO for the purposes of biocompatibility.

Briefly, in order to tune the crosslink density of the material with minimal alteration to the drug content, an uncrosslinked polyurethane was prepared (*DPY-PU 1* in Scheme 1). The dipyrindamole units in these uncrosslinked chains still contained an average of two available hydroxyl groups as sites for further reaction with isocyanates. The addition of an aliquot of HDI enabled the formation of crosslinks in the material with the density of bonds between chains determined by the ratio of remaining hydroxyl groups to isocyanate functionalities added. Theoretically, an overall feed ratio of NCO/OH = 1 results in full crosslinking of the material due to complete reaction of all available functional groups, whereas a ratio of NCO/OH < 1 results in only partial consumption of the available OH groups and lower crosslink density in the resulting polyurethane network. Experimentally, the ability of the feed ratio of hydroxyl to isocyanate groups to tune the crosslink density of the resulting

polyurethanes was supported by the observed trends in mechanical properties (Figure 2) and degradation profiles (Figure 3) of the variously crosslinked materials.

Fully crosslinked samples with varying dipyridamole content were prepared to explore the effect of pharmaceutical dose on the material cytocompatibility and platelet resistance without unduly impacting other crosslink related properties of the materials. In this study, in order to simplify the design, a feed ratio of $\text{NCO/OH} = 1$ was used to define theoretically complete crosslinking of the polymer network. For examination of the antiplatelet-containing materials' interaction with biological entities, three concentrations of drug were investigated, as seen in Table 2. Depending on the desired drug concentration, additional HDI could be added during the DPY addition step (*Step 2* in Scheme 1) in order to maintain the NCO/OH feed ratio at 1. Although not described in this study, different doses of drug incorporation could also be achieved for any desired crosslink density by altering the molecular weight of the initial PCL diol component.

2.2. Polymer mechanical properties

Current polymers used for engineering cardiovascular scaffolds generally possess suboptimal mechanical properties to match the native tissue dynamics, which often leads to the development of long term complications in grafts. A material for fabricating cardiovascular implants needs to be strong enough to withstand the forces exerted by the surgeon during implantation and elastic enough to match the compliance of the surrounding healthy native tissues to accommodate hemodynamics. Our initial hypothesis was that by covalently crosslinking our polymers, we could impart greater strength and elasticity to the polyurethane materials. Mechanical characterization by tensile analysis revealed that the materials are capable of significant elastic extension beyond their original length (Figure 2A). Tensile strain at failure for the polyurethanes ranged from $72.5 \pm 13.1\%$ when $\text{NCO/OH} = 1$ to $551.2 \pm 34.1\%$ when $\text{NCO/OH} = 0.75$, with all samples breaking at the grip. Although we expected to observe an increase in the material stiffness as we increased the crosslinking density, surprisingly tensile analysis revealed that the Young's moduli of our materials decreased dramatically with the formation of more crosslinks (Figure 2B). To account for this effect, differential scanning calorimetry (DSC) was used to analyze the polyurethane chain morphology, which revealed larger melting phase transitions in the less crosslinked polymers, indicating higher proportions of crystalline domains in those materials (Figure 2C). The decreasing polymer crystallinity content with increasing crosslink density likely results from the crosslinked polymer chains having less freedom to rearrange into an ordered crystal structure. As such, we have developed highly extensible strong elastomers with a tunable range of Young's moduli much better suited to recreating the natural compliance of native cardiovascular tissue compared with current clinically applied biocompatible polymers (Figure 2D).

2.3 Degradation and drug release

In addition to tuning the elastomeric tensile properties, covalent incorporation of dipyridamole into the polymer backbone also gave rise to homogenous distribution of the antiplatelet agent in the bulk materials to achieve locally sustained release at the site of regenerating cardiovascular tissue throughout the polymers' duration in the body.

Dipyridamole has previously been shown to improve the thromboresistance of polyurethane grafts when covalently tethered to the polymer surface^[35] or loaded into polymer scaffolds for diffusion-mediated drug delivery.^[30] However, surface coatings are susceptible to loss of pharmacological activity due to surface erosion, and depletion of untethered drug depots prior to scaffold degradation can leave the polymer grafts at risk of late stage thrombosis. Covalent incorporation of dipyridamole throughout the bulk polymer avoids the risk of losing the protective drug content prior to polymer degradation, as the drug component is only released when the surrounding polymer network breaks down, ensuring the presence of the antiplatelet drug at the material surface until the graft is fully resorbed.

Quantification of the polymer degradation rate and drug release at 37 °C in PBS (Figure 3) revealed that the materials degrade slowly in buffered aqueous conditions at physiological pH, as is typical of PCL and related polyurethanes.^[43, 47–50] Both the dipyridamole release and polymer degradation rate over the first 130 days diminish with increasing crosslink density in the materials, suggesting that the number of covalent linkages to the bulk network has a greater influence than the degree of polymer crystallinity on the loss of polymer structural components during this time. The level of dipyridamole in the PBS solution was quantified by UV-Vis spectroscopy, which found that the drug release curves were consistent with degradation mediated delivery. Mass spectrometry identified the unmodified dipyridamole unit as the major observable form of the drug present in the surrounding aqueous media during degradation, with several minor analogues also present (Figure S2, Supporting Information). The level of drug released daily into the PBS during the initial week of the trial (excluding the small burst release observed on the first day) ranged from $2.91 \times 10^{-3} \pm 1.60 \times 10^{-3}$ µg per mg of the fully crosslinked material to $6.40 \times 10^{-2} \pm 9.22 \times 10^{-3}$ µg per mg of the uncrosslinked polymer. The approximate mass of a thin-walled PTFE clinical vessel graft of 6 mm diameter and 20 cm length is 5000 mg.^[51] Based on our *in vitro* results, an equivalent mass of our polymers would yield a daily released dipyridamole concentration ranging between 3.1×10^{-3} µg mL⁻¹ to 6.8×10^{-2} µg mL⁻¹ when diluted throughout the adult human circulatory system (approximately 5 L in volume^[52]). These dipyridamole levels fall below the clinical systemic dose of 0.5–1.9 µg mL⁻¹,^[53] preventing the likelihood of off-target pharmacological effects outside the region of localized delivery. Broader ranges of drug doses could be achieved by increasing or decreasing the drug content in the polymer backbone. It should be noted that implants made from our polyurethanes might demonstrate altered degradation profiles in the body compared to *in vitro* conditions, as PCL and related polymers have been known to degrade faster *in vivo* than under simple hydrolytic conditions.^[54, 55] Although the degradation studies were not continued past 130 days, the initial mass loss behavior of the dipyridamole-containing polyurethanes is consistent with previous reports of PCL and related polymers, where an initial stage of degradation is dominated by chain scission and gradual mass loss which accelerates once the average chain length becomes short enough for the material to begin to lose structural stability.^[56] While some control over the initial rate of degradation of our polyurethanes was achievable through modification of the crosslink density, incorporating units of faster degrading polyester chains or enzyme-cleavable peptide sequences could further tune the degradation rate and drug release from the materials to meet the demands of different cardiovascular implants.

2.4 Cytocompatibility and cell proliferation

To explore the suitability of our materials as scaffolds for cardiovascular tissue regeneration, we confirmed their non-toxicity and ability to support cell adhesion and proliferation for two types of human vascular cells: human umbilical vein endothelial cells (HUVEC) and human umbilical vein smooth muscle cells (HUVSMC). For both cell types, the cells were seeded onto non-porous polymer disks (Figure S3, supporting information) punched from fully crosslinked materials representing a range of dipyridamole concentrations. The cells adhered well to the materials without the need for surface treatments like NaOH, which are often required to encourage cell adhesion on PCL.^[57–59] Over the course of one week, the cells showed consistent proliferation until they had reached confluence (Figure 4). The polyurethane surfaces supported full coverage by the HUVEC and HUVSMC, with no visible bare patches of the underlying substrates, which is important for full cellular repopulation of porous scaffolds and for minimizing the long term exposure of blood tissue to foreign surfaces. No differences were observed in the morphology of cells cultured on the polyurethane materials or on tissue culture polystyrene well plates.

To further confirm the lack of material cytotoxicity, HUVEC and HUVSMC were grown in cell culture media supplemented with PBS from the degradation and drug release study taken at 7, 30, and 120 days. Vascular cells cultured in a 1:1 mixture of cell culture media and degradation release media for 2 days showed no difference in overall viability from cells cultured in standard cell culture media as determined by live/dead staining. All cells were above 98% viable (Figure 5). The non-toxicity of the polyurethane degradation media is consistent with the materials' slow degradation (Figure 3) and observed cytocompatibility (Figure 4), suggesting that any potentially negative degradation products are released in low enough levels to not harm the cells in the culture. We further characterized the behavior of HUVEC cultured on the surface of our materials and subsequently embedded in a collagen environment. It is clear that HUVEC seeded onto our polymers can sprout and form capillary-like networks in the surrounding collagen gel (Figure S4, Supporting Information), further supporting the non-toxicity of our polymers and their promise as scaffolds that can maintain the viability and functionality of vascular cells.

2.5. Platelet adhesion and activation

While endothelial cell coverage provides the most promising method to ensure a fully hemocompatible scaffold surface, endothelialization can be a lengthy process and so any potentially blood-contacting material should demonstrate thromboresistance in case of scaffold exposure to the blood. In order to determine the ability of our antiplatelet-containing materials to resist platelet adhesion and activation, polymers containing a range of dipyridamole concentrations were exposed to a solution of platelet rich plasma (PRP) for 2 hours. PTFE, which is frequently used in cardiovascular implants, was used as a reference material and glass was used as a positive control.^[31, 33] After incubation of the samples in the PRP, a lactate dehydrogenase assay was used to measure the relative number of adhered platelets on the polymer disks, finding that the dipyridamole containing materials displayed values similar to the PTFE. The polymer with the highest drug content out of the materials tested showed a significant decrease in number of adhered platelets compared with the PCL-HDI no drug control, suggesting that the addition of the dipyridamole component to the

polymer chain can augment the surface thromboresistance. Examination of the samples by scanning electron microscope (SEM) after platelet adhesion revealed the degree of platelet activation as determined by platelet spreading and the number and length of pseudopodia (Figure 6).^[60, 61] In the case of the dipyridamole-loaded polymers, the number of pseudopodia and the degree of extension and flattening were relatively low, with many platelets still adopting a somewhat spherical morphology, as was also observed on the PTFE surface. Glass and PCL-HDI controls on the other hand showed high levels of platelets in more advanced stages of spreading, with extremely extended membranes. It is important to note that highly activated platelets were also observed on the dipyridamole-containing polymers and PTFE, however in both of these cases the relative number of platelets displaying higher levels of activation was small. These results suggest that the inclusion of dipyridamole decreases the activation of platelets on the polyurethane surfaces, supporting the suitability of our polyurethane substrates for blood-contacting interfaces in implants and devices. In our previous studies, we have characterized the *in vitro* platelet adhesion, protein adsorption, and hemolytic potential of related polycaprolactone-based polyurethanes and observed statistically equivalent or improved behavior compared to commercially available vessel graft materials.^[31] We anticipate that our new family of dipyridamole-containing elastomers has the potential for even greater hemocompatibility due to the tethering and slow release of the antiplatelet component. However, the effects of a sustained, localized low dose release of dipyridamole on the interaction between cardiovascular implants and their surrounding biological tissues can only be tested *in vivo*, which will be the subject of future investigations.

3. Conclusion

We have designed and synthesized a family of novel biodegradable polyurethanes with improved biocompatibility and mechanical properties for cardiovascular tissue engineering and regenerative medicine. Our novel polyurethanes are fabricated from readily available and cost effective reagents via a straightforward one-pot, catalyst-free synthetic method, well suited for benchtop production. The polyurethanes are significantly more elastic compared to most common materials used for cardiovascular implants, with a tunable range of moduli to better match native cardiovascular tissues. Covalent incorporation of dipyridamole directly into the polymer backbone enables sustained drug release regulated by polymer degradation, ensuring the localization of an antiplatelet drug at the blood-polymer interface throughout the lifetime of the implant. The polyurethanes support the attachment and proliferation of HUVEC and HUVSMC, while showing decreased adhesion and activation of platelets exposed to the surface, making these materials promising candidates for application in blood-contacting tissue engineered constructs. The modular synthetic approach could be easily adapted to include other hydroxyl and amine functionalized small molecules, peptides, or polymer units to further tune the material properties or to develop specialized materials uniquely suited for other niche applications. We anticipate that our polyurethanes, which were specifically designed to display a suite of characteristics not fully replicated by other common graft materials, will be useful substrates to supplement the cardiovascular tissue engineer's limited toolkit of biocompatible polymers suitable for blood-contacting applications.

4. Experimental Section

Materials:

Polycaprolactone (PCL; 2000 Da) was purchased from Sigma-Aldrich. Hexamethylene diisocyanate (HDI) was purchased from Fluka. Dipyridamole and dibutylamine were purchased from TCI. Dimethylformamide (DMF, anhydrous) was purchased from Fisher. All chemicals were used as received.

HUVEC were a gift from the laboratory of the late Dr J. Folkman (Children's Hospital, Boston)^[62] and were cultured in EBM-2 (endothelial basal media) supplemented with an EGM-2 (endothelial growth media) kit from Lonza, as well as 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine (PSG) from Life Technologies. Immortalized HUVERMC were purchased from ABM and were cultured following the recommendations using their prigrrow X medium. Rat tail high concentration collagen type I was purchased from Corning. Live/Dead viability/cytotoxicity kit was purchased from Life Technologies and LDH cytotoxicity assay kit was purchased from Thermo Scientific.

Synthesis of polyurethanes:

PCL (998.8 mg, 0.5 mmol, 1 eq) and HDI (0.16 mL, 1.0 mmol, 2 eq) were dissolved in DMF (1.0 mL) in a 20 mL vial at 60 °C under a dry N₂ atmosphere and the reaction was stirred for 2 hours. Then, dipyridamole (252.0 mg, 0.5 mmol, 1 eq) and additional DMF (1.0 mL) were added to the reaction vessel, mixed well, sealed under N₂, and placed in a 70 °C oven for 18 hours, yielding a highly viscous yellow solution at room temperature. To form crosslinks in the final product, an additional aliquot of HDI was added at this stage (between 0 – 1 eq). The reaction mixture was mixed thoroughly, then left 18 hours at 70 °C under N₂ atmosphere. A range of crosslink densities (DPY-PU 1–5) was achieved with the final ratio of NCO/OH in the product ranging between 0.67 – 1.

Alternatively, to produce a range of fully crosslinked polyurethanes with varying dipyridamole concentrations (DPY-PU 5–7), the quantity of dipyridamole added during the dipyridamole addition step can be varied between 0.5 – 1 equivalents of DPY per equivalent of PCL in the reaction mixture. An additional aliquot of HDI might be needed to maintain a final NCO/OH ratio of 1. The reaction mixture is then mixed thoroughly and left 18 hours at 70 °C under N₂ atmosphere.

To produce the PCL-HDI no drug control material, PCL (998.8 mg, 0.5 mmol, 1 eq) and HDI (0.16 mL, 1.0 mmol, 2 eq) were dissolved in DMF (1.0 mL) in a 20 mL vial at 60 °C under a dry N₂ atmosphere and the reaction was stirred for 2 hours. Then, additional DMF (1.0 mL) was added and the reaction vessel was mixed well, sealed under N₂, and placed in a 70 °C oven for 18 hours.

After the reactions were completed, the samples were placed in a 70 °C vacuum oven for 24 hours to remove the solvent with no further purification, producing non-porous polymer sheets (Figure S3, supporting information).

Structural characterization:

FTIR spectra were recorded with a Nicolet iS50 attenuated total reflectance (ATR)-FTIR spectrometer. Molecular weights of the prepolymers and uncrosslinked polyurethanes were analyzed by GPC using a Thermo Scientific UltiMate 3000 system equipped with Jordi Resolve DVB column and ERC RefractoMax 520 refractive index detector. DMF was used as the eluent with a flow rate of 1 mL min⁻¹. Samples were calibrated against polyethylene glycol standards. The active isocyanate content in the reaction mixture was determined by quenching the reaction with an excess of dibutylamine followed by back titration using hydrochloric acid (0.1 N), as recommended by the ASTM standard D2572–97. DSC analysis was performed on a TA Instruments Q2000 differential scanning calorimeter. Cyclical temperature scans at a rate of 10 °C min⁻¹ were performed to examine the thermal behavior of the polymers between –10 and 120 °C. Nuclear magnetic resonance (NMR) spectra were obtained for the uncrosslinked polymers on a Varian Inova 500 MHz instrument using deuterated chloroform as solvent (Figure S1, Supporting Information).

Mechanical characterization:

To investigate the relationship between the polymer crosslink density and Young's modulus, polyurethanes with a range of crosslink densities were examined by tensile analysis. The solid polymer samples were removed from the reaction flasks and cut into strips. Tensile measurements were performed on an Instron 5565 fitted with a 50 N load cell following the ASTM standard D412–16. Samples were extended until failure. All samples broke at the grips.

Degradation and drug release:

To measure the polyurethane degradation rate due to hydrolysis, polymer samples weighing approximately 30 mg were incubated in 1 mL of PBS at 37 °C. At designated time points, the samples were removed from the solution, patted dry, and weighed.

To measure the drug release profile of the polymers, the release media was removed and replaced with fresh PBS at designated time points. 100 µL of the collected release medium was mixed with 100 µL of ethanol and read at 405 nm using a Tecan Infinite F50 UV-Vis plate reader. Mass spectrometry was used to identify the presence of the dipyrindamole molecule and related degradation products released from the polymer into the surrounding PBS. The degradation solutions were first run on an Agilent 1100 high-performance liquid chromatograph before being analyzed on a Thermo LTQ XL linear ion trap mass spectrometer.

Cell proliferation:

Cells were cultured using the recommended culture media listed above and were incubated under standard conditions (37 °C, 5% CO₂, 95% humidity). For proliferation studies, 6 mm polymer disks were punched from a non-porous sample sheet using a biopsy punch. The samples were sterilized by submersion in a 1% anti-anti solution (Life technologies)^[63, 64] followed by drying in a sterile culture hood. The disks were then fixed to the bottom of a 24-well culture plate. HUVEC (passage 3) or HUVMSC (passage 3) were seeded in the wells at a cell density of 2.6×10^4 cells mm⁻² and 1.3×10^4 cells mm⁻², respectively. The cells were

allowed to adhere for 20 minutes before being moved to a cell culture incubator. Cell media was changed every 2 days. At the designated time points (days 1, 4, and 7), the cell media was removed and the cells were rinsed gently with PBS prior to detachment using trypsin. The cells were counted by Beckman Coulter Z2 particle counter with size thresholds set to exclude smaller debris particles. For determination of HUVEC sprouting behavior from the polyurethane surfaces, HUVEC were seeded on the surface of polymer blocks and allowed to grow to confluence. Collagen (3 mg mL^{-1}) was then cast around the polymer blocks and cell sprouting was examined by visible light microscopy over the course of 7 days.

Degradation product cytocompatibility:

To determine whether the degradation products of the polymers demonstrate any toxicity, aliquots from the degradation release study from day 7, day 30 and day 120 were saved and mixed with HUVEC or HUVSMC cell culture media in a 1:1 ratio. Cells were then cultured in the corresponding mixtures for 2 days, stained using a live (calcein AM)/dead (ethidium homodimer-1) viability cytotoxicity kit and counted by fluorescent microscopy to quantify the ratio of living to dead cells.

Platelet adhesion and activation:

Medical grade platelet rich plasma (PRP) was collected by the Stanford Blood Center in accordance with AABB standards. The PRP was prepared by centrifugation apheresis and contained 1.7×10^6 platelets μL^{-1} suspended in plasma and anticoagulant citrate dextrose. The white blood cell count (WBC) was below 23 WBC μL^{-1} . The active PRP was obtained 5 days after collection and used immediately, as received, within the clinical shelf-life of the product.^[65] The activity of the platelets was further confirmed by swirling.^[66] 5 mm disks were punched out of polymer sheets using a biopsy punch and were then submerged in PRP. The samples were gently agitated at room temperature for 2 hours, after which the polymer disks were rinsed two times with PBS. Any adhered platelets on the polymer samples were then lysed and the lactate dehydrogenase content measured by spectrophotometer using an LDH cytotoxicity kit (Pierce, Thermoscientific) following the suggested protocol. Serial dilutions of the PRP in PBS were concurrently lysed and analyzed for their LDH content in order to create a standard curve correlating LDH to the platelet count. To observe platelet morphology, samples were fixed overnight in a 0.1 M sodium cacodylate buffer (pH 7.3) with 2% glutaraldehyde and 4% paraformaldehyde (PFA), treated with 1% aqueous OsO_4 for 1 hour, dehydrated in graded ethanol series, dried by critical point drying (Tousimis Autosamdri 814), sputter coated with Pd/Au (Denton DeskII), and observed by scanning electron microscopy (Zeiss Sigma FESEM).

Statistical Analysis:

Data was analyzed using SPSS software (IBM). Homogeneity of variance was confirmed by Levene's test, followed by one-way ANOVA with Tukey's post hoc test to determine statistical significance. $P < 0.05$ was considered significant. Data are presented as means $\pm$ the standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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5. References

- [1]. Brisbois EJ, Handa H, Meyerhoff ME, Recent advances in hemocompatible polymers for biomedical applications, *Advanced Polymers in Medicine*, Springer, 2015.
- [2]. Kang H-W, Lee SJ, Ko IK, Kengla C, Yoo JJ, Atala A, *Nat. Biotechnol* 2016, 34, 312. [PubMed: 26878319]
- [3]. Rippel RA, Ghanbari H, Seifalian AM, *World J Surg* 2012, 36, 1581.
- [4]. Wu W, Allen RA, Wang Y, *Nat. Med* 2012, 18, 1148. [PubMed: 22729285]
- [5]. Everett W, Scurr DJ, Rammou A, Darbyshire A, Hamilton G, De Mel A, *Sci. Rep* 2016, 6, Article ID 26848.
- [6]. Claiborne TE, Slepian MJ, Hossainy S, Bluestein D, *Expert Rev. Med. Devices* 2012, 9, 577. [PubMed: 23249154]
- [7]. Greenwald S, Berry C, *J. Pathol* 2000, 190, 292. [PubMed: 10685063]
- [8]. Goldman M, McCollum C, Hawker R, Drolc Z, Slaney G, *Surgery* 1982, 92, 947. [PubMed: 6216621]
- [9]. Johnson WC, Lee KK, *J. Vasc. Surg* 2000, 32, 268. [PubMed: 10917986]
- [10]. Dodge JT, Brown BG, Bolson EL, Dodge HT, *Circulation* 1992, 86, 232–246. [PubMed: 1535570]
- [11]. Peck M, Gebhart D, Dusserre N, McAllister TN, L'Heureux N, *Cells Tissues Organs* 2012, 195, 144–158. [PubMed: 21996786]
- [12]. Pashneh-Tala S, MacNeil S, Claeysens F, *Tissue Eng. Part B* 2015, 22, 68–100.
- [13]. Sarkar S, Sales KM, Hamilton G, Seifalian AM, *J. Biomed. Mater. Res., Part B* 2007, 82, 100–108.
- [14]. Braune S, Gross M, Walter M, Zhou S, Dietze S, Rutschow S, Lendlein A, Tschöpe C, Jung F, *J. Biomed. Mater. Res., Part B* 2016, 104, 210–217.
- [15]. Trubel W, Moritz A, Schima H, Raderer F, Scherer R, Ullrich R, Losert U, Polterauer P, *ASAIO* 1994, 40, 273.
- [16]. Crapo PM, Wang Y, *Biomaterials* 2010, 31, 1626. [PubMed: 19962188]
- [17]. Ghista DN, Kabinejadian F, *Biomed. Eng. Online* 2013, 12, 129. [PubMed: 24330653]
- [18]. Hiob MA, She S, Muiznieks LD, Weiss AS, *ACS Biomater. Sci. Eng* 2016, 3, 712–723.
- [19]. Walker DK, Brendzel AM, Scotten L, *J. Heart Valve Dis* 1999, 8, 687. [PubMed: 10616249]
- [20]. Svennevig JL, Abdelnoor M, Nitter-Hauge S, *Circulation* 2007, 116, 1795. [PubMed: 17893279]
- [21]. Roudaut R, Serri K, Lafitte S, *Heart* 2007, 93, 137. [PubMed: 17170355]
- [22]. Dasi LP, Simon HA, Sucusky P, Yoganathan AP, *Clin. Exp. Pharmacol. Physiol* 2009, 36, 225. [PubMed: 19220329]
- [23]. Vlahakes G, *Circulation* 2008, 117, 302.
- [24]. Gott VL, Alejo DE, Cameron DE, *Ann. Thorac. Surg* 2003, 76, 2230.
- [25]. Cheung DY, Duan B, Butcher JT, *Expert Opin. Biol. Ther* 2015, 15, 1155. [PubMed: 26027436]
- [26]. Klopffleisch R, Jung F, *J. Biomed. Mater. Res., Part A* 2017, 105, 927–940.
- [27]. Hibino N, McGillicuddy E, Matsumura G, Ichihara Y, Naito Y, Breuer C, Shinoka T, *J. Thorac. Cardiovasc. Surg* 2010, 139, 431. [PubMed: 20106404]

- [28]. Schmidt D, Dijkman PE, Driessen-Mol A, Stenger R, Mariani C, Puolakka A, Rissanen M, Deichmann T, Odermatt B, Weber B, J. Am. Coll. Cardiol 2010, 56, 510. [PubMed: 20670763]
- [29]. Allen B, Sparks R, Welch M, Mason N, Mathias C, Clark R, J. Surg. Res 1984, 36, 80. [PubMed: 6228692]
- [30]. Punnakitikashem P, Truong D, Menon JU, Nguyen KT, Hong Y, Acta Biomater 2014, 10, 4618. [PubMed: 25110284]
- [31]. Mercado-Pagán ÁE, Stahl AM, Ramseier ML, Behn AW, Yang Y, Mater. Sci. Eng. C 2016, 64, 61.
- [32]. Mercado-Pagán ÁE, Kang Y, Findlay MW, Yang Y, Mater. Sci. Eng. C 2015, 49, 541.
- [33]. Mercado-Pagán ÁE, Ker DFE, Yang Y, J. Biomater. Appl 2014, 29, 557. [PubMed: 24913612]
- [34]. Eisert WG, Platelets, 3rd edition (ed. Michelson A), Elsevier, London, UK 2013, pp. 1155–1170.
- [35]. Aldenhoff YB, van der Veen FH, ter Woorst J, Habets J, Poole–Warren LA, Koole LH, J. Biomed. Mater. Res. Part A 2001, 54, 224.
- [36]. Pereira MJN, Ouyang B, Sundback CA, Lang N, Friehs I, Mureli S, Pomerantseva I, McFadden J, Mochel MC, Mwizerwa O, Adv. Mater 2013, 25, 1209. [PubMed: 23239051]
- [37]. Dey J, Xu H, Shen J, Thevenot P, Gondi SR, Nguyen KT, Sumerlin BS, Tang L, Yang J, Biomaterials 2008, 29, 4637. [PubMed: 18801566]
- [38]. Tatai L, Moore TG, Adhikari R, Malherbe F, Jayasekara R, Griffiths I, Gunatillake PA, Biomaterials 2007, 28, 5407. [PubMed: 17915310]
- [39]. Laurent S, Girerd X, Mourad J-J, Lacolley P, Beck L, Boutouyrie P, Mignot J-P, Safar M, Arterioscler. Thromb. Vasc. Biol 1994, 14, 1223.
- [40]. Wesley R, Vaishnav RN, Fuchs J, Patel DJ, Greenfield JC, Circ. Res 1975, 37, 509. [PubMed: 1182942]
- [41]. Mavrilas D, Missirlis Y, J. Biomech 1991, 24, 331. [PubMed: 1904875]
- [42]. Karimi A, Navidbakhsh M, Shojaei A, Faghihi S, Mater. Sci. Eng. C 2013, 33, 2550–2554.
- [43]. Suchkova G, Maklakov L, Vib. Spectrosc 2009, 51, 333.
- [44]. Guan J, Sacks MS, Beckman EJ, Wagner WR, J. Biomed. Mater. Res. Part A 2002, 61, 493.
- [45]. Materials Data Book Cambridge University Engineering, 2003.
- [46]. Eshraghi S, Das S, Acta biomater 2010, 6, 2467. [PubMed: 20144914]
- [47]. Elomaa L, Kang Y, Seppälä JV, Yang Y, J. Polym. Sci. Part A: Polym. Chem 2014, 52, 3307.
- [48]. Ratheesh G, Venugopal JR, Chinappan A, Ezhilarasu H, Sadiq A, Ramakrishna S, ACS Biomater. Sci. Eng 2017, 3, 1175.
- [49]. Woodruff MA, Hutmacher DW, Prog. Polym. Sci 2010, 35, 1217.
- [50]. Gorna K, Gogolewski S, Polym. Degrad. Stab 2002, 75, 113.
- [51]. Jørgensen C, Paaske W, Eur. J. Vasc. Endovasc. Surg 1998, 15, 416. [PubMed: 9633497]
- [52]. Marieb E, Hoehn K, Human Anatomy & Physiology, 9th edition. Pearson, Boston, USA 2013, p. 632.
- [53]. Rao GH, Handbook of platelet physiology and pharmacology Springer Science & Business Media, 2012.
- [54]. Pulkkinen M, Malin M, Böhm J, Tarvainen T, Wirth T, Seppälä J, Järvinen K, Eur J Pharm. Sci 2009, 36, 310–319.
- [55]. Chen J, Ma A, Lai Y, Chen Y, Cui M, J. Biomed. Eng 1997, 14, 334.
- [56]. Sun H, Mei L, Song C, Cui X, Wang P, Biomaterials 2006, 27, 1735–1740. [PubMed: 16198413]
- [57]. Serrano MC, Portolés MT, Vallet-Regí M, Izquierdo I, Galletti L, Comas JV, Pagani R, Macromol. Biosci 2005, 5, 415. [PubMed: 15895476]
- [58]. Recek N, Resnik M, Motaln H, Lah-Turnšek T, Augustine R, Kalarikkal N, Thomas S, Mozeti M, Int. J. Polym. Sci 2016, 2016, Article ID 7354396.
- [59]. Tan P, Teoh S, Mater. Sci. Eng. C 2007, 27, 304.
- [60]. Loftus JC, Albrecht R, Cell Biol J. 1984, 99, 822–829.
- [61]. Neffe AT, von Ruesten-Lange M, Braune S, Lützow K, Roch T, Richau K, Krüger A, Becherer T, Thünemann AF, Jung F, J. Mater. Chem. B 2014, 2, 3626–3635.

- [62]. Freedman DA, Folkman J, Cell Cycle 2004, 3, 809–814.
- [63]. Hofmann S, Stok KS, Kohler T, Meinel AJ, Müller R, Acta biomater 2014, 10, 308–317. [PubMed: 24013025]
- [64]. Shearer H, Ellis MJ, Perera SP, Chaudhuri JB, Tissue Eng 2006, 12, 2717–2727. [PubMed: 17518641]
- [65]. Reddoch KM, Pidcoke HF, Montgomery RK, Fedyk CG, Aden JK, Ramasubramanian AK, Cap AP, Shock 2014, 41, 54–61. [PubMed: 24169210]
- [66]. Maurer-Spurej E, Chipperfield K, Transf. Med. Rev 2007, 21, 295–306.

Figure 1.

FTIR transmittance spectra of the reaction mixture at $t = 0, 2,$ and 20 hours showing the loss of isocyanate peak and growth of urethane peak during polymerization.

Figure 2.

(A) Representative stress-strain curves of the polyurethanes of different feed ratios in Table 2 (DPY-PU 1–5). (B) The Young's modulus decreases with increasing crosslinking ($n=6$). (C) DSC curves of the polymers normalized by polymer mass reveal a T_m for all but the most crosslinked polymers. The area of the T_m peak increases with decreasing crosslinking, which reveals a larger crystalline content in the less crosslinked polymers. (D) Range of Young's moduli of our dipyrindamole-containing polyurethanes compared with moduli of PET and PTFE;^[45] unmodified PCL;^[46] and human radial artery (RA),^[39] saphenous vein (SV),^[40] aortic heart valve (AV; circumferential and radial),^[41] and coronary arteries (CA; healthy and atherosclerotic).^[42]

Figure 3.
(A) In vitro degradation of polymers with varying crosslink density carried out in PBS and
(B) concurrent release of dipyradomole measured by UV-Vis spectroscopy (n=4).

Figure 4.

(A) HUVEC and (C) HUVSMC proliferation over 7 days on polymer samples (n=6) containing different concentrations of dipyridamole: DPY-PU 5 (16.8 wt%), DPY-PU 6 (13.5 wt%), or DPY-PU 7 (9.7 wt%), PCL-HDI (no drug, 0 wt%); TCPS well plates were used as a control; (*) denotes statistical difference ($p < 0.05$). Bright-field microscopy showing confluent (B) HUVEC and (D) HUVSMC on the high drug polymer sample.

Figure 5. Representative images from live/dead staining of HUVEC and HUVSMC cultured in media supplemented with degradation products collected at various time points of the polymer degradation. No cytotoxic effects were observed (n=3).

Figure 6.

(A) Platelet adhesion on various surfaces measured by LDH assay ($n=6$); (*) denotes statistical difference ($p < 0.05$). (B) Representative platelet morphology revealing platelets in the early stages of adhesion and activation on the dipyridamole-containing polyurethanes (DPY-PU) and PTFE and in intermediate stages of spreading on glass,^[60, 61] observed by scanning electron microscopy. Scale bars 1 μm .

Scheme 1.

Synthesis of dipyradamole-containing polyurethanes. (*) denotes hydroxyl groups available for crosslinking.

Table 1.

Molecular weight data obtained by GPC relative to polyethylene glycol standard.

<i>Polymer</i>	<i>Time (h)</i>	<i>M_n (kDa)</i>	<i>M_w (kDa)</i>	<i>PDI</i>
<i>PCL</i>	<i>0</i>	<i>1.67</i>	<i>2.25</i>	<i>1.35</i>
<i>PU 1</i>	<i>2</i>	<i>4.05</i>	<i>6.57</i>	<i>1.61</i>
<i>DPY-PU 1</i>	<i>20</i>	<i>16.68</i>	<i>61.61</i>	<i>3.69</i>

Table 2.

Monomer feed ratios in the dipyridamole-containing polyurethanes (DPY-PU).

<i>Polymer</i>	<i>DPY content (wt%)</i>	<i>PCL : DPY : HDI (molar equivalents)</i>	<i>NCO/OH</i>
<i>Tuning crosslink density</i>			
<i>DPY-PU 1</i>	<i>17.8</i>	<i>4 : 4 : 8</i>	<i>0.67</i>
<i>DPY-PU 2</i>	<i>17.5</i>	<i>4 : 4 : 9</i>	<i>0.75</i>
<i>DPY-PU 3</i>	<i>17.3</i>	<i>4 : 4 : 10</i>	<i>0.83</i>
<i>DPY-PU 4</i>	<i>17.0</i>	<i>4 : 4 : 11</i>	<i>0.92</i>
<i>DPY-PU 5</i>	<i>16.8</i>	<i>4 : 4 : 12</i>	<i>1.0</i>
<i>Tuning dipyridamole content</i>			
<i>DPY-PU 5</i>	<i>16.8</i>	<i>4 : 4 : 12</i>	<i>1.0</i>
<i>DPY-PU 6</i>	<i>13.5</i>	<i>4 : 3 : 10</i>	<i>1.0</i>
<i>DPY-PU 7</i>	<i>9.7</i>	<i>4 : 2 : 8</i>	<i>1.0</i>