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Controlled Release of Salicylic Acid from Biodegradable Crosslinked Polyesters

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ABSTRACT

The purpose of this work was to develop a family of crosslinked poly(xylitol adipate salicylate)s with a wide range of tunable release properties for delivering pharmacologically active salicylic acid. The synthesis parameters and release conditions were varied to modulate polyester properties and to understand the mechanism of release. Varying release rates were obtained upon longer curing (35 % in the non-cured polymer to 10 % in the cured polymer in seven days). Differential salicylic acid loading led to the synthesis of polymers with variable crosslinking and the release could be tuned (100 % release for the lowest loading to 30 % in the highest loading). Controlled release was monitored by changing various factors and the release profiles were dependent on the stoichiometric composition, pH, curing time and presence of enzyme. The polymer released a combination of salicylic acid and disalicylic acid and the released products were found to be non-toxic. Minimal hemolysis and platelet activation indicated good blood compatibility. These polymers qualify as "bioactive" and "resorbable" and can, therefore, find applications as immunomodulatory resorbable biomaterials with tunable release properties.

Keywords: Polyester; Salicylic acid; Controlled release; Biodegradable polymers

1. INTRODUCTION

Biodegradable polymers constitute a popular class of biomaterials that are widely studied for use in a variety of resorbable implants and medical products such as sutures, fracture fixation devices, tissue scaffolds and drug/gene delivery vehicles,[1] etc. These polymers are typically prepared from biochemicals endogenous to the human body such that the degradation products can be rapidly metabolized and eliminated with minimal cytotoxicity. Yet these biodegradable polymers are observed to elicit inflammatory and foreign body responses *in vivo*.[2, 3] Other common causes of failures of such implants include bacterial infections,[4] poor blood compatibility,[5] and insufficient vascular growth. The controlled release of drugs[6, 7] and biomolecules such as growth factors[8] loaded in the biomaterial are used to overcome such potential complications and to enhance the performance of the implant. An increasingly popular strategy is the direct incorporation of the drug into the polymer backbone containing hydrolysable bonds to aid in controlled drug release complemented with gradual degradation of the polymer.[9] A recent review discusses this important aspect of release accompanied with polymer degradation.[10]

Non-steroidal anti-inflammatory drugs or NSAIDS are a class of Cox-1 and/or Cox-2 inhibitors[11] that suppress inflammation. Aspirin, a salicylic acid derivative, is one of the most popular NSAIDs that acts as an analgesic, anti-pyretic, anti-thrombotic, and anti-inflammatory agent.[12] Salicylic acid (SA) has received significant attention because of its anti-bacterial, anti-fungal, and anti-inflammatory activities. It has also been shown to be a promising drug for preventing cardiovascular diseases and cancer.[13, 14] Thus, a rapid release of SA may be desired for anti-microbial and anti-inflammatory applications such as treatment of infections and as coating on biomedical devices. A more sustained release may be appropriate for reducing the

risk of cancer and cardiovascular diseases. However, its low half- life (2-30 h)[15] and stability are major concerns that need to be addressed in order to use this as a potent drug.

A number of studies have been conducted on controlled release of SA. SA has been incorporated into poly(anhydride-esters),[16, 17] dendrimers,[18] biopolymers,[19] micelles,[20] polyurethanes[21] etc. for use as an anti-inflammatory, anti-oxidative, antibacterial and/or anti-biofilm formation agent.[21] SA is mostly encapsulated or entrapped in these polymers and its release is directed by the nature and degradation of the polymer.[6] It is desirable that SA is released in a controlled fashion at a rate commensurate with its functional/biological activity from the polymeric matrix and the polymer simultaneously degrades inside the body. There is a need to tailor the release of SA over a wide range depending on the intended application. The incorporation of SA in the polymer backbone is a particularly promising approach because the release of SA occurs only by cleavage of the bonds. This release is, therefore, accompanied by a simultaneous erosion and mass loss of the polymer, leading to its resorption. However, to achieve this objective, the synthetic chemistry should be suitably modified to preserve the chemical nature of the drug and to ensure that the functional role is retained after release. Although studies have been reported on the successful incorporation of SA, the nature of the released product has often not been fully characterized.

Motivated by prior studies[9, 22] that have investigated release of SA, we have synthesized a group of SA releasing polymers based on adipic acid and xylitol that can release SA over a period ranging from a few hours to days. These polymers are crosslinked polyesters that are capable of releasing SA in the body by hydrolysis of their ester bonds. We have recently reported a group of crosslinked polyesters including crosslinked poly(xylitol adipate).[23] Poly(xylitol adipate) is a fast degrading polymer (58 % in 7 days) and was, hence, chosen as a suitable

candidate for delivery of an anti-inflammatory agent like SA. The objectives of the work are three-fold and (i) involve the synthesis of the polymer wherein SA is incorporated into the polymer backbone to make it an immunomodulatory biomaterial; (ii) develop and tailor the controlled release of SA over a broad range by hydrolysis of its polymeric chains by varying the synthesis parameters, namely the SA loading, and the post–polymerization time during synthesis, and (iii) examine the release as a function of curing time, SA loading, varying pH environments and by the action of lipase.

2. EXPERIMENTAL SECTION

2.1 Materials. Adipic acid (SRL Laboratories), xylitol (Sigma Aldrich), SA (Merck) and pyridine (Merck) were used in the synthesis of the crosslinked polyesters. Solvents such as tetrahydrofuran (THF, Merck), N,N-Dimethylformamide (DMF, Merck), ethanol (Emsure) and acetone (Merck) were used during various stages of the synthesis. Lipase from *Candida Antarctica* immobilized on acrylic resin, Novozym 435 (Sigma Aldrich) (with enzyme activity of 5000 U/g) was used.

2.2 Synthesis. The synthesis of the polyesters reported herein is a two-step process (Scheme I) where the first step involves the synthesis of a diacid of SA and adipoyl chloride. The second step is a melt condensation polymerization involving the diacid and xylitol to form the crosslinked polyesters.

Step I: 1 molar equivalent of SA (7 g, 50.7 mmol) was dissolved in THF (10 mL). To this solution, 2 molar equivalents of pyridine (8.3 mL, 101.4 mmol) were added and allowed to stir for 10 min at 37 ˚C. 0.5 molar equivalent of adipoyl chloride (3.7 mL, 25.35 mmol) was added dropwise to the stirred solution and the reaction was conducted for 16 h. Hydrochloric

acid was then added to the reaction mixture at 0 ˚C (ice bath) to precipitate the diacid until a pH of 2 was achieved. The precipitate was washed with water, vacuum filtered and dried before proceeding to the next step. The presence of the diacid was confirmed by mass spectrometry. This step was based on a previously reported work.[24]

Step II: The dried diacid and xylitol were taken in specific stoichiometric ratios. Polymerization was achieved by a melt condensation process where the diacid (5 g, 12.9 mmol) and appropriate amounts of xylitol were melted at 150 ˚C under nitrogen environment and connected to a -700 mm Hg vacuum line to remove the byproduct (water). This reaction reached completion between 12-16 h depending on the stoichiometry. The lower stoichiometric ratio of diacid to xylitol required higher reaction time of 16 h. The product obtained was a sticky, highly viscous, semi-solid prepolymer (uncured). These prepolymers were subsequently post-polymerized at 130 ˚C and -700 mm Hg vacuum for 6 days to yield completely cured (crosslinked) polymers. The nomenclature used for the different polymers are PXASA11, PXASA21, PXASA12 and PXASA14, wherein the numbers at the end indicate the ratio of xylitol to diacid (for example, xylitol: diacid at ratio of 2:1 is represented as PXASA21). Note that PXA refers to poly(xylitol adipate). Unless mentioned otherwise, the characterization was performed only for cured polymers.

An alternative technique was attempted initially which involved the simultaneous melt condensation polymerization (at 150 ˚C) of SA, xylitol and adipic acid (Scheme II) in a single step similar to the synthesis of PXA. Scheme II was not found suitable for this synthesis due to the degradation of SA in the released product, as further discussed below.

2.3 Material Characterization

2.3.1 FTIR spectroscopy

Fourier transform infrared (FTIR) spectroscopy was used for chemical characterization. FTIR spectra of the polymers were acquired on a Perkin-Elmer Frontier FT-NIR/MIR spectrometer in the Universal attenuated total reflectance (uATR-FTIR) mode. Each spectrum obtained was an average of 32 scans taken with a resolution of 4 cm^{-1} over the range 4000-650 cm^{-1}.

2.3.2 Dynamic mechanical Analysis (DMA)

Mechanical properties of all the 6 day cured samples of PXA, PXASA11, PXASA12 and PXASA14 were characterized by dynamic mechanical analysis (DMA, TA Instruments Q800). An isothermal frequency sweep at 37 ˚C was used to analyze the samples (30 mm × 5 mm × 2 mm) using a tension clamp. A frequency sweep from 0.1-1 Hz, amplitude of 15 μm and a preload of 0.01 N was applied.

PXASA21 could not be characterized by DMA because of its sticky, viscous nature. Therefore, the viscoelastic properties of PXASA21 were analyzed using a stress-controlled Discovery Hybrid Rheometer (TA Instruments, DHR-3) with Peltier cone and plate geometry (40 mm diameter plate, cone angle 1 degree and gap distance 50 μm). A fixed strain (0.1 %), was applied to it (this is within the linear viscoelastic region). Oscillation frequency measurements were performed at 37 ˚C from 0.1-1 Hz.

2.3.3 Differential scanning calorimetry

Thermal characterization of the polyesters was obtained by a differential scanning calorimeter (DSC, TA Instruments Q 2000). Samples (3-5 mg) were subjected to a temperature range of –50 ˚C to 150 ˚C in nitrogen environment (flow rate of 50 mL/min). A temperature ramp of 10 ˚C/ min during the heating cycle and 5 ˚C/ min during the cooling cycle was used. The first heating cycle of the heat-cool-heat temperature program removed the thermal history due to prior processing of the samples. The thermal properties of the polyesters were obtained from the second heating curve.

2.3.4 Surface water wettability

Water contact angle was measured to characterize the surface water wettability of the samples using a contact angle goniometer (Dataphysics). Static contact angle measurements were made by placing a 1 μL droplet of ultrapure water (Sartorius) on polyester discs (4.5 mm in diameter). Measurements were taken after equilibrium was attained. The data is depicted as mean ± standard deviation (S.D) based on three independent samples.

2.3.5 Measurement of swelling ratio and gel content

Swelling ratio and gel content of the polymers was obtained by measuring the swelling-deswelling behavior of these polyesters. For determination of swelling ratio, small polymer discs (diameter 4.5 mm and thickness 1 mm) were immersed in non-solvent (ethanol) at 37 ˚C and allowed to swell until a constant weight was achieved. The samples were then dried at 37 ˚C until constant weight was achieved again. The swelling ratio (S in %) is given by:

$$\% \ \boldsymbol{S} = \frac{\boldsymbol{W_s - W_d}}{\boldsymbol{W_d}} \times \mathbf{100} \quad \textbf{(1)}$$

In equation (1), W_s is the sample weight after swelling in ethanol and W_d is the dried polymer weight.

The % gel content was calculated according to ASTM standard D-2765. The initial dry weight of the polymer discs were measured (W_{d1}). These polymers were allowed to swell in N,N-DMF for 24 h. After the polymers attained constant weight and no further increase in weight was observed, the polymers were removed from DMF and dried under vacuum. After the polymer discs re-attained a constant weight and no mass loss occurred due to drying, the samples were weighed again (W_{d2}). The gel content was obtained by the following equation:

$$\% \ \boldsymbol{gel\ content} = \mathbf{100} - \left(\frac{\boldsymbol{W_{d1} - W_{d2}}}{\boldsymbol{W_{d1}}} \times \mathbf{100}\right) \quad \textbf{(2)}$$

2.3.6 Hydrolytic degradation studies

Polymer films (thickness 1 mm) were formed by drop casting the molten prepolymer on a petridish. These films were then cured at 130 ˚C for six days. Circular discs (4.5 mm diameter) were punched out from these films. These discs were placed inside nylon mesh bags, which were then immersed in tubes containing 20 mL phosphate buffered saline (PBS) at pH 7.4 and 37 ˚C. These tubes were kept in an incubator shaker at 100 rpm. The bags were dried and weighed every 24 h. The percentage weight loss of the samples was calculated as follows:

$$\% \ \boldsymbol{weight\ loss} \ = \frac{\boldsymbol{M_o - M_t}}{\boldsymbol{M_o}} \times \mathbf{100} \quad \textbf{(3)}$$

In equation (3), M_o is initial weight of the sample and M_t is the weight of the degraded sample after hydrolysis in PBS for a specific time period t.

2.3.7 Salicylic acid release studies

Circular discs (4.5 mm diameter, 1 mm thickness) were prepared as above. These discs were placed in nylon mesh bags and immersed in 20 mL PBS (pH 7.4) at 37 ˚C. These tubes were placed in an incubator shaker maintained at a shaking speed of 100 rpm. The release profiles of all polyesters were recorded by measuring the sample absorbance at 297 nm (characteristic absorption peak for SA) in a Shimadzu 1700 PharmaSpec UV-visible spectrophotometer. The completely cured (6 day cured) samples of PXASA11, PXASA21, PXASA12, and PXASA14 were characterized. In addition to the 6 day cured PXASA11 sample, the PXASA11 prepolymer and 2 day cured PXASA11 polymers were also characterized. A similar SA release study was performed by varying the pH of the buffer. SA release was recorded at pH 3.2 (acidic, ionic strength 1 mM), 7.4 (physiological, ionic strength 1.5 mM), and 9.0 (basic, ionic strength 3 mM) to understand the role of varying pH on the release profiles. The release of SA from PXASA11 and PXASA14 by enzymatic degradation was observed in the presence of lipase. Lipase (Novozym 435) at a concentration of 1 mg/mL was used. The use of immobilized enzyme allowed separation of the enzyme by centrifugation so as to measure SA release spectrophotometrically without interference from the soluble protein spectra. Free porcine lipase interfered with the 297 nm absorption maxima of SA and was, hence, not used.

The total loading concentration of SA was calculated by dissolving the polymer discs in PBS (pH 9.0). The absorbance at 297 nm gave an indication of the concentration of SA in the polymer. This concentration is taken as the total loading concentration and all % release values are calculated based on this.

2.3.8 Mass spectrometry

Mass spectrometry was performed on Micromass ESI-TOF Mass Spectrometer (Waters) to analyze the mass spectra of the release product. The release product was injected by direct injection with an autosampler, using water as the mobile phase. The run time was 0.5 min and the cone voltage was 130-140 V. The molecular weights obtained were matched with that of the expected compounds, using a standard database.

2.3.9 Cytocompatibility studies

NIH3T3 cells (ATCC, USA), a well established line of mouse embryonic fibroblasts, were used to assess cytocompatibility. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies) supplemented with 10 % (v/v) fetal bovine serum (FBS, Gibco, Life Technologies) and 1 % (v/v) penicillin-streptomycin at 37 ˚C and 5% CO_2. Cells were passaged with Trypsin-EDTA (Gibco, Life Technologies). All studies were performed on cells of Passage 12. Prior to cell seeding, polymer discs of PXA and PXASA11 (4.5 mm diameter, thickness of 1 mm) were ethanol and UV sterilized for 30 min. Discs were immersed in 5 mL media for 24 h such that the conditioned medium contained all the degraded products of the polymer and the released drug (including adipic acid, xylitol, several oligomers along with SA and di-SA). NIH3T3 cells were treated with this conditioned media for 1 day and 3 days to assess cytocompatibility. 10^4 cells were initially seeded per well in a 48-well plate in fresh medium for 24 h. The medium was replaced with 500 μL of conditioned media. Fresh medium was used as the control. The media (both fresh and conditioned) was replenished after every 24 h.

Cell viability was assessed 1 day and 3 days post treating with conditioned media using WST-1 (water soluble tetrazolium salts, Roche). The oxidoreducates present only in viable cells can react with the WST reagent to produce a water soluble formazan (colored product). 20 µL of WST-1 was dissolved in 200 µL of complete culture medium. This solution was added to each well and incubated at 37 ˚C in 5% CO_2 for 4 h. The formation of formazan crystals was monitored by measuring the absorbance at 440 nm in a well plate reader (Synergy HT, Biotek). The absorbance value provides a direct correlation to the number of cells in the well. The measurements were obtained by averaging the data from four independent wells and the data is presented as mean ± S.D. for n= 4. Analysis of variance (ANOVA) with Tukey's test for $p<0.05$ was used to evaluate statistically significant differences.

2.3.10 Hemolysis assay

Blood compatibility tests were performed through a commercial contract (TheraIndx Lifesciences, India) with approval from the Animal Ethics Committee. Whole blood from New Zealand white rabbits was collected in tubes containing sodium citrate (anticoagulant). Polymer discs of PXA and PXASA11 (4.5 mm diameter, 1 mm thick) were equilibrated and leached in saline water (0.9 % NaCl in water) for 6 h. Anticoagulated whole blood (0.2 mL) was added to each of the samples and kept at 37 ˚C for 30 min. 4 mL of saline was added to each of the samples to stop hemolysis, and incubated for 60 min at 37 ˚C. Each tube was then inverted to mix the contents homogenously, and then centrifuged to collect the supernatant. Negative and positive controls were prepared by adding 0.2 mL of whole blood to 4 mL of saline and distilled water, respectively. The optical densities (OD) of the supernatant were measured at 541 nm and percentage hemolysis was calculated as

$$\%\ \boldsymbol{Hemolysis} = \frac{(\boldsymbol{OD_{test} - OD_{neg}})}{(\boldsymbol{OD_{pos} - OD_{neg}})} \times \mathbf{100} \quad \textbf{(4)}$$

In equation (4), OD_{neg} and OD_{pos} denote OD of supernatant of negative and positive control, respectively. OD_{test} is the absorbance value of the test sample.

2.3.11 Platelet adherence assay

Blood was collected from rabbits as above. Platelet rich plasma (PRP) was collected by centrifuging this blood at 2500 rpm for 10 min. Platelet count obtained was nearly 5×10^5/ μL.

PXA and PXASA11 discs (4.5 mm diameter, 1 mm thick) were sterilized in absolute ethanol and dried for 5 min. The discs were placed in 12 well TCPS plates. All samples were assayed in quadruplicates. The samples were equilibrated in 3 mL phosphate buffered solution for 6 h at room temperature. After 6 h, PBS was removed and 3 mL of freshly prepared PRP was added to each disc for 90 min at 37 ˚C. The PRP was removed with an aspirator, and each disc was rinsed three times with 3 mL of PBS. After washing, the cells were fixed in 2.5% glutaraldehyde for 30 min. The films were washed with PBS and subsequently dehydrated by gradient washing in a series of ethanol water solutions (50, 60, 70, 80, 90, 95, and 100 % (v/v)). The samples were then imaged using scanning electron microscopy.

3. RESULTS AND DISCUSSION

3.1 FTIR spectroscopy

FTIR spectroscopy was performed at all steps of synthesis. It is observed that the precursors SA and adipic acid both show –C=O stretching (corresponding to –COOH) near 1690 cm^{-1}. From the FTIR spectra (Figure 1a), it is evident that after the completion of **Step I** of synthesis of PXASA, FTIR spectra displays the appearance of an additional –C=O stretching

(aliphatic ester formation) peak at 1740 cm^{-1}. A very prominent 1690 cm^{-1} peak is also present in the diacid due to the presence of free –COOH groups of SA. This is in agreement with previously reported literature.[25] The formation of the diacid was further confirmed by ESI-MS. The mass spectrum reports a peak at 386 Dalton, confirming the synthesis of the diacid.

Consequently, after **Step II** of the reaction, the 1690 cm^{-1} peak gradually decreases with a corresponding increase in the phenyl ester peak. An ester bond is also formed between SA and xylitol. The peak due to the –C=O stretching of a carboxylic acid[25] observed at ~1680 cm^{-1} in the prepolymer, shifts slightly to 1706 cm^{-1} (indicating phenyl ester)[25, 26] and becomes less prominent in the cured polyester. This shift is observed as curing progresses. The aliphatic ester peak is observed at 1740 cm^{-1} for both the prepolymer and cured polyester. Asymmetric and symmetric –CH stretching vibrations are observed at 2926 cm^{-1} and 2853 cm^{-1}, respectively, for both prepolymer and cured polymer. Peaks corresponding to $–CH_2$ bending vibrations are observed at 1465 cm^{-1}.

FTIR spectra were also recorded for the varying stoichiometric ratios of xylitol: diacid (Figure 1b). PXASA21 shows a very prominent –OH stretching peak near 3300 cm^{-1} indicating the excess of hydroxyl groups owing to the excess of the polyol (xylitol). PXASA21 also shows a very small phenyl ester peak at 1680 cm^{-1} due to very less esterification. All other cured polyesters (PXASA12 and PXASA14) show sharp peaks at 1740 cm^{-1} indicating the formation of the aliphatic ester. The PXA polyester shows a peak only at 1740 cm^{-1} and no phenyl ester peak at 1706 cm^{-1} because of the absence of SA.

3.2 Differential scanning calorimetry

Thermal characterization of the samples shows that all the crosslinked polyesters are amorphous. No melting or crystallization peaks are observed. The glass transition temperatures (T_g) are obtained (Figure 2). It is observed that all the polymers have a T_g below physiological temperature and hence, may be suited as elastomeric biomaterials *in vivo*. T_g values followed the order of PXASA21< PXASA14< PXASA11< PXASA12. The recorded T_g values for PXA, PXASA11, PXASA12, PXASA14 and PXASA21 are 17 ˚C, 22 ˚C, 30 ˚C, 18 ˚C, and 13 ˚C, respectively. The increase in T_g value from PXASA11 to PXASA12 may be attributed to the increased crosslinking (discussed below) in PXASA12 as compared to PXASA11. Increased crosslinking leads to the formation of more rigid networks, thereby restricting motion of the polymeric chains.[27, 28] Moreover, the inclusion of cyclic structures within the polymer has also been reported to increase T_g as it reduces flexibility of the polymer backbone, thereby, hindering bond rotation and raising the T_g.[29] This is observed in all the cases where inclusion of SA into the polymer has a higher T_g than PXA, except for PXASA21, which has a very low degree of esterification. T_g was also recorded for PXASA11 as a function of curing time. T_g increases from 17 ˚C in the prepolymer to 22 ˚C in the 6 day cured PXA. This increase in T_g is accompanied by a broadening of the transition. This broadening is due to enhanced crosslinking and heterogeneity in the length of crosslinks.

3.3 Dynamic Mechanical Analysis

The values of storage and loss moduli are tabulated in Table 1. Mechanical characterization of the polyesters shows that the storage modulus follows the trend: PXASA14 < PXASA11 < PXASA12. The storage modulus increases from PXASA11 to PXASA12 because

of increasing crosslinking (as evidenced by swelling data discussed below).[30] However, the storage modulus of PXASA14 decreases as compared to PXASA11 or PXASA12. This may be attributed to the excess of SA diacid as compared to the alcohol. This results in many unreacted groups and, therefore, a more loosely crosslinked network. Lower degree of crosslinking yields less stiff polymer networks, thereby decreasing the storage modulus.[31] The rheological analysis of PXASA21 shows that it has a storage modulus of 7 kPa. The increase in the crosslinking in PXASA11 and PXASA12 led to the enhancement of mechanical properties of the material. Increased crosslinking makes the polymer network stiffer and, therefore, raises the moduli of the samples.[30, 32] Lower esterification and consequently lower crosslinking (increased swelling ratio) result in lower mechanical properties of PXASA21. This is also corroborated by the increase in the T_g values, as discussed earlier. Polyesters PXA, PXASA11, PXASA12 and PXASA14 have T_g lower than the physiological temperature and, therefore, are elastomeric in nature at 37 ˚C.

3.4 Surface water wettability

Water contact angles are indicative of the hydrophobicity of the material. Surface water wettability studies show that all the synthesized polyesters are hydrophobic (except for PXASA21) in nature and have comparable water contact angles (Table 1). However, contact angle of PXASA21< PXASA14< PXA< PXASA11= PXASA12. The excess –OH groups present in PXASA12 are responsible for the higher hydrophilicity of this polymer. However, as crosslinking increases (discussed below), the number of unreacted groups in the polymer backbone decreases and lesser –OH groups are available to interact with water. This results in an increase of hydrophobicity in PXASA11 and PXASA12. However, lower polymerization due to excess acid[23] in PXASA14 leads to a slight decrease in the hydrophobicity. The lesser

crosslinking (increased swelling) could also be attributed to the decreased water contact angle in PXASA14.

The hydrophobicity is dependent on the crosslinking of the polymers. However, the hydrophobicity cannot increase beyond a certain contact angle due to thermal curing. Since the polymers have variable stoichiometric composition, the extent of reaction changes and this consequently alters its interaction with water.

3.5 Measurement of swelling ratio and gel content

The swelling ratios obtained from the polymers can be directly correlated to the extent of crosslinking in the polymers. The swelling ratio increases in the following order where PXASA14< PXASA11< PXASA12. The results obtained (Table 1) show that swelling ratio decreases in PXASA11 as compared to PXA, indicating higher crosslinking. Further increase in crosslinking was observed in PXASA12. The increased crosslinking in the SA loaded polyesters as compared to PXA is most likely due to the availability of more functional groups in the former. In the SA loaded polyesters, two kinds of ester bonds may form: an aliphatic ester and a phenyl ester. This increased probability of ester linkage formation may be responsible for the enhanced crosslinking. Moreover, in PXASA12 the functionality ratio of the free –COOH to the free –OH groups is closer to 1 than in PXA or PXASA11. The extent of conversion is, therefore, high in PXASA12. PXASA14 shows a much higher swelling and is less crosslinked compared to the other polyesters. The presence of excess –COOH groups in PXASA14 leads to a lower polymerization and consequently increases the swelling ratio indicative of lower crosslinking.

Consequently, the gel content measurement data shows that the % gel content follows an order of PXASA12> PXASA11>PXASA14>PXASA21. The sol content in the polymer is the

fraction of the polymer that can be extracted from the polymer network by a solvent. Since the degree of esterification is less in PXASA21, 84 % of the polymer mass is lost by extraction in DMF. Subsequently, the esterification and crosslinking being higher in the other polyesters, the gel content was higher in these polyesters. Both the swelling ratio and gel content follow the same trend as shown in Table 1.

3.6 Hydrolytic degradation studies

Hydrolytic degradation studies (Table 1) show that the rate of degradation decreases as the crosslinking increases. The degradation rate exhibits the following trend: PXASA21> PXASA14> PXASA11> PXASA12. The degradation slows down from PXASA21 (which degrades ~ 100 % in six days) to PXASA14 (~ 65 % in seven days) to PXASA11 (~ 30 % in seven days) to PXASA12 (4% in 7 days), underscoring the broad range of release obtained through the control of stoichiometry. The trend is the same as that of contact angle, swelling ratio and % gel content. These results indicate that hydrophobicity and crosslinking of the polymers play an important role in the degradation of polyesters. The crosslinking governs the rate of water permeation into the polymer. As is evident from the SEM micrographs, the mass loss due to degradation is not due to predominant surface erosion (Figure 3). No surface cracks are observed on the polymer discs. The erosion, therefore, is primarily controlled by the water diffusing into the bulk of the polymer. Compared to 30 % mass loss in the completely cured polymer in 7 days, the uncured prepolymer of PXASA11 degrades 80 % (Figure 4a). Hydrophobicity increases as the crosslinking increases.[33] This is because higher crosslinking leads to lesser free –OH groups in the polymer. Therefore, lesser unreacted –OH groups are available to interact with water and makes the surface substantially more hydrophobic.

Hydrophobicity directs the amount of water that is present and/or retained in the vicinity of the hydrolysable ester bonds.

In all cases, it is observed that the rate of degradation follows a first order process which is fast at the beginning and gradually slows down as the availability of hydrolysable ester bonds decreases (Figure 4b). The hydrolytic degradation data show that ~26 % of the mass loss occurs in the first 96 h, after which the weight loss becomes almost constant at ~30 % for the degradation of PXASA11. This can be modeled by first order degradation kinetics with a limiting mass, as observed for the degradation of PXA.[23]

The effect of lipase on polymer degradation was evaluated. The completely cured PXASA11 undergoes 30 % and 45 % mass loss in the absence or presence of lipase, respectively. A wound is generated at the implant site and the biological milieu can be significantly altered from normal physiological conditions. The pH at a wound site can be significantly altered.[34] A number of enzymes are present in the human body. Lipases, a subset of esterases, are present throughout the body and particularly abundant at the sites of inflammation.[35] FTIR spectroscopy (Figure S1 in supplementary information) of samples degraded for seven days shows that degradation is governed by external pH conditions and the presence of enzyme. The graph clearly shows that the acid to ester peak ratio increases with an increase in the rate of degradation. The data were normalized and the acid to ester peak ratio was calculated by taking the ratio of the peak intensities at 1690 cm^{-1} and 1740 cm^{-1}. Under physiological pH conditions, the acid to ester peak ratio is 1.04, compared to 0.90 and 1.40 under acidic and basic conditions, respectively. This is because the rate of ester hydrolysis is markedly higher as compared to neutral or acidic pH under alkaline conditions. This may also be due to the

accumulation of adipic acid, SA and xylitol on the surface of the polymer. All of these components are more soluble in alkaline pH.[36]

The FTIR spectra of PXASA11, after seven days of degradation in the presence of lipase, shows an appreciable decrease in the –C=O stretching peak for ester at 1740 cm^{-1}, where almost all the ester bonds have hydrolyzed. The acid to ester peak ratio increases to 2.76. This indicates that the presence of lipase quickens the degradation process as compared to when the polyesters are subjected to hydrolytic degradation, as is reported for other polymers.[37, 38]

Kinetics of degradation

The degradation kinetics may be governed by equations as reported earlier[23] and may be fitted to the following equation:

$$\ln\left(\frac{M_t - M_{lim}}{M_0 - M_{lim}}\right) = -kt \qquad (5)$$

The semi-log plot of $\left(\frac{M_t - M_{lim}}{M_0 - M_{lim}}\right)$ with time is linear (Figure 4b). The rate constants of degradation, k, are obtained from the slope of the plot. The k values ($\times 10^{-3}\ h^{-1}$) are 13.7, 13.3, 17.9, and 20.1 for PXASA11, PXASA12, PXASA14, and PXASA21, respectively. The prepolymer of PXASA11 (designated as PXASA11_prepol) has a rate constant of 19.1 $\times 10^{-3}\ h^{-1}$, which is higher than that of PXASA11. Under the influence of lipase, the rate of degradation is further enhanced and the k value ($\times 10^{-3}\ h^{-1}$) obtained for PXASA11 (under lipase) is 27.6.

3.7 Salicylic acid release studies

The major focus of the present work is to develop a SA releasing polymer matrix that may be used *in vivo*. A number of factors have, therefore, been varied to observe the influence of these factors on the release mechanism of the polymer. The extent of crosslinking directly controls drug release from a crosslinked polymer. The SA release from the matrix is slower if the network is stiffer. A number of other factors that also influence the degradation and simultaneous SA release include the nature of the drug and the matrix, hydrophobicity, geometry, surface area,[39] polymer degradation and the interaction between the drug and the matrix.

We confirmed that the polymer release product contains SA and not a degraded derivative by analyzing its entire UV-visible spectrum. When the release from the polymer synthesized by Scheme II was studied, the UV-visible spectrum of the released product showed an additional peak at 280 nm along with the reported SA peak at 297 nm (Figure 5). This product is either a phenyl salicylate or phenol formed due to the high temperature of the reaction (150 $^{\circ}$C) as reported earlier.[40] As phenol is cytotoxic,[41, 42] no further studies were conducted on these polymers. The UV-visible spectrum from the released products of all PXASA polymers (synthesized by Scheme I) confirm the presence of SA in the release medium and display its characteristic peak at 297 nm. However, when further analyzed by MS (Figure S2 in supplementary information), the mass spectra reveal that SA is indeed released from the polymer along with another compound whose molar mass is 258 Dalton, which is most likely di-salicylic acid. The release products also included adipic acid and xylitol, along with other oligomers that were released due to chain scission in the polymer. This is not revealed by the UV-visible spectra since di-salicylic acid has a similar absorption maximum at 300 nm. As both SA and di-salicylic

acid are known to exhibit anti-inflammatory properties,[43] this system was considered a potential candidate as an immunomodulatory polymer and is characterized further.

3.7.1 Curing time dependent SA release

SA release was studied as a function of curing time for PXASA11. It is observed that with an increase in the curing time, the crosslinking (swelling ratio) increases and there is a subsequent slowing down in the release of the drug (Figure 6a). It is observed that, as the curing time increases, the number of free –OH groups decreases in the polymer. The hydrophobicity of the polymer increases and therefore, the water penetration into the polymer decreases. This leads to reduced hydrolysis of the ester bonds resulting in a consequent slow drug release. Hydrolysis of the ester bonds would typically cause the release of SA from the polymer releasing SA and other associated oligomers of the polymer chain. The release of SA would involve the hydrolysis of both the phenyl and the aliphatic ester linkages. The rates of hydrolysis of these two bonds differ and combine to give the actual release rate of SA. The non-cured prepolymer releases approximately 35 % of the SA loaded onto the polymer after 7 days, whereas the 2 day cured samples release nearly 27% SA in 7 days. The completely cured PXASA11 (6 day cured) releases at a much slower rate and only <10 % of the loaded SA is released from the polymer. This curing dependent release has been shown for other crosslinked polymer systems like polyesters[44] and hydrogels.[45] Increased crosslinking (evident from swelling studies) results in slower release rates in all cases.

3.7.2 pH dependent SA release

SA release was also studied as a function of the pH of the surrounding environment (Figure 6b). This is of particular interest since the hydrolysis of ester bonds is accelerated under basic conditions. It is observed that hydrolysis occurs slightly faster under basic conditions,

where 13 % SA is released in 7 days whereas, acidic and neutral pH result in comparable SA releases of ~ 10 %. We have previously reported that alkaline pH accelerates ester hydrolysis in PXA substantially with an increase in the pH.[23] However, in this case, two types of ester bonds are present and the breakage/ hydrolysis of the phenyl ester may be hindered due to steric hindrance. Although the aliphatic ester hydrolyzes under basic conditions, the phenyl ester may not hydrolyze to release the SA from the polymer backbone. This is most likely the reason why pH does not appreciably change the rate of SA release and the release is almost pH independent.

3.7.3 SA loading dependent SA release

It is important to study the dosage dependence of SA release from the polymer. It is observed that PXASA21 releases 100 % of the loaded drug in 6 days (Figure 6c). This is primarily because PXASA21 is a loosely crosslinked network. Since SA is a hydrophilic molecule, a major driving force of SA release is its solubility in PBS. The lower esterification and resultant low crosslinking makes it easier for water to penetrate into the network and hydrolyze the ester bonds. Another reason why it releases faster is because this is more hydrophilic than the other polyesters discussed herein owing to the free –OH groups as is supported by their FTIR spectra. PXASA11 and PXASA12 show comparable rates of SA release. Even though the loading of SA is higher in PXASA12 than PXASA11, their releases are comparable (10 % in 7 days) owing to the higher crosslinking and lower gel content in PXASA12. PXASA14 releases nearly 30 % SA in 7 days. This is due to lower crosslinking in PXASA14 due to an excess of acidic groups. Higher gel content in this polymer allows greater water permeation and is, therefore, causes SA release from the polymer. This study shows that the loading concentration of the SA affects the release profile of the polymer. The drug loading in PXASA11 and PXASA12 were 11 % and 18 %, respectively. The drug loading does not

significantly affect the release profile of these two polymers at later time points (discussed below in section 3.7.5).

3.7.4 Lipase mediated release

The primary motivation for incorporating SA into the polymer backbone is to prepare an anti-inflammatory polymeric biomaterial that can reduce inflammation at the implant site without the aid of external immunosuppressors. These inflammation sites are particularly rich in enzymes such as lipases or esterases. It is also pertinent for cancer treatment, another potential target application of sustained release of SA, as cancer is associated with increased expression of different degradative enzymes including lipases.[46] It is, therefore, important to study the effect of these enzymes on the degradation and consequent SA release from the polymers. It is observed that the presence of lipase accelerates the breakage of the ester bonds and causes a much faster release from the polymer. PXASA11 and PXASA14 release 30 % and 75 %, respectively, in 7 days in the presence of lipase. This is higher than 10 % and 30 % release observed in the absence of lipase (Figure 6d).

As indicated by the above studies, it is evident that the crosslinking plays a pivotal role in controlling the drug release from the polymer. As gel content decreases, the release becomes faster as the sol fraction allows easier water permeation into the polymer. As supported by SEM analysis (Figure 3), the initial degradation takes place from the bulk of the polymer and is, therefore, dependent on the water permeation into the bulk of the sample. If the degradation of the polymer had been a surface erosion dependent process, it would not directly depend on the swelling ratio or crosslinking. The polymer, however, begins to develop surface cracks after 5

days of being immersed in PBS. The release profile of the six day cured PXASA11 shows that the release becomes slow and has a sustained release after 96 h.

3.8 Cell cytocompatibility studies

The effect of the presence of polymers loaded with SA was characterized (Figure 7). WST-1 readings after 1 and 3 days show that there is no statistically significant difference between the control (fresh medium) and the conditioned medium from the different samples PXA, PXASA11. PXA was tested for cytocompatibility in order to eliminate any cytotoxic effect of the polymer degradation products. Both monomers xylitol and adipic acid can be metabolized by the body[47, 48] and are utilized by the fatty acid oxidation and carbohydrate metabolism, respectively. Both PXA and PXASA11 display comparable increase in the cell population at the end of 1 and 3 days. Microscopic investigation revealed that all cells appeared healthy and reached confluency by day 2. The pH of the medium is not significantly affected by the release of SA into the medium. As obtained from the release studies of the polymer, the concentration of SA is ~40 μg/mL. SA is known to be non-toxic at this concentration. However, all release studies are done in PBS whereas this release is done in growth medium and thus the actual concentration released may differ. Importantly, this data proves that the polymers are all cytocompatible and may, hence, find applications in biomedical applications. No statistical significant differences are observed in the cell proliferation assays of control TCPS, PXA, and PXASA11 polymers.

3.9 Hemolysis assay

Hemolysis is a quantitative measure of blood compatibility of biomaterials. Hemolysis occurs when excessive fluid diffusion into the cells causes it to swell and eventually rupture. The consequent release of adenosine diphosphate enables it to recruit increased number of platelets to

form a thrombus or clot. It is, therefore, essential to test the response of biomedical materials to blood. The assay shows that PXA and PXASA11 result in low hemolysis of 0.58± 0.07 % and 0.69 ± 0.09 %. This is similar to other commercially available polymers used in biomedical applications like polycaprolactone (PCL) (1.40 % hemolysis).[49] In general, hemolysis of up to 5 % is permissible for biomaterials. These polymers may, therefore, find applications in biomedical applications as blood contacting devices *in vivo*.

3.10 Platelet adherence assay

Blood coagulation occurs on biomaterial surfaces with the onset of platelet aggregation due to exposure to tissue factor upon injury. A fibrin network is formed as a response to the exposure to blood. Platelet activation induces their fusion. A clot/ thrombus forms by the agglomeration of these fused platelets with the fibrin network in the presence of thrombin. Figure S3 (in supplementary information) shows the adhered platelets on PXA and PXASA11 and the platelets show no increased activation, spreading or thrombus formation. Similar observations have been reported for other polymers used clinically.[49, 50] These polymers are, therefore, hemocompatible and may be used as potential materials for biomedical applications.

4. CONCLUSIONS

We have successfully synthesized a family of crosslinked polyesters based on xylitol and adipic acid for controlled release of SA. These biodegradable polymers are shown to be cytocompatible *in vitro* and thus could potentially find use as biomaterials that elicit minimal inflammation. The SA released is pharmacologically active making these polymers functionally bioactive, resorbable polymers. The wide range of tailored SA release is demonstrated through control of stoichiometry and post-polymerization time. The rate of SA release has been tuned

from 10 % to 35 % of the initial loading for cured and non-cured polymers. Simultaneously PXASA11 and PXASA12 have significantly different degradation rates (30 % and 4 % in seven days, respectively) but have very similar release profiles where 9 to 10 % of the loaded SA is released in seven days. These two polymers may, therefore, be used for different tissue applications where similar dosage of SA is required. Variable mechanical properties ranging from 0.5 MPa to 15.3 MPa are displayed by these polyesters. The tunability of SA release has been reported herein under varying release conditions and synthesis parameters. The functionally active SA release slows down as curing and swelling ratio increases.

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Table 1: Physical properties of the PXASA polyesters cured for 6 days at 130 ˚C.

Polyester	T_g (˚C)[a]	Storage modulus (MPa)[b]	Loss modulus (MPa)[b]	Contact angle[c]	Swelling ratio (%)	% gel content[d]	Mass loss (%) in 7 days[d]	Degradation rate constant, k× 10^{-3} (h^{-1})	SA release (%) in 7 days[d]
PXA	17	0.8	0.6	95			58		-
PXASA11	22	1.4	0.4	100	14	86.0	30	13.7	10
PXASA12	30	13.6	2.7	101	9	92.5	4	13.3	9
PXASA14	18	0.9	0.7	90	25	65.0	65	17.9	30
PXASA21	13	7 × 10^{-3}	3× 10^{-3}	38	93	16.0	100	20.1	100

[a] Within a range of ± 2 ˚C

[b] Recorded at frequency of 1 Hz

[c] Error ±1

[d] Error ±1 %

Figure Captions

Scheme 1: Synthesis scheme of PXASA polymer.

Scheme 2: Synthesis scheme of PXASA polymer where salicylic acid is degraded.

Figure 1a: FTIR spectra of PXASA11 at different steps of synthesis.

Figure 1b: FTIR spectra of PXASA with different salicylic acid loading.

Figure 2: DSC thermogram showing representative spectra of PXASA11 and PXASA21. The numbers on the dotted lines indicate T_g.

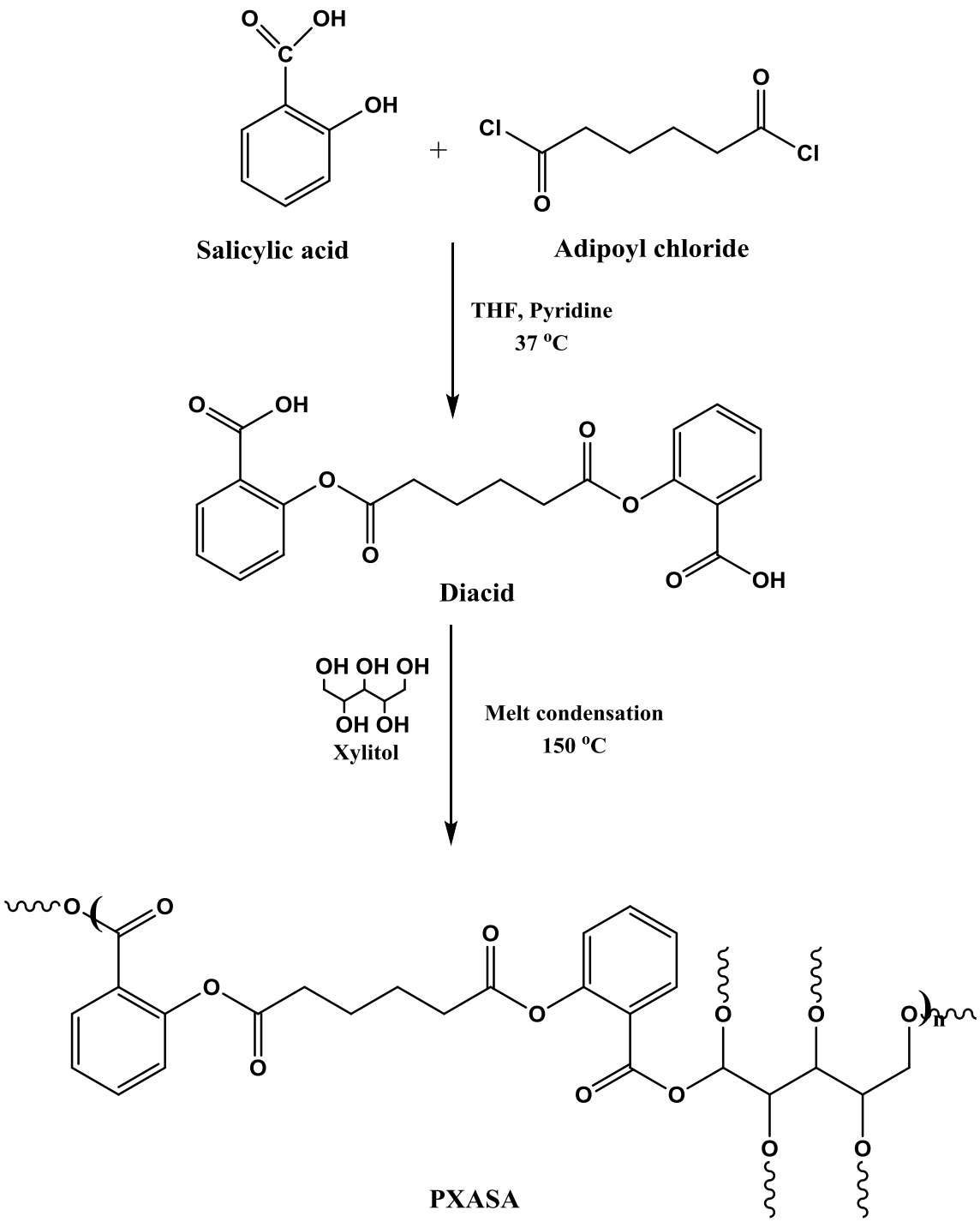
Figure 3: SEM micrographs showing degradation after (a) one day (b) 7 days.

Figure 4: (a) Mass loss of PXASA_prepol, PXASA11, PXASA12, PXASA14, PXASA21 and PXASA11_lipase with time. (b) Variation of mass with time of the polyesters linearly fitted to obtain rate constants.

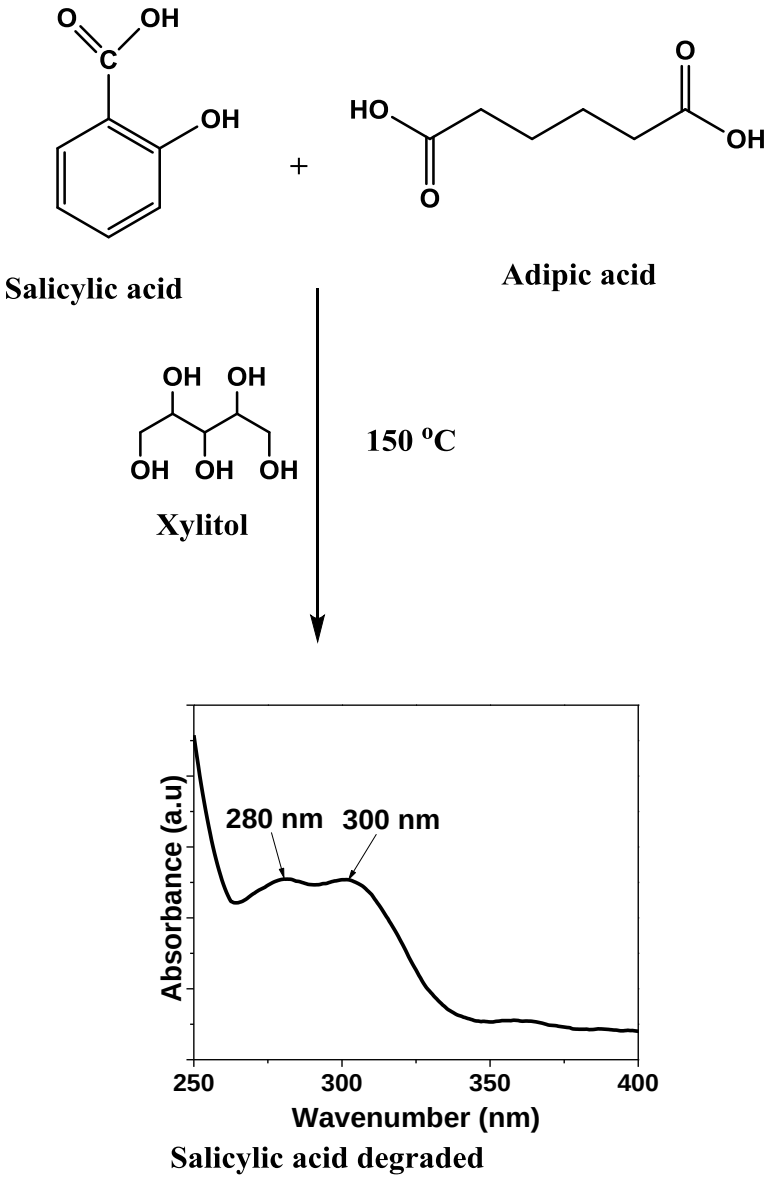
Figure 5: Representative UV-visible spectra of SA and release products from PXASA synthesized by Scheme I and II.

Figure 6: Variation of SA release % with time (a) for different curing time (b) at varying pH conditions (c) at different SA loading concentration (d) in the presence of lipase.

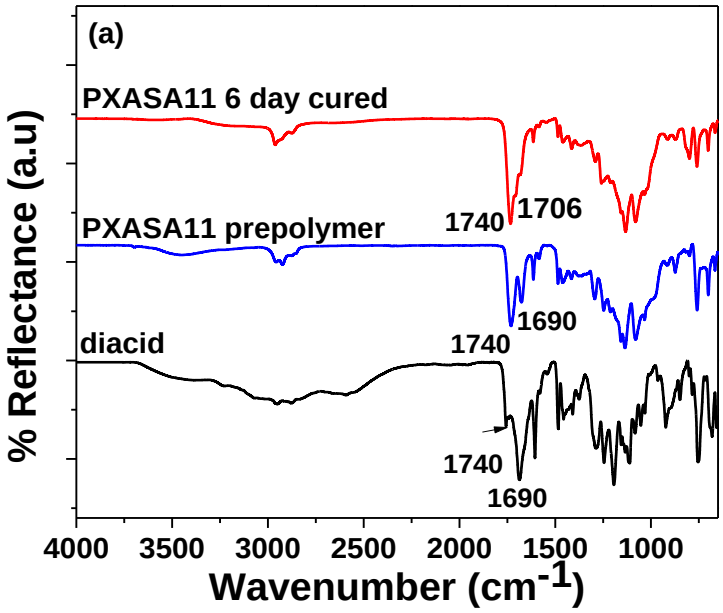
Figure 7: Cytocompatibility results. WST-1 data showing absorbance at 440 nm with respect to time.


Salicylic acid
Adipoyl chloride
THF, Pyridine
37 °C
Diacid
Xylitol
Melt condensation
150 °C
PXASA

Scheme 1


O
OH
C
OH
HO
O
OH
O
+
Salicylic acid
Adipic acid
OH OH
OH OH OH
Xylitol
150 °C
Absorbance (a.u)
280 nm
300 nm
250
300
350
400
Wavenumber (nm)
Salicylic acid degraded

Scheme II


(a)
PXASA11 6 day cured
PXASA11 prepolymer
diacid
1740
1706
1690
1740
1740
1690
% Reflectance (a.u)
4000 3500 3000 2500 2000 1500 1000
Wavenumber (cm-1)

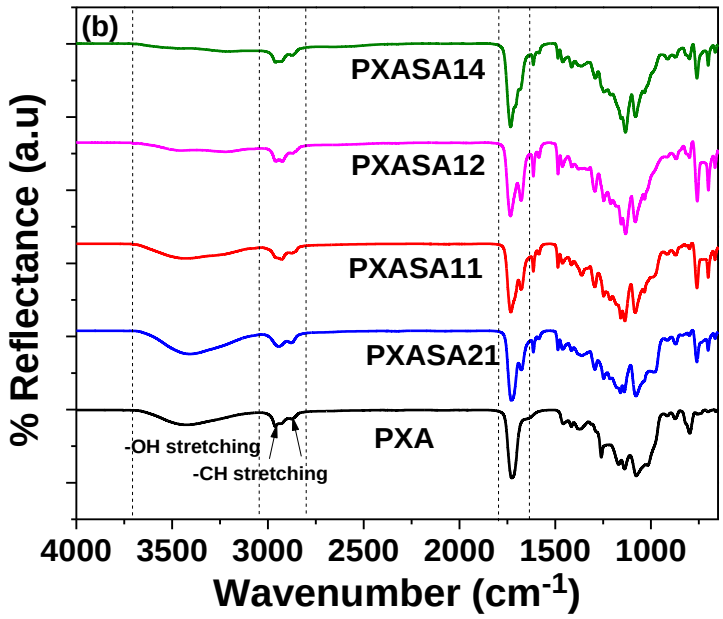

(b)
PXASA14
PXASA12
PXASA11
PXASA21
PXA
-OH stretching
-CH stretching
% Reflectance (a.u)
4000 3500 3000 2500 2000 1500 1000
Wavenumber (cm-1)

Figure 1

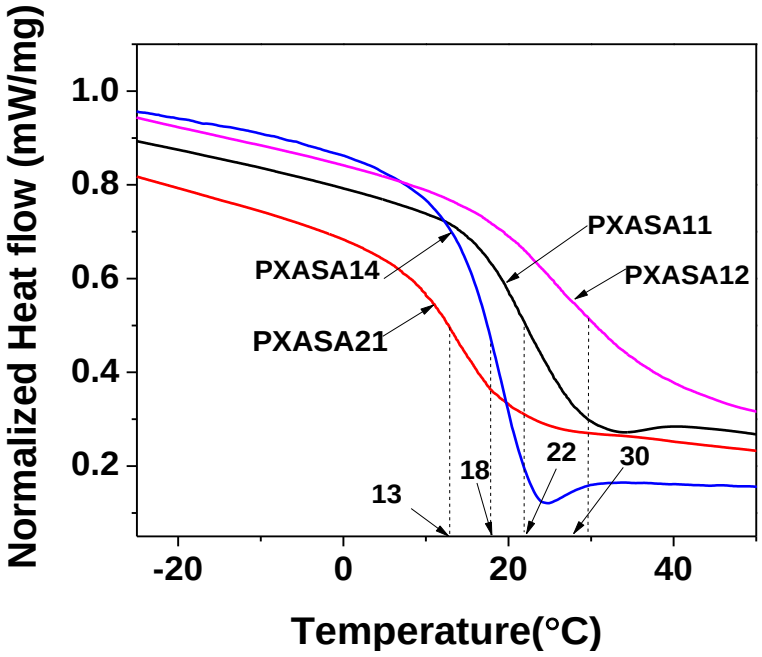
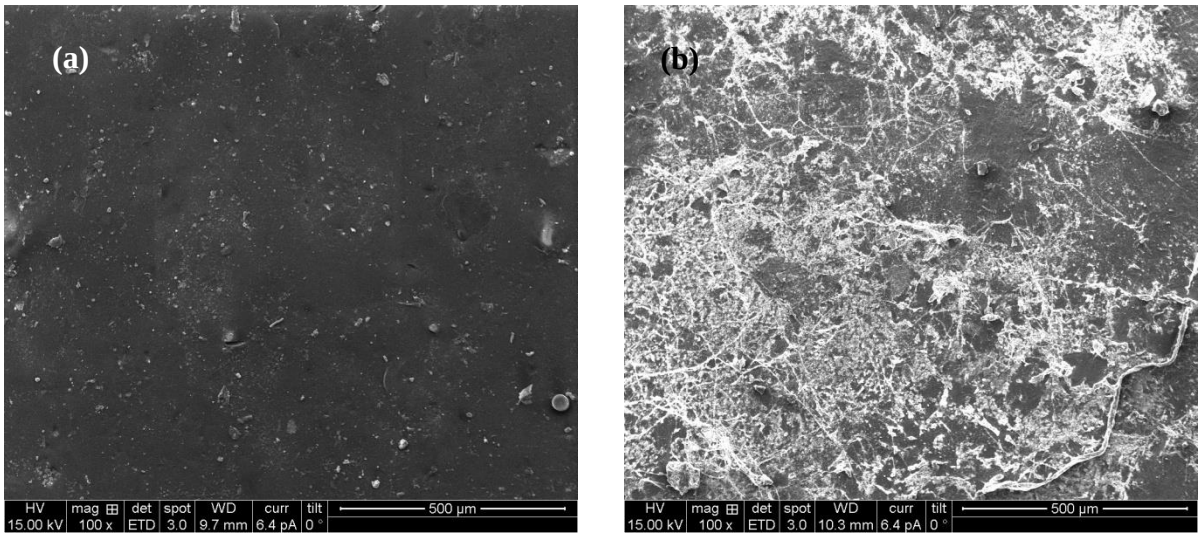

Normalized Heat flow (mW/mg)
1.0
0.8
0.6
0.4
0.2
-20
0
20
40
Temperature(°C)
PXASA11
PXASA12
PXASA14
PXASA21
13
18
22
30

Figure 2


(a)
HV 15.00 kV
mag 100 x
det ETD
spot 3.0
WD 9.7 mm
curr 6.4 pA
tilt 0 °
500 µm
(b)
HV 15.00 kV
mag 100 x
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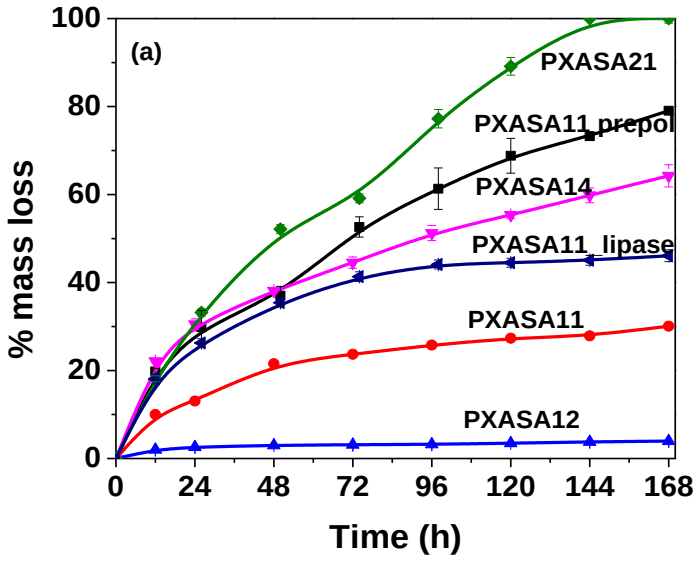
spot 3.0
WD 10.3 mm
curr 6.4 pA
tilt 0 °
500 µm

Figure 3


(a)
PXASA21
PXASA11_prepol
PXASA14
PXASA11_lipase
PXASA11
PXASA12
% mass loss
Time (h)
0
20
40
60
80
100
0
24
48
72
96
120
144
168

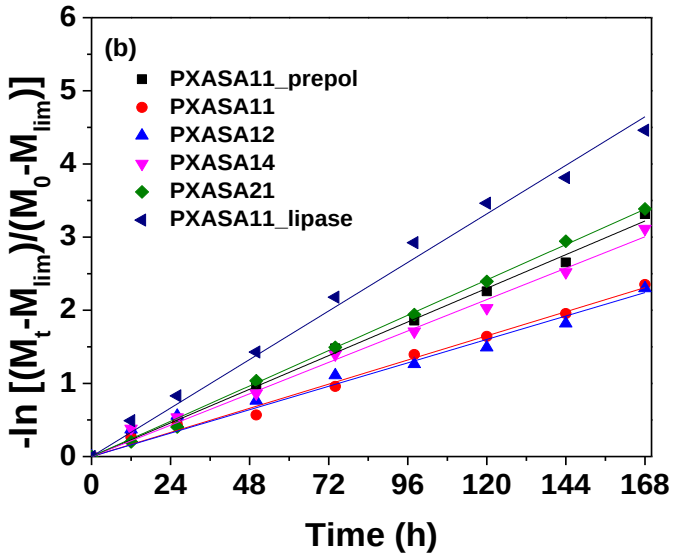

(b)
PXASA11_prepol
PXASA11
PXASA12
PXASA14
PXASA21
PXASA11_lipase
-ln [(M_t-M_lim)/(M_0-M_lim)]
Time (h)
0
1
2
3
4
5
6
0
24
48
72
96
120
144
168

Figure 4

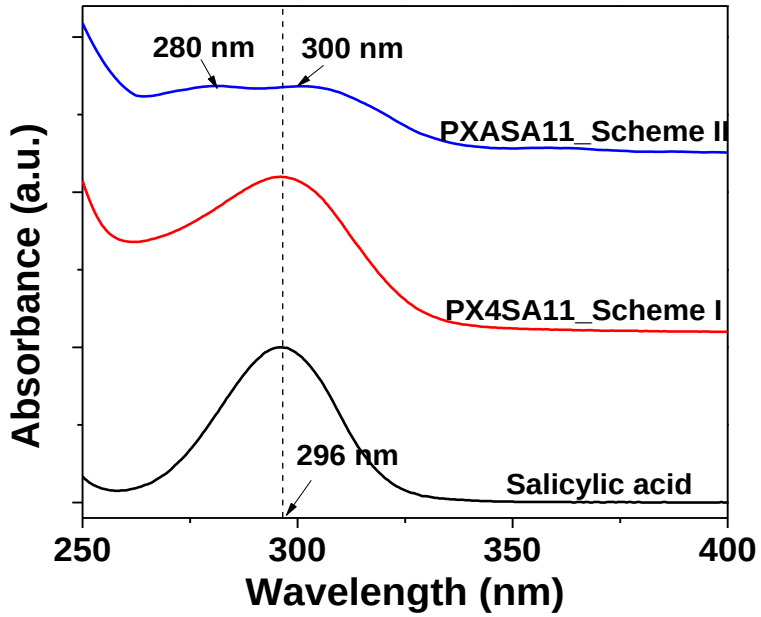

280 nm
300 nm
PXASA11_Scheme II
PX4SA11_Scheme I
296 nm
Salicylic acid
Absorbance (a.u.)
250
300
350
400
Wavelength (nm)

Figure 5

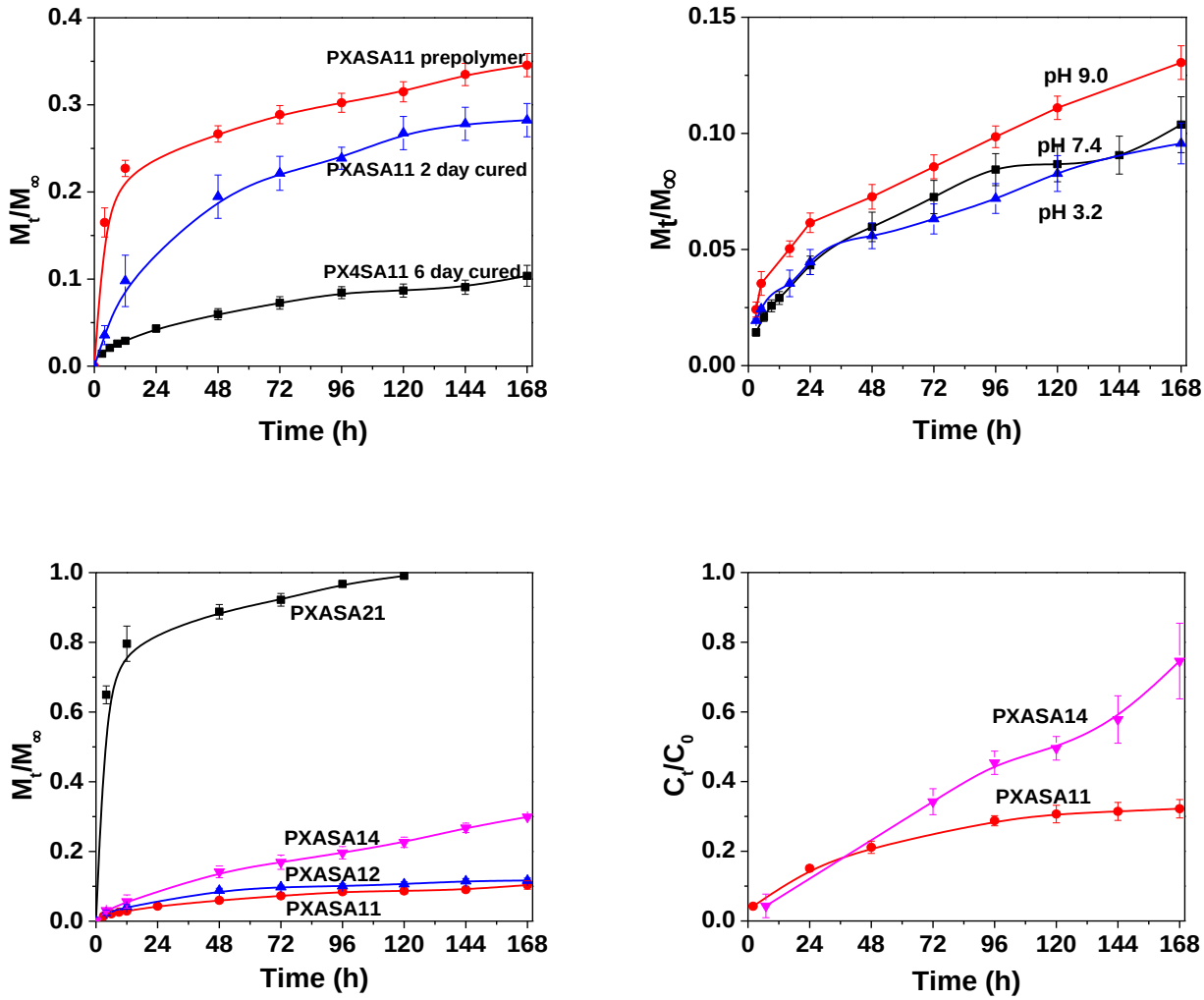

PXASA11 prepolymer
PXASA11 2 day cured
PX4SA11 6 day cured
M_t/M_∞
Time (h)
pH 9.0
pH 7.4
pH 3.2
M_t/M_∞
Time (h)
PXASA21
PXASA14
PXASA12
PXASA11
M_t/M_∞
Time (h)
PXASA14
PXASA11
C_t/C_0
Time (h)

Figure 6

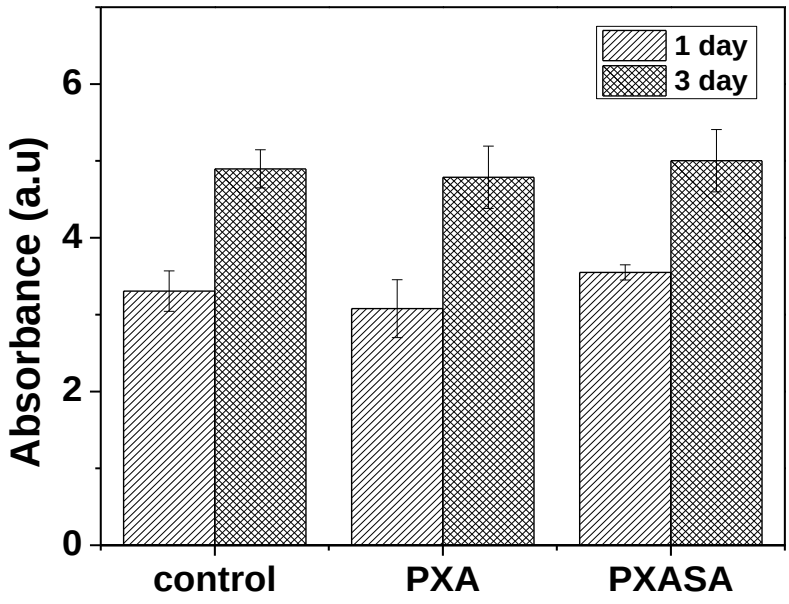


Figure 7

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Controlled Release of Salicylic Acid from Biodegradable Crosslinked Polyesters

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