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Tyramine functionalization of poly(glycerol sebacate) increases the elasticity of the polymer

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Abstract

Poly(glycerol sebacate) (PGS) is an elastomer used widely in tissue engineering studies due to good biocompatibility. Hereby we report a tyramine functionalized PGS called PGS-TA. Tyramine adds a stronger physical bonding capability to PGS-TA. Tensile tests showed that the softness and toughness of the material were similar to PGS. However, PGS-TA demonstrated 16-folds increase of elastic deformations compared to PGS processed under identical conditions. The *in vitro* studies demonstrated that the viability, and metabolic activity of baboon smooth muscle cells were the same as those on tissue culture polystyrene. Porous subcutaneous implants of PGS-TA substantially degraded *in vivo* over two weeks, showing good biodegradability and biocompatibility. We expect PGS-TA to be useful for applications in tissues and organs that are subjected to large reversible mechanical deformations.

TOC image

Tyramine-functionalized PGS shows strong physical interactions that make the material more elastic to recover from large deformations.

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Keywords

Polyester; elastomer; biocompatible and biodegradable; tissue engineering; scaffold

Introduction

Many soft tissues such as ligaments, myocardium, veins and arteries are elastic and undergo reversible mechanical deformations repetitively.^{1–4} Repair and regeneration of these tissues can benefit from an elastomer that can sustain and recover from deformations reversibly without adverse impacts on the surrounding tissues.^{5–7} Here we report an elastomeric polyester, tyramine-functionalized poly(glycerol sebacate) (PGS-TA). The material is completely biodegradable and bioresorbable *in vivo*. Compared to PGS, PGS-TA shows significantly higher elasticity to restore from large deformations. We attribute these to the hydrogen bonding and π - π stacking interactions from the tyramine functionalities pendent on the PGS backbone. This material is potentially very useful for tissue engineering applications that require high tolerance of large mechanical deformations.

Many elastomers have been designed for biomedical applications, such as polyurethane, poly(ester urethane), polyester, poly(dimethylsiloxane) and hydrogels.^{8–13} The principles behind these elastic materials have their own features for each design. Generally, the crosslinking types, crosslinking density, the length of polymer chains between the crosslinking points, and physical interactions between the polymer chains play important roles in manipulating the mechanical properties and elasticity. PGS is a polyester bioelastomer with a Young's modulus typically ranging from 0.02 to 1.2 MPa and a maximal elongation up to approximately 300%, depending on the composition and curing condition.^{5, 14–17} This elastomer has been widely used for soft tissue engineering, such as repair of infarcted myocardium, synthetic vascular graft, cartilage regeneration, nerve conduits and retina transplantation.^{5–7, 14–16, 18, 19} A PGS prepolymer is synthesized by polycondensation between glycerol and sebacic acid. The prepolymer is then thermally crosslinked at 110 to 160 °C for a certain time to generate PGS elastomers for different uses.^{14, 15, 17} Both monomers are endogenous and bioresorbable molecules; glycerol is the basic building unit for lipids and sebacic acid is the natural metabolic intermediate of medium- to long-chain fatty acids.¹⁴ The elasticity of PGS is attributed to the covalent crosslinks and the hydrogen bonds between the random coils.¹⁴ Under reversible mechanical deformations, the hydrogen bonds repeatedly undergo association and dissociation to dissipate the stress. This reversible process prevents chemical bonds from breaking down, thus helping retain the integrity of the elastomer. Because the hydroxyl groups are directly attached to PGS backbone, a portion of them will be sterically hindered to bond under

dynamic deformations. Therefore, the goal of this research is to introduce functional groups that are pendent on the PGS backbone and more accessible to reach each other to form physical bonds. We hypothesize that these additional physical interactions will further enhance the elasticity of PGS, enabling the derivative to sustain larger mechanical deformations.

PGS has been modified to undergo different types of crosslinking or tune mechanical properties for different applications. For example, norbornene-functionalized PGS was made for photo-triggered crosslinking;²⁰ urethane as linkage was employed for crosslinking at room temperature.²¹ 2-ureido-4[1*H*]-pyrimidinone (UPy) has been employed to graft on the polyester backbone to introduce additional hydrogen bonds and tune the mechanical properties. However, UPy is not an endogenous molecule and the strong hydrogen bonding interactions it introduced necessitate special processing conditions of the derivative.^{22, 23} Thus, we designed the tyramine-functionalized PGS to introduce additional non-covalent interactions between tyramine moieties to reinforce the elasticity. In this case, we chose biocompatible molecules of succinic acid and tyramine for PGS modification.^{24, 25} Tyramine is a natural metabolic compound from the tyrosine amino acid that bears phenol group for π - π stacking interactions.^{26–28} In addition, phenol group is a proton donor that can establish hydrogen bonding interactions with proton acceptors such as phenolate ion, aromatic ring, and carboxylic acid or carboxylate groups.^{29, 30} The immobilized tyramine moieties are pendent on the PGS backbone with succinate as a spacer. In this way, the tyramine moieties are more accessible and more flexible and it is easier to establish physical interactions between them. Therefore, PGS-TA is expected to form multiple non-covalent interactions such as hydrogen bonding and π - π stacking interactions in the material. These physical bonds would enhance the elasticity and thus increase the capability to tolerate mechanical deformations of the elastomer. To our knowledge, tyramine is typically used to functionalize a biopolymer for horseradish peroxidase triggered oxidation to form a covalent crosslink.^{25, 31, 32} This work will demonstrate the utilization of tyramine to form physical interactions for biomaterial design.

Result and Discussion

1. Design and characterization of PGS-TA

The PGS-TA is synthesized in two steps using a commercially sourced PGS as a starting material (Fig. 1A). The initial PGS bears secondary hydroxyl groups directly attached to the backbone that are reacted with succinic anhydride to yield an intermediate succinate-functionalized PGS (PGS-SA). The PGS-SA is made to impart more carboxylic acid groups that are then coupled with tyramine molecules using a DCC/NHS coupling reaction to generate the resultant PGS-TA. We synthesized 15 and 25 mol% tyramine-functionalized PGS (PGS-TA₁₅ and PGS-TA₂₅) to compare the effects of tyramine content on material properties (Table 1). Different from the starting PGS and the intermediate PGS-SA, PGS-TA appears yellowish and is highly adhesive and elastic (Fig. 1B). We speculate that this is due to the introduction of tyramine moieties that bear phenol groups and easily form non-covalent interactions between them. Our study revealed that further increasing tyramine content yielded a PGS-TA that was very difficult to dissolve in organic solvent even with

sonication, likely due to excessive physical interactions between the polymer chains. This makes the polymer inconvenient for use. It is noted that the intermediate PGS-SA was obtained with a relatively low yield of 58.8% because of its loss during the purification process by precipitating in diethyl ether. However, both PGS-TA products were produced with yields of approximately 90–95% after a similar purification process (Table 1).

The coupling of tyramine to PGS-SA is examined by FT-IR spectroscopy (Fig. 2A). Compared with the spectra of PGS-SA and the tyramine control, both PGS-TA₁₅ and PGS-TA₂₅ show new absorptions of amide I and II bands at 1655 and 1550 cm⁻¹, indicating a successful coupling reaction between PGS-SA and tyramine. In addition, the resultant PGS-TA prepolymers also show spectra from both PGS-SA and tyramine components. The absorptions at 2926 and 2853 cm⁻¹ for methylene C-H stretching, 1732 and 1162 cm⁻¹ from ester bonds of C=O and C-O stretching are contributed by the PGS-SA component. The absorptions at 1516 and 830 cm⁻¹ are attributed to tyramine moieties because of the aromatic C=C stretching and aromatic C-H bending vibrations.^{30, 31} Furthermore, the unreacted secondary hydroxyl groups in PGS-SA show absorption at 3467 cm⁻¹ that turns into a broader band at 3367 cm⁻¹ in both PGS-TA spectra. It is likely that the absorptions of phenolic hydroxyl and amide groups from the immobilized tyramine moieties are merged with the unreacted secondary hydroxyl groups in the resultant PGS-TA. These FT-IR spectroscopic characteristics are consistent with the successful coupling reaction between PGS-SA and tyramine to yield PGS-TA products.

Proton NMR analysis further reveals the chemical compositions of PGS-SA and PGS-TA. The detailed chemical shifts of PGS-SA and PGS-TA are listed in Fig. 2B. Compared to PGS-SA and tyramine (Fig. S2), both PGS-TA₁₅ and PGS-TA₂₅ exhibit chemical shifts from PGS-SA and tyramine components. For example, chemical shifts (δ) at 6.97, 6.67, 3.18 and 2.58 ppm are assigned to the aromatic protons (H_n and H_o) and methylene protons (H_l and H_m) from tyramine moieties respectively. The integral area ratio of $H_n:H_o:H_l:H_m$ is approximately 1:1:1:1, matching well with the chemical structure of tyramine. The δ at 2.27, 1.51 and 1.24 ppm are attributed to methylene protons (H_d , H_e and H_{f+g}) from the sebacate component with integral area ratio of nearly 1:1:2, also showing agreement with the chemical structure of sebacate. In addition, the δ from 3.86 to 4.29 ppm are contributed by methylene protons (H_a and H_c) from glycerol component. The integral area ratio of H_{a+c} to H_d is approximately 0.85:1 by mole, representing the composition ratio of glycerol to sebacate in the starting PGS. The immobilized succinate shows methylene protons (H_j and H_i) at 3.35 and 3.49 ppm respectively. All these chemical shifts from different components demonstrated the desired chemical structure of the intermediate PGS-SA and resultant PGS-TA products.

To evaluate the actual contents of succinate and tyramine in the intermediate PGS-SA and the resultant PGS-TA prepolymers, the integral area ratios of H_i+H_j to H_d and H_n+H_o to H_d are used to quantify their compositions (Table 1, Fig. S1). The results show that PGS-SA bears approximately 13 mol% of actual succinate, indicating that about 52 % succinic anhydride was reacted with PGS to yield the intermediate. However, both PGS-TA₁₅ and PGS-TA₂₅ contain actual tyramine contents of 17 and 26 mol%, close to the theoretical contents of 15 and 25 mol%. The slight deviation between the actual and theoretical

tyramine contents might reflect the limited accuracy of the integration measurement of the proton NMR spectra. Nonetheless, these results demonstrated a nearly quantitative coupling reaction between tyramine and PGS-SA. Considering only 13 mol% of the actual succinate is in the intermediate, we speculate that tyramine molecules were also reacted with some terminal carboxylic acid or carboxylate groups from sebacate in PGS, not just coupled with the immobilized succinate moieties.

This is evidenced by the change of molecular weight distribution characterized by GPC analysis (Fig. S3). The GPC data demonstrate that the intermediate PGS-SA shows a single distribution band with molecular weight above 106 kDa referring to PEG standard. However, after coupling with tyramine, both PGS-TA products show a high molecular weight band along with a side band of low molecular weight between 1840 to 22100 Da. We estimate that the low molecular weight products are likely produced by aminolysis of the ester bonds with tyramine molecules that leads to the breaking down of some polyesters into short chains. This is why 15 and 25 mol% tyramine could be quantitatively coupled to the PGS-SA intermediate that has only approximately 13 mol% succinate functionalities. Unfortunately, we failed to find a suitable polymer standard to calibrate the molecular weight of the resultant PGS-TA that possesses pendent tyramine with strong physical interactions between the polymer chains. Here we use PEG standards as references primarily to identify the change of molecular weight distribution before and after PGS is coupled with tyramine.

Hydrophilicity is an important property for applications in tissue scaffolding of the as-made PGS-TA elastomer. We made a crosslinked PGS-TA film to examine the contact angle with deionized water in comparison to the PGS control (Fig. 3). The results demonstrate nearly the same contact angles among PGS-TA₁₅, PGS-TA₂₅, and PGS control. This result indicates that PGS functionalized with tyramine up to 25 mol% does not alter the hydrophilicity. This is because the hydrophilicity is related to the hydroxyl groups in the polymer. Although some secondary hydroxyl groups in the starting PGS are replaced with tyramine moieties, the tyramine moieties bear phenolic hydroxyl groups. Thus, the overall hydrophilicity of PGS-TA is nearly identical to the PGS control.

2. PGS-TA shows high elasticity and sustains large mechanical deformations

The main objective of introducing tyramine functionalities to PGS is to enhance the elasticity and improve PGS' capacity at sustaining larger mechanical deformations. On the other hand, the softness remains similar to that of PGS. To simplify, here the elasticity represents the ability of the designed elastomers to undergo reversible elastic deformations. The elasticity and the ability to recover from mechanical deformations are very important for an elastomeric scaffold. It will help the scaffold retain its integrity for use in a mechanically dynamic environment upon implantation.^{7, 33} To examine the elasticity and ability to recover from reversible deformations, cyclic loading mechanical (hysteresis) tests were performed on dog-bone samples of the PGS control, PGS-TA₁₅, and PGS-TA₂₅ elastomers (Fig. 4A and Fig. S4). The loading speed was 30 mm·min⁻¹ and strain was kept between 5 to 50%. Compared to the PGS control that finished only 1 cyclic loading, both PGS-TA₁₅ and PGS-TA₂₅ are able to undergo 9 and 16 cycles without hysteresis loop under the same testing conditions. This is approximately 9 to 16-folds increase of the capability recovering from

elastic deformations as the tyramine content increases to 15 and 25 mol%. Please note that these samples were cured at 150 °C for 24 h that is a longer time than what was typically used and reported in literature, thus the strain at fracture was lower for these samples.¹⁴ We further compared the elasticity of the PGS control and PGS-TA₂₅ cured for a shorter time (8 h). The PGS alone could undergo the cyclic loading up to 595 cycles, but the stress-strain curves demonstrate hysteresis loops with a reduced stress as the cyclic loading number increases (Fig. S4C). This indicates damages occurred in the crosslinked PGS network by the cyclic loading. However, PGS-TA₂₅ cured at the same condition is able to undergo the cyclic loading more than 1000 cycles with less hysteresis loop, indicating little damage in the PGS-TA₂₅ network during the cyclic loading test (Fig. S4D). These results indicate that the elasticity and ability to undergo mechanical deformations of both PGS and PGS-TA are highly related to the curing conditions and tyramine functionalities. Compared to the PGS alone processed under identical conditions, the tyramine functionalities could more efficiently dissipate the stress and protect the elastomer network from breaking down by dynamic deformations. This is the most notable and desirable property for the tyramine functionalization of PGS.

The elasticity of the PGS control is contributed by the covalent crosslinks between the random chains and the hydrogen bonds between the secondary hydroxyl groups on the backbone.¹⁴ However, PGS-TA possesses tyramine moieties to establish additional hydrogen bonding and π - π stacking interactions. FT-IR spectra show that both PGS control and PGS-TA elastomers demonstrate a nearly identical adsorption at 3467 and 3367 cm^{-1} before and after a thermal crosslinking (Fig. S7). The FT-IR data indicate that most secondary hydroxyl groups and phenolic hydroxyl groups remain intact for physical interactions between them. Compared to the secondary hydroxyl groups in the PGS control, the flexible pendent tyramine functionalities make the physical bonds association and dissociation easier under deformations during the hysteresis tests. As a result, the loading stress could be efficiently dissipated by the dynamic bonding, thus enabling PGS-TA elastomers to tolerate more deformations than the PGS control (Fig. 4A and Fig. S4). We would like to note that the hysteresis test was done with strain setup between 5 to 50%, which is much larger than many soft tissues such as ligaments and arteries typically suffer (typically < 20%).³⁴ We expect that the tolerance to cyclic deformations would further increase if the strain were set up between 5 to 20%. Furthermore, it is worth noting that both PGS control and PGS-TA elastomers show strain at fracture of approximately 50% when the materials are cured at 150 °C for 24 h (Fig. 4B). When the curing time is reduced to 8 h, the strain at fracture of the PGS control and PGS-TA₂₅ elastomers goes up to approximately 200 and 160% respectively (Fig. 4B). In this way, the hysteresis tests demonstrate a significant increase of elastic deformations from 1 to 595 cycles for the PGS control and 16 to more than 1000 cycles for PGS-TA₂₅ (Test was stopped at 1000 cycles without break of the sample.) (Fig. S4). We speculate that this is because the shorter curing time leads to a lower crosslinking density and thus a longer polymer chains between the crosslinking points. This makes the polymer network more flexible and easier to form more physical interactions between the polymer chains. As a result, both PGS and PGS-TA are able to undergo much more elastic deformations. More notably, tyramine functionalities further enhance the elastic performance of PGS-TA compared to PGS alone. Here, we used a commercial PGS to make

PGS-TA. The curing condition, molecular weight and polymer architecture are different with the previously reported PGS.¹⁴ Thus, this work demonstrated a different strain at fracture compared to prior PGS that was able to go up to approximately 300% if a lab-made PGS was used.¹⁴ We set up strain at 50% for hysteresis tests in order for an easier comparison of the enhanced elasticity by tyramine functionalization of PGS. Therefore, the hysteresis tests confirmed a significant enhancement of elasticity and the ability to restore from large mechanical deformations by tyramine functionalization. This notable property will undoubtedly promote the PGS-TA elastomer to retain its integrity and thus reduce potential mechanical irritation to the host due to the material damage during tissue regeneration process.

In addition, we want the as-made PGS-TA elastomers to be suitable for soft tissue engineering applications like PGS control. To compare other mechanical properties between the PGS-TA and PGS control, tensile tests were performed to examine the strain at fracture (%), ultimate tensile strength (UTS) and Young's modulus (E) (Fig. 4B, Fig. S5 and Table S1). Similar to PGS control, both PGS-TA₁₅ and PGS-TA₂₅ elastomers demonstrate a strain at fracture of ca. 50% and UTS of ca. 500 kPa when they are crosslinked at 150 °C for 24 h. The E values are increased approximately from 800 kPa for PGS control to 1000 kPa for both PGS-TA₁₅ and PGS-TA₂₅. It appears that the tyramine functionalization up to 25 mol% do not alter the mechanical properties significantly (Fig. 4B, 1, 2, 3). Combined with hysteresis test data, the results suggest that the tyramine functionalities would significantly reinforce the elasticity (Fig. 4A), but have little impact on the mechanical properties of strain at fracture, UTS and E. The latter are likely determined by the chemical crosslinks and crosslinking density.

Furthermore, the polymers crosslinked at 150 °C for a different time period are also investigated to compare the impacts of crosslinking density on the mechanical properties. As expected, both PGS control and PGS-TA₂₅ elastomers demonstrate a reduced strain approximately from 200% to 50% and 160% to 50% as the crosslinking time increases from 8 to 24 h (Fig. 4B, 4). Contrarily, the E values are elevated as the crosslinking time increases (Fig. 4B, 6). This is because a longer crosslinking time generates more crosslinks and thus shorter chains between the crosslinking points. This leads to a reduced strain and increased modulus. The UTS values of both PGS control and PGS-TA₂₅ show a change approximately from 380 to 500 kPa, but not statistically different, as the crosslinking time increases (Fig. 4B, 5). The UTS is typically determined by the ultimate strain and modulus of the elastomer that demonstrates a nearly linear elongation. In this case, the strain at fracture and modulus at each crosslinking time point show an opposite relationship (Fig. 4B 4, 6). Thus, the UTS at different crosslinking times remain relatively stable. Again, the strain at fracture, UTS and E values at three different crosslinking time points show no statistical differences between PGS control and PGS-TA₂₅ elastomers. Overall, these data further demonstrate that PGS-TA possesses similar mechanical properties to the PGS control for soft tissue engineering applications, but more importantly, the tyramine functionalization makes PGS stronger for uses.

3. *In vitro* cytocompatibility study

We designed the PGS-TA elastomer for potential applications in a synthetic vascular graft. Thus, the cytocompatibility is evaluated by directly seeding and proliferating baboon smooth muscle cells (BaSMCs) on a PGS-TA₂₅ coating. SMCs are chosen because they are important for remodeling the synthetic vascular graft to form a blood vessel.^{7, 35} Biocompatibility of PGS has been well established in prior work.^{14, 36} Here we chose PGS-TA₂₅ to represent tyramine modified PGS with a higher tyramine content to examine the impacts on the cell activity and viability. After culturing for 24 and 48 h with the coating, the cell metabolic activity is evaluated by MTT assay (Fig. 5A). In addition, live/dead assays are performed to compare the cell viability on PGS-TA coated surface and tissue culture polystyrene (TCPS) control (Fig. 5B–D).

The MTT assay demonstrates nearly the same cell metabolic activity on both PGS-TA₂₅ coating and TCPS control (Fig. 5A). Compared to the TCPS control, increasing the incubation time over 48 h leads to a slightly reduced, but not statistically significant, cell metabolic activity on PGS-TA₂₅ coating. Furthermore, live/dead assay microscopic images display nearly the same cell proliferating and spreading behavior on both cultures (Fig. 5B–D). After culturing for 48 h, both the PGS-TA₂₅ coating and the TCPS control show an increased cell death in a similar trend (Fig. 5C, E, red fluorescent spots). The *in vitro* studies demonstrate that the cell metabolic activity and viability are nearly the same on the PGS-TA coating as those on the TCPS control, indicating good cytocompatibility.

4. Morphologies and mechanical properties of the porous scaffolds

The porous scaffolds of the PGS control and PGS-TA₂₅ were made for *in vivo* biocompatibility study using a salt-leaching method (Fig. 6A, B).³⁵ We made the scaffolds by curing at 150 °C for 24 h because this condition has been established to make PGS vascular graft in our lab.^{7, 33} 75–150 µm NaCl salt was used as the template to give a nearly identical porous structure and pore size of both scaffolds (Fig. 6C, D). The mechanical stability of the porous scaffolds is important for applications. Thus, we evaluated the mechanical properties by compressive test before and after incubating the porous scaffolds in PBS (1×, pH 7.4) at 37 °C for 75 h (Fig. 6E). The stress-strain curves show elastic deformations of both PGS control and PGS-TA porous scaffolds (Fig. S6). The results indicate that both scaffolds demonstrate no statistical difference of the compressive modulus before and after incubation in PBS for 75 h at body temperature, indicating a stable structure for use.

5. *In vivo* biocompatibility study

Subcutaneous implantation of PGS-TA₂₅ and PGS control porous scaffolds in mice were used to investigate the host responses. All animals survived throughout the study without any malignancy, infection, or abscess at the implanted sites. At days 7 post-implantation, cells infiltrated in both scaffolds that made the implants partially degraded, particularly near the surface regions. But the scaffolds are still able to maintain the integral shape at this stage. Both implants nearly completely degraded over two weeks post-implantation. The PGS-TA₂₅ scaffold showed a slightly slower degradation than the PGS control (Fig. 7).

Tissues around the implantation show mild adverse responses such as inflammation and fibrosis, but no necrosis or muscle degeneration (Fig. 8, 9, 10). H&E staining shows phagocytic inflammatory infiltrates near the implants at days 3 post-implantation (Fig. 8A, B (1, 4)). The inflammatory infiltrates increase significantly at days 7 post-implantation and the cells migrate into and proliferate inside the implants (Fig. 8A, B (2, 5)). At this time point, more cells are observed in PGS control than in PGS-TA₂₅ implant. However, at days 14 post-implantation, it appears that more cells are observed in the PGS-TA₂₅ implantation site than in the PGS control (Fig. 8A, B (3, 6)). Furthermore, the surrounding tissues on both PGS-TA₂₅ and PGS control implantation sites show little inflammation. The native tissue alignment and muscle cell morphologies remain intact.

In addition, the cell infiltrates led to collagen deposition along with the implant degradation. MTS staining shows a relatively loose layer of collagen and a minimal deposition on the surface of PGS-TA₂₅ implant at days 7 post-implantation (blue fibrous structure) (Fig. 9A (1, 3), arrow pointed sites). However, at this time point, the collagen deposition around the PGS implant is somewhat denser, but the thickness shows no statistical difference compared to that around the PGS-TA implant (Fig. 9C). At days 14 post-implantation, both collagen depositions turn into a thicker network structure surrounding the two types of implant residues (Fig. 9A, B (2, 4)). The thickness of both collagen depositions increases significantly compared to those at days 7 post-implantation (Fig. 9C). A strip of PGS-TA₂₅ residue retains an integral form of the implant, whereas the PGS residue is degraded into small pieces at this stage (Fig. 9A, B (2, 4)). These differences indicate that the PGS-TA₂₅ implant likely undergoes a slower degradation than PGS control and proceeds mostly by surface erosion. The PGS-TA implant appears more stable to retain the scaffold shape due to the non-covalent interactions by the tyramine functionalization.

To further investigate the cell activities along with the implant degradation, we use CD68 and DAPI staining to detect the macrophage distribution around the implants (Fig. 10A–F). CD68 is a pan-macrophage marker and DAPI is used to stain the cell nuclei. The macrophages are quantified for comparison by randomly selecting six areas of the immunohistochemically stained sections around each implant (Fig. 10G). It is noted that both implants induce a mild inflammatory cell recruitment in the tissues adjacent to the implants by day 3 after implantation as revealed by CD68 and DAPI staining. This is typically attributed to a non-specific inflammatory response to the implanted material.³⁷ Interestingly, throughout the *in vivo* study, less macrophages are detected around the PGS-TA implant compared to the PGS control group, but it is not statistically different (Fig. 10G). At day 14 after implantation, the CD68 positive macrophages around the PGS-TA implant demonstrate a significant increase along with the implant degradation. However, the macrophage population remains relatively steady relative to the PGS implant. We speculate that the different macrophage distributions related to the two implants are likely determined by their different *in vivo* degradation behavior. The more stable PGA-TA implant gave a slower degradation that induced a relatively mild inflammatory response to the implant and its degradation components.

It is known that the inflammatory response to an implanted biomaterial activates the immune cells (e.g. macrophages) to initiate the production of inflammatory cytokines and

chemokines. These secreted peptides typically recruit more immune cells to the implantation site.^{37, 38} In this case, the recruited cells accumulate on the implant edge, then migrate into and proliferate inside the implants and digest the implants. The recruited cells secreted certain amounts of collagen locally that formed a network structure in the implantation sites during the implant degradation, leading to mild fibrosis. The subcutaneous studies showed no scar tissue and necrosis formed around the implantation sites. The fibrous collagen network does not appear to stop inflammatory cell infiltration and degradation of the implants (Fig. 8, 9, 10). We would like to note that PGS-TA₂₅ represents the PGS derivative with a higher tyramine content. Thus, the host responses represent a more severe result, but it still resembles the PGS control. Therefore, the good biocompatibility and biodegradability of PGS-TA are similar to the PGS control for utility.

Conclusion

We have reported a tyramine-functionalized PGS as an elastomeric material for soft tissue engineering applications. The PGS-TA elastomer demonstrated a 9 to 16-folds increase of elastic deformations depending on the contents of the tyramine motifs in PGS when the elastomers were cured at 150 °C for 24 h. Tensile tests showed that the softness and toughness of the PGS-TA were similar to the PGS control. The *in vitro* and *in vivo* studies demonstrated good biocompatibility, biodegradability and bioresorbability of the as-made PGS-TA elastomer. This elastomeric material is expected for tissue engineering applications where a scaffold is needed to tolerate reversible mechanical deformations, such as a synthetic graft for vascular regeneration, a conduit for nerve regeneration and a patch for myocardial repair. For example, in our lab, we use this material to make a PGS-TA graft for vascular regeneration. The PGS-TA graft is used to compare with the PGS control to examine if the enhanced elasticity would affect the regeneration behavior and the properties of the neoartery. We would like to note that this study demonstrated the strong physical bonding ability of tyramine for the material design, instead of using tyramine's enzymatically triggered oxidation for a covalent crosslinking. This feature would likely expand tyramine utilities to design other elastic biomaterials.

Experimental Section

1. Synthesis of tyramine-functionalized poly(glycerol sebacate)

Tyramine-functionalized poly(glycerol sebacate) (PGS-TA) was synthesized in two steps. First, a commercial PGS (Regenerez™ RG-100 Series, Secant Medical®, PA, USA) (19.28 g, 75 mmol based on PGS repeat unit) and succinic anhydride (Alfa Aesar, 99%) (1.88 g, 18.8 mmol, 25 mol% related to PGS repeat unit) were mixed in 50 mL of 1,4-dioxane anhydrous (ACROS, 99.0%) in a round bottom flask with magnetic stirring for approximately 1 h to yield a clear solution. Then 1.55 mL (19.2 mmol) of pyridine anhydrous (Alfa Aesar, 99.5+%) was added to the solution. The reaction was performed in a glovebox at room temperature for 3 h under nitrogen atmosphere to yield PGS-SA₂₅ (25 mol % succinate). The reaction solution was precipitated in 2 L anhydrous diethyl ether with gentle magnetic stirring for 6 h and then settled in a -20 °C fridge overnight before decanting the supernatant. The solvation and precipitation were repeated twice. Finally, the

PGS-SA₂₅ solution was precipitated in six centrifugation tubes loaded with 40 mL of chilled diethyl ether in each tube. The solids were collected by centrifugation at 3000 rpm for 20 min and dried under reduced pressure to obtain PGS-SA₂₅ with yield of 58.8 wt.%.

In the second step, the PGS-SA₂₅ was coupled with tyramine (ACROS, 97%) using a DCC/NHS coupling reaction (ALDRICH, 99% and Alfa Aesar, 98+%) in the presence of catalyst amount of 4-(Dimethylamino)pyridine (DMAP) (AVOCADO, 99%) to yield tyramine-functionalized PGS (PGS-TA₁₅ or PGS-TA₂₅, 15 or 25 mol% of tyramine). Specifically, 4.20 g of PGS-SA₂₅ (containing approximately 7.1 mmol succinate) was dissolved in 10 mL DMF anhydrous (ACROS, 99.8%) in a glass vial. Then, the coupling agents, DCC/NHS/DMAP (1.60/0.815/0.009 g, 7.8/7.1/0.071 mmol), were dissolved in 3 mL DMF and added to react for approximately 15 min to activate the carboxylic acid groups on PGS-SA₂₅. A pre-dissolved tyramine in DMF solution (0.972 g in 13 mL, 0.55 mmol/mL) was then slowly added with magnetic stirring to perform a coupling reaction for 21 h in the glovebox to yield cloudy PGS-TA₂₅ solution. The product solution was then transferred into six 15 mL centrifuge tubes and performed centrifugation twice at 3500 rpm for 15 min to remove the insoluble byproducts. The clear supernatant was collected and precipitated in 1 L of diethyl ether with gentle magnetic stirring for 6 h and settled in a -20 °C fridge overnight before decanting the supernatant. The solvation and precipitation were repeated twice. Finally, the product solution was precipitated in four 50 mL centrifuge tubes containing 40 mL of chilled diethyl ether each tube. The viscous sediments were collected by centrifugation at 3500 rpm for 15 min and washed twice using diethyl ether (approximately 12 h per wash) with orbital rotation to remove DMF residue. The viscous PGS-TA₂₅ was dried at room temperature under reduced pressure for three days with yield of 95.6%. The PGS-TA₁₅ was similarly synthesized using 15 mol% tyramine relative to PGS-SA₂₅ unit. All chemical agents were used as received.

2. Polymer characterization

The molecular weights of the as-made PGS-SA₂₅, PGS-TA₁₅ and PGS-TA₂₅ were characterized by gel permeation chromatography (GPC, Viscotek GPCmax VE2001, Malvern Instruments Ltd, UK), equipped with Viscotek 270 dual detector and VE 3580 RI detector.) Two Gram GPC Analytical columns of 1000 Angstrom, 10 Micron and dimension of 8 × 300 mm (Polymer Standard Service-USA Inc.) and DMF were used as stationary and mobile phases respectively. 1 mg/mL of each polymer sample in DMF solution was prepared and filtered through a 0.2 µm syringe filter before testing. Poly(ethylene glycol) was used as a standard for molecular weight calibration with concentration prepared at 1 mg/mL.

FTIR (NICOLET iS10, Thermo SCIENTIFIC, USA) and ¹H-NMR (400 Hz, Bruker, MA) were used to examine the coupling reaction between PGS-SA₂₅ and tyramine and verify the chemical structure and the tyramine composition.

3. Contact angle measurement to evaluate the hydrophilicity of PGS-TA and PGS control

The polymer films were made by casting prepolymer solutions (0.5 mL, 10 % w/v in acetone/THF 5:1 v/v) on glass slide surface and air-dried for 24 h, followed by vacuum drying at 60 °C for 24 h. The films were then crosslinked at 150 °C for 24 h under reduced

pressure and then cooled down to room temperature for 3 h before test. The contact angle was measured using attension (by KSV Instruments).

4. Prepare crosslinked PGS and PGS-TA films for mechanical study

The PGS control, PGS-TA₁₅, and PGS-TA₂₅ were separately dissolved in acetone/THF (5:1, v/v) with help of sonication to yield 10 % w/v solution for use. A rectangular silicone rubber mold (4×1.5×0.1 cm) was mounted on a glass slide for a film preparation. For easy detachment of the crosslinked film from the mold, a thin layer of hyaluronic acid gel (1 wt %) was applied to the glass surface and dried under reduced pressure overnight. 1 mL of the PGS or PGS-TA solutions was transferred into the mold and air-dried for about 4 h each time until 4 mL of each polymer solution was subsequently transferred into the mold. The prepolymer films were air-dried in a fume hood for approximately 24 h and further dried under reduced pressure for 3 days before a thermal crosslinking.

The PGS and PGS-TA films were cross-linked at 150 °C for 8, 16 and 24 h under reduced pressure at 30 in. Hg vac in a vacuum oven (Thermo SCIENTIFIC). The crosslinked polymer films on the molds were then immersed into deionized water for 24 h and gently peeled off from the substrates. After air-drying, the transparent films with thickness of ca. 500 µm were obtained for mechanical tests.

5. Mechanical properties tests

Dog-bone samples were punched from the crosslinked PGS and PGS-TA films using a dog-bone cutter (3.0×0.5 cm). Tensile and hysteresis tests were performed according to the standard method (ASTM D412) using MTS Insight™ instrument (MTS Systems Corp., MN, USA) equipped with a 50 N loading cell. An elongation rate was set up at 10 mm per minute for tensile test and 30 mm per minute for hysteresis test. The hysteresis tests were repeated thrice to obtain an average cyclic loading numbers for each sample; whereas the tensile tests for each sample were replicated at least five times to obtain the mean values with standard deviation of ultimate tensile strength (UTS), young's modulus (E) and strain at fracture (Strain %).

6. Prepare PGS and PGS-TA porous scaffolds using a salt template-leaching method

10 % w/v solutions of PGS or PGS-TA₂₅ in acetone/THF (5:1, v/v) were prepared for use. NaCl salt was ground into fine particles with diameter between 75 to 150 µm. 4 g of the salt was evenly spread into a circular metal mold (D=6 cm, thickness=1 mm). Then 13.3 mL of the polymer solution was slowly dropped onto the center of the salt template. The polymer solution was evenly diffused throughout the salt template. After evaporation of solvent at room temperature for approximately 12 h, the sample was further dried under reduced pressure for 24 h to yield PGS or PGS-TA₂₅/salt composites (polymer/salt mass ratio=1:3). The composite samples were crosslinked at 150 °C for 24 h. After cooling down to room temperature, both samples were immersed in deionized water for at least 48 h to wash away salt with replacement of deionized water every 24 h. The washed PGS-TA₂₅ and PGS foams were freeze dried for use.

7. Mechanical tests of the porous scaffolds

The mechanical properties of the as-made porous PGS and PGS-TA₂₅ scaffolds were examined by compressive test using MTS Insight™ instrument equipped with a 50 N load cell as previously reported.³⁹ Disc-shaped samples of the porous scaffolds (9.52 mm in diameter and 1 mm in thickness) were punched, autoclaved 20 min and subsequently washed with 70, 50, 25 and 0 % w/v of ethanol in PBS, 15 min each. One set of the scaffold samples were evaluated immediately after the washing, and another set of the samples were tested after they were incubated in PBS at 37 °C for 75 h. Each sample was preloaded at 0.0 N, and compressed from 0 to 30% strain at a rate of 1.00 mm/min. The test for each sample was replicated three times (n=3) to obtain an average compressive modulus. The compressive modulus was determined according to the slope of the stress-strain curve between 5 – 20% strain.

8. *In vitro* cytocompatibility study

An *in vitro* cytotoxicity assay was performed on PGS-TA₂₅ coating using primary baboon smooth muscle cells (BaSMCs) according to the standard protocol (ISO 10993). BaSMCs were obtained from fresh carotid arteries of 17–20 kg juvenile male baboons and thoroughly characterized.³⁶ BaSMCs (passage 4) were cultured in MCDB131 (Mediatech, Manassas, VA) containing 10% FBS (Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (Mediatech, Manassas, VA), and supplemented with 1% L-glutamine at 37 °C with 5% CO₂ until sufficient quantities were obtained.^{36, 40} The cells were diluted in cell media to 5×10⁴ cell mL⁻¹ for use.

1 % w/v of PGS-TA₂₅ in DMF was prepared and 22 µL of the solution was evenly spread on a 15 mm diameter circular cover glass. A PGS-TA₂₅ coating with thickness of approximately 1000 nm was formed on the cover glass after the slide was air-dried in a fume hood overnight and further dried in a vacuum oven at 90 °C for 4 h. The coated slides were placed into a 24-well culture plate with the coating layer orientated upward. The coating was subsequently washed with 75, 50, 25% w/v of ethanol in deionized water solutions and DPBS 1× (20 min per wash). Then 5×10⁴ cells per well were seeded on the PGS-TA₂₅ coating. The plate was incubated at 37 °C with 5% CO₂. After incubation for 24 and 48 h, cell viability (MTT assay, n=3) was determined by a CellTiter-Blue Cell Viability Assay Kit (Promega, Madison, WI). Live/dead assay (n=3) was performed using LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Carlsbad, CA). The absorbance and fluorescence were recorded by a SynergyMX plate reader (Biotek, Winooski, VT). The fluorescent microscopic images of the live/dead assay were recorded using a Nikon ECLIPSE Ti Fluorescence Microscope (Nikon Instruments Inc., NY, USA). The BaSMCs cultured in a 24-well tissue culture polystyrene plate (TCPS) were used as a control.

9. *In vivo* biocompatibility study

Male BALB/cJ mice (Jackson Laboratory) with an average age of 8–9 weeks were used and cared for in compliance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

The PGS-TA₂₅ and PGS foams prepared in section 5 were punched into circular foams with a diameter of 6 mm and thickness of 1 mm. The foams were sterilized by autoclave prior to the subcutaneous implantation. Under isoflurane anesthesia, the PGS-TA₂₅ and PGS foams were implanted in the left and right back of BALB/cJ mice. At days 3, 7 and 14 after implantation, the animals were sacrificed and the tissues were harvested at the implantation sites. Tissues were fixed in 10% formalin for 15 min, and then soaked in 30% sucrose and embedded in the Tissue-Tek optimum cutting temperature (O.C.T) compound (Sakura Finetek USA). Cross sections (6- μ m thick, longitudinal axial cut) were stained with hematoxylin and eosin (H & E), Masson's trichrome stain (MTS) and ED-1 to examine host responses such as inflammation, collagen deposition or any adverse effects. For ED-1 immunohistochemical analysis, 6- μ m thick sections of the tissues were dried and fixed in histology-grade absolute ethanol for 15 min, air dried, and incubated with rat monoclonal anti-CD68 (1:200, Abcam, Cambridge, MA) for ED-1 identification. The slides were then incubated with a goat anti-rat-Alexa 594 (1:400, Life technologies, Carlsbad, CA) for 1 hour. ED-1 stained sections were analyzed for the density of newly recruited macrophages. Five to ten 200 $\times$ magnification images were obtained for each specimen. The images were taken using an inverted microscope Eclipse Ti (Nikon, Melville, NY) equipped with a digital camera (QImaging, BC, Canada).

The number of CD68 positive macrophages was counted and confirmed by DAPI-positive nuclei. The value was divided by the area of the imaged tissue to obtain macrophage number per unit area (MP per mm²) and a mean value was calculated based on the six images per group.

10. Statistic analysis

Statistic analysis was performed using one-way ANOVA with post-hoc Bonferroni correction. A *p* value < 0.05 is considered significant. Data represent mean $\pm$ standard deviation (SD).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

(A) Chemical reaction to synthesize a tyramine-functionalized PGS, PGS-TA_x (x=15 or 25 mol%). A commercial PGS is reacted with succinic anhydride to yield an intermediate PGS-SA. The PGS-SA bears more carboxylic acid groups from the immobilized succinate moieties that are then coupled with tyramine to yield the resultant PGS-TA. (B) Photographs of the three prepolymers. (I) the commercial PGS, (II) PGS modified by succinic anhydride, PGS-SA and (III) PGS derivative with tyramine, PGS-TA₂₅. Different from the starting PGS and the intermediate PGS-SA, PGS-TA₂₅ is very adhesive and displays high elasticity due to the introduction of tyramine functionalities.

Fig. 2.

(A) Comparison of FT-IR spectra of tyramine (TA), PGS-SA and the resultant PGS-TA products. Both PGS-TA₁₅ and PGS-TA₂₅ show new absorptions of amide I and II at 1655 and 1550 cm⁻¹, indicating a successful coupling reaction between PGS-SA and tyramine. (B) Proton NMR spectra identify the chemical composition of the as-made PGS-TA. Compared to the spectrum of PGS-SA, both PGS-TA prepolymers show the chemical shifts from PGS-SA and tyramine moieties. The actual succinate and tyramine contents in PGS-

SA and PGS-TA products are determined using the integral area ratios of H_{i+j} to H_d and H_{n+o} to H_d (Supporting Information, Fig. S1).

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Fig. 3. Comparison of contact angles among the crosslinked PGS, PGS-TA₁₅ and PGS-TA₂₅ films. Both PGS-TA₁₅ and PGS-TA₂₅ show nearly identical contact angles to the PGS control, indicating the tyramine functionalization does not impact the hydrophilicity.

Fig. 4.

Comparison of mechanical properties of PGS-TA and PGS control elastomers. (A) Hysteresis tests ($n = 3$). (1) PGS control, (2) PGS-TA₁₅, and (3) PGS-TA₂₅. All elastomers demonstrate nearly no hysteresis loop over cyclic loading. PGS control finished only 1 cyclic test, indicating weak resistance to large deformation. However, PGS-TA₁₅ and PGS-TA₂₅ can undergo 9 and 16 cycles, indicating 9 to 16 folds increase of the capability resistant to the mechanical deformations. The hysteresis data were handled by averaging 21 data points to obtain smooth curves. (B) Tensile tests to compare the mechanical properties of PGS control and PGS-TA elastomers ($n = 4$). The relationship of tyramine content and curing time to strain at fracture (1, 4), ultimate tensile strength (UTS) (2, 5), and Young's modulus (E) (3, 6) of PGS and its tyramine derivatives. Compared to the PGS control, all PGS-TA elastomers show similar properties of strain at fracture, UTS and E with no statistic difference, indicating suitability for soft tissue engineering applications. One-way ANOVA followed by Bonferroni correction is performed for a statistic analysis. $P < 0.05$ is considered significant. Data represent mean $\pm$ SD.

Fig. 5.

(A) MTT assay to evaluate the metabolic activity of BaSMCs on the PGS-TA₂₅ coating and TCPS control. After culturing for 24 h, the cells on PGS-TA₂₅ coating show a nearly identical metabolic activity to TCPS control. Further incubating over 48 h, PGS-TA₂₅ coating demonstrates a slightly reduced cell metabolic activity, but not statistically significant, compared to the TCPS control. Live/dead assay microscopic images are recorded on (B, C) PGS-TA₂₅ coating and (D, E) TCPS control at 24 and 48 h, respectively. The BaSMCs were able to proliferate and spread on PGS-TA₂₅ coating in a similar fashion to those on the TCPS control. After incubating for 48 h, the dead cells (red spots) increased

slightly on both PGS-TA₂₅ coating and TCPS control. These results demonstrated little impact of the PGS-TA₂₅ coating on the cell viability and metabolic activity, indicating good cytocompatibility. One-way ANOVA followed by Bonferroni correction is performed for a statistic analysis of MTT assay. $P = 0.1346$, $P < 0.05$ is considered significant. Data represent mean $\pm$ SD ($n = 3$).

Fig. 6.

(A, B) Photographs of the PGS control and PGS-TA₂₅ porous scaffolds made by salt-leaching technique. (C, D) SEM micrographs show the porous morphologies of the scaffolds (A and B) from the cross-sectional view. Both scaffolds show nearly identical porous structures. (E) The compressive modulus (E, kPa) of the porous PGS and PGS-TA₂₅ scaffolds before and after incubating in PBS (1×, pH 7.4) at 37 °C for 75 h. The original stress-strain curves are shown in Fig. S6. The compressive modulus of both scaffolds shows no statistic difference before and after incubation, indicating a stable porous structure for use. One-way ANOVA followed by Bonferroni correction is performed for a statistic analysis of the compressive modulus. $P = 0.3533$, $P < 0.05$ is considered significant. Data represent mean $\pm$ SD (n = 3).

Fig. 7.

In vivo degradation of PGS-TA₂₅ and PGS control implants in BALB/cJ mice by subcutaneous implantation. Both implants substantially degraded over two weeks post-implantation. No malignancy, infection or abscess were observed at the implanted sites. The PGS-TA₂₅ implants degraded slightly slower than the PGS control.

Fig. 8.

In vivo biocompatibility study. Micrographs of H&E stained sections surrounding the implants, (A) PGS-TA₂₅ and (B) PGS control. White star marks the implanted sites. Low magnification of images (1–3) represent the H&E stained tissues harvested at days 3, 7 and 14 respectively, (Scale bar, 500 μm). Rectangular frames indicate the region chosen for higher magnifications (4–6), (Scale bar, 100 μm).

Fig. 9.

MTS stained tissues illustrate collagen deposition after implantation, (A) PGS-TA₂₅, and (B) PGS control. The arrows point to the collagen deposition on the surface of the implants. Low magnification of images (1, 2) are the tissues harvested at days 7 and 14 post-implantation (Scale bar, 500 μm). Rectangular frames indicate the regions chosen for higher magnifications (3, 4), (Scale bar, 100 μm). (C) The thickness of collagen deposition (Col. Thick., μm) around both implants are quantified for comparison at days 7 and 14 post-implantation. For each group, 8 locations are randomly selected from three MTS stained

micrographs and measured to obtain the mean value of thickness with standard deviation. One-way ANOVA followed by Bonferroni correction is performed for a statistic analysis of the compressive modulus. $P^{***} = 0.0001$, $P < 0.05$ is considered significant. Data represent mean $\pm$ SD (n = 8).

Fig. 10.

Representative micrographs of immunohistochemically stained sections of CD68 positive macrophages merged with DAPI staining (Scale bar, 100 μm). (A-C) PGS-TA implant, and (D-F) PGS implant. Tissues were harvested at days (A, D) 3, (B, E) 7 and (C, F) 14. (G) The number of CD68 positive macrophages (MP per mm²) at different time points after implantation. The population of CD68 positive cells increases significantly from day 3 to day 14 along with the PGS-TA implant degradation; whereas PGS control implant induces somewhat more and a relatively steady inflammatory response throughout the *in vivo* study. Images from 6 random areas around the implantation site are used for quantification. $P^{***} = 0.0006$, $P < 0.05$ is considered significant. Data represent mean $\pm$ SD (n = 6).

Table 1

Summary of reactant ratio, actual tyramine content and yield of each polymer.

Polymer	Reactant ratio by mole ^a	Yield, %	SA or TA ^b Theo./Act., mol%
PGS-SA	SA:PGS 0.25:1	58.8	25/13
PGS-TA ₁₅	TA:PGS-SA ₂₅ 0.15:1	90.0	15/17
PGS-TA ₂₅	TA:PGS-SA ₂₅ 0.25:1	95.6	25/26

^aThe reactant ratio is calculated based on succinic anhydride (SA) or tyramine (TA) to the repeat unit of PGS or PGS-SA. When calculating the ratio of TA to PGS-SA, succinic anhydride is assumed to completely react with PGS.

^bThe actual SA or TA contents in the PGS-SA or PGS-TA are determined by proton NMR analysis (Supporting Information, Fig. S1).