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Human Mesenchymal Stem Cell Behavior on Segmented Polyurethanes Prepared with Biologically Active Chain Extenders

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Abstract

The development of elastomeric, bioresorbable and biocompatible segmented polyurethanes (SPUs) for use in tissue-engineering applications has attracted considerable interest because of the existing need of mechanically tunable scaffolds for regeneration of different tissues, but the incorporation of osteoinductive molecules into SPUs has been limited. In this study, segmented polyurethanes were synthesized from poly (ϵ -caprolactone)diol, 4,4'-methylene bis(cyclohexyl isocyanate) (HMDI) using biologically active compounds such as ascorbic acid, L-glutamine, β -glycerol phosphate, and dexamethasone as chain extenders. Fourier Transform Infrared Spectroscopy (FTIR) revealed the formation of both urethanes and urea linkages while Differential Scanning Calorimetry, Dynamic Mechanical Analysis, X-ray Diffraction and mechanical testing showed that these polyurethanes were semi-crystalline polymers exhibiting high deformations. Cytocompatibility studies showed that only SPUs containing β -glycerol phosphate supported human mesenchymal stem cell (hMSC) adhesion, growth, and osteogenic differentiation, rendering them potentially suitable for bone tissue regeneration, whereas other SPUs failed to support either cell growth or osteogenic differentiation, or both. This study demonstrates that modification of SPUs with osteogenic compounds can lead to new cytocompatible polymers for regenerative medicine applications.

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Keywords

Segmented polyurethane; osteogenic chain extender; bone regeneration

1. INTRODUCTION

Biodegradable segmented polyurethanes (SPUs) represent a promising class of polymers for bone tissue reconstruction due to their controlled degradation, wide range of mechanical properties and acceptable biotolerance achieved through the use of biodegradable soft segments or labile linkages on the rigid segment [1, 2]. Biodegradable SPUs with appropriate mechanical properties have been synthesized by using 1,4-diisocyanatobutane and poly (ϵ -caprolactone) (PCL) as the biodegradable macrodiol and 1,4-diisocyanatebutane tyramine as the biodegradable chain extender [3]. Improvements in mechanical properties of polyurethanes for bone tissue regeneration via increases in hard segment led also to an increase in cell proliferation with a decrease in the osteogenic potential of human bone-derived cells [4]. The mechanical and biological properties of these polymeric matrices can be further improved by incorporating different types of bioactive fillers, such as hydroxyapatite (HA), β -tricalcium phosphate (β -TCP) as well as various bioactive glasses [5–12].

An attractive alternative to the use of SPU with varying mechanical properties but limited capacity of bone regeneration exhibited by differentiate cell is the culture of multipotent stem cell and then differentiate them into an osteoblast lineage. The fate of MSCs is greatly influenced by both intrinsic and extrinsic signals, including cell-cell and cell-substrate interactions, the gradients of oxygen, nutrient, protein concentrations and mechanical properties of the 2D or 3D substrate. For instance, Zanetta *et al.*[13] have demonstrated that MDI based polyurethane foams support cell proliferation and differentiation of MSC into osteoblasts. You *et al.*[14] showed that there is an enhanced osteogenic differentiation of hMSC on nanostructured UV curable polyurethanes. More recently, Alves *et al.*[15] grafted sulfonic groups on a commercial polyurethane (Elastollan) and studied the adhesion and spreading of human bone marrow stem cell. They found good cell adherence and spreading that developed a well established actin cytoskeleton on unmodified and modified PU. Finally, Kuo *et al.*[16] showed that single hMSC proliferation slightly increased with fiber diameter, while hMSC aggregates had similar proliferative capacity [16].

Despite these modifications of polyurethanes for the development of scaffolds for engineering bone tissue, biologically active components that support osteoblast proliferation and collagen synthesis [17] and that are commonly found in cell culture media have not been used in the synthesis of segmented polyurethanes except for the work of Zhang *et al.*[18] This group synthesized a polyurethane foam from lysine-di-isocyanate (LDI) and glycerol. Ascorbic acid (AA) was copolymerized with LDI-glycerol i.e. AA was used as part of the prepolymer but not as a chain extender. In the present study, we synthesized polyurethanes formulated with ascorbic acid, L-glutamine, dexamethasone, and β -glycerol phosphate as candidate polymeric materials for bone applications. In this regard, functional groups such as hydroxyl, amine and carboxylic present in the biologically active compounds were used

for the chain extension reaction with isocyanate terminated PCL prepolymers. Even when these biologically active molecules are taken up directly by the cells it is hypothesized that their presence in the backbone of the polymer will allow a continuous release to induce cell proliferation and differentiation. Therefore, we also characterized the osteogenic activity of human mesenchymal stem cells (hMSCs) on these new polymers to identify suitable formulations for future *in vivo* studies.

2. MATERIALS AND METHODS

2.1. Polyurethane synthesis

Poly (ϵ -caprolactone) diol (PCL diol, $M_n=2000$), 4,4(methylene-bis-cyclohexyl)isocyanate (HMDI) and stannous octoate (SnO) were purchased from Sigma-Aldrich (Milwaukee, USA). Dimethyl formamide (DMF) from Sigma-Aldrich (Steinheim, Germany) and tetrahydrofuran (THF) from JT Baker (Phillipsburg, USA) were used as solvents in the synthesis or Im preparation. Ascorbic acid (ASC), L-glutamine (GLU), dexamethasone (DEXA) and sodium β -glycerol phosphate (BETA) used as chain extenders were also obtained from Sigma-Aldrich (Milwaukee, USA).

Segmented polyurethanes (SPUs) with a molar ratio of 1:2:1 (PCL:HMDI:chain extender) were prepared by a two-step procedure in nitrogen atmosphere at 60°C with 0.3% w/w stannous octoate (SnO) as catalyzer[19]. In the first step, PCL diol terminated (4 mmol, $M_n=2,000$) and SnO were dissolved in DMF. Next, the solution was mixed with an excess of HMDI (8.4 mmol) and stirred during 4 hours in order to form an NCO-terminated prepolymer. In the second stage, the chain extender (4.4 mmol) was dissolved in DMF and added to the reaction and stirred during 2 hours to extend the polymer. To stop the reaction, product was precipitated and washed with distilled water. Finally, the polymer was dried at 60°C at reduced pressure. Scheme of Figure 1 depicts the suggested structure of the synthesized polyurethanes. Table 1 shows the designation used in this study and the theoretical amount of soft and rigid segments [20].

2.2. Physicochemical Characterization of SPUs

All characterization was conducted using solvent casting films except GPC that made use of the polymer obtained after synthesis.

2.2.1. Fourier transform infrared (FTIR) spectroscopy—Infrared spectra of the SPUs were obtained after casting a film on KBr disc with a Thermoscientific Nicolet 8700 FTIR (Madison, WI) in the spectral range from 4000 cm^{-1} to 600 cm^{-1} averaging 50 scans with a resolution of 4 cm^{-1} .

2.2.2. Molecular weight measurement—Gel Permeation Chromatography (GPC) was used for determining the average molecular weight of the synthesized polyurethanes (5 mg/ml), using an Agilent 1100 GPC-SEC system equipped with Zorbax PSM (60S and 1000S) columns and a refractive index detector (Agilent Technologies, Germany). HPLC grade DMF without LiBr was used as eluent with a flow rate of 1 mL/min at 50°C and the

calibration curve was obtained with 1 mg/mL polystyrene molecular weight standards in the range from 1,050 g/mol to 420,600 g/mol.

2.2.3. Thermal properties—The thermal behavior was evaluated with a differential scanning calorimetry (DSC) on a DSC 7 from Perkin Elmer (Norwalk, CT) using 5 mg of the polymer films encapsulated on aluminum pans. For determining the glass transition temperature (T_g) samples were heated from -80°C to 50°C and the onset used to measure the T_g during the second heating cycle. Melting temperature (T_m) was obtained during the first heating cycle from 40°C to 160°C at $5^\circ\text{C}/\text{min}$ under nitrogen atmosphere. Relative percent crystallinity (X_c) of the PCL in the SPUs was determined from the enthalpy of fusion using equation 1:

$$\%X_c = \frac{\Delta H_f}{w_{ss} \times \Delta H_f^\circ} \times 100 \quad (1)$$

Where H_f is the enthalpy of melting of SPUs obtained experimentally, w_{ss} is the theoretical mass fraction of the flexible segment and H_f° is the enthalpy of 100% crystalline PCL taken as 136 J/g [21].

T_g was also obtained by dynamic mechanical analysis with a Perkin Elmer DMA 7 (Norwalk, CT) in the extension mode. Strips obtained by THF casting of $20 \times 3 \times 0.1$ mm were heated from -100°C to 100°C at $5^\circ\text{C}/\text{min}$ using a static force of 90 mN and a dynamic force of 70 mN at 1 Hz.

For thermogravimetric analysis (TGA), 20 mg of the SPU films were heated from 50°C to 700°C at $10^\circ\text{C}/\text{min}$ under nitrogen atmosphere using a TGA 7 from Perkin Elmer (Norwalk, CT). From the first derivative curve, decomposition temperatures (T_d) were obtained.

2.2.4. Microstructure determination

X-ray diffraction (XRD): X ray diffraction measurements were carried out with a D-5000 Siemens diffractometer (Karlsruhe, Germany) using monochromatic radiation ($\text{CuK}_\alpha \lambda = 1.5418 \text{ \AA}$) at 35 kV and 24 mA. For these experiments, 1 cm^2 films were used and registered in the range $5^\circ < 2\theta < 60^\circ$ with a step count of 3 s and a step size of $0.02^\circ (2\theta)$.

Scanning electron microscopy (SEM): Morphology of the SPUs was observed by SEM using a JEOL 6360 LV (Tokyo, Japan). Films were gold coated and observed using an accelerating voltage of 20 kV.

2.2.5. Mechanical properties—Tensile mechanical properties were measured according to ASTM D-882 standard, using a Shimadzu precision universal testing machine (Model Autograph AGS-X, Kyoto, Japan). Samples ($n=5$), obtained by solvent casting, of $30 \times 5 \times 0.11$ mm were tested with a 100 N load cell, a cross-head speed of 100 mm min^{-1} and a gauge length of 12.7 mm. The Young's modulus at 100% of strain (E^{100}), tensile strength (σ) and strain to failure (ϵ) are reported.

2.3 Cytocompatibility studies

2.3.1. Polymer samples—The polymers examined were films of SPUGLU, SPUASC, SPUBETA and SPUDXA obtained by THF casting. Thin sample discs (thickness of 0.11 mm) used for biocompatibility studies were cut out using a 12 mm biopsy punch, soaked in PBS overnight, sterilized in 200 proof ethanol, and then rinsed with PBS. Polymeric samples were stored in PBS until use.

2.3.2. Cell Culture—Human mesenchymal stem cells (hMSCs) were obtained from Lonza. Cells were maintained on tissue culture polystyrene using Mesenchymal Stem Cell Growth Medium (MSCGM; Lonza pt3001) per manufacturer's instructions. Early passage hMSCs (<5) were used for all studies.

2.3.3. Cell number assay—hMSCs were seeded on polymer samples and tissue culture polystyrene (TCP, reference material) at 1,000 cells/cm². Cell number was analyzed at days 1 and 7 (n=4) using the CyQUANT Cell Proliferation Assay (Life Technologies) per the manufacturer's instructions. Briefly, cells were washed with Ca/Mg-free PBS and frozen at -80°C until analyzed. Frozen cells were thawed and treated with 200 µL of the CyQUANT GR dye/lysis buffer for 2–5 minutes at room temperature and protected from light. Fluorescence was measured at 480 nm excitation and 520 nm emission. Cell number was determined using a cell standard curve prepared using known cell numbers.

2.3.4. Osteogenic differentiation assays—hMSCs were seeded at 10,000 cells/cm² with four replicates [22, 23]. Controls included cells cultured on tissue culture plastic in osteogenic induction media (TCP ind) or growth media (TCP non ind). Cells were cultured in hMSC Mesenchymal Stem Cell Osteogenic Differentiation Medium (Lonza).

For alkaline phosphatase (ALP) activity assay, protein content was quantified using the Pierce BCA Protein Kit (Thermo) per the manufacturer's instructions. Briefly, cells were rinsed with Ca/Mg-free PBS at day 9. Cells were treated with ice-cold 50 mM Tris-HCl (pH 7.4), transferred to a centrifuge tube and sonicated to lyse the cells. Samples were centrifuged at >10,000g for 5 minutes. The supernatant was removed and used for further analysis. Samples were incubated at 37°C for 1–2 hours with BCA working reagent and protein content was quantified by measuring absorbance at 562 nm in triplicate. ALP activity was quantified by reacting cell lysate with MUP substrate (50 mM NaHCO₃, 10 mM diethanolamine, 500 µM MgCl₂, 200 µM 4-methylumbelliferyl phosphate in H₂O, pH 9.5). Samples were protected from light and incubated for 1 hour at 37°C. Fluorescence was read at an excitation of 360 nm and an emission of 465 nm. ALP activity was normalized by the protein content in each sample.

For mineralization assays, cultures at 21 days post-osteogenic induction were fixed with 10% paraformaldehyde for 15 minutes at room temperature. Cells were rinsed with excess water and treated with 40 mM Alizarin red (pH 4.1) for 20 minutes at room temperature. Excess alizarin red was rinsed with water, and samples were transferred to 1.5 mL tubes. Acetic acid (10% v/v) was added to each tube or well and incubated while shaking for 30 minutes. Samples were heated to 85°C for 10 minutes, centrifuged for 15 minutes at

20,000g, and supernatant was neutralized with ammonium hydroxide. Sample absorbance was read at 405 nm.

2.4 .Statistical analysis

Cell number, ALP activity and Alizarin red quantification were analyzed using a one-way ANOVA followed by a Tukey's multiple comparisons test performed in GraphPad using an alpha value = 0.05. Significance was defined as $P < 0.05$.

RESULTS

Spectroscopic studies

The chemical structure of the polyurethane was elucidated by FTIR. The IR spectra of SPUs are shown in Figure 2. The absorption peaks at 3373 cm^{-1} and 3525 cm^{-1} was assigned to N-H vibrations, while CH_2 asymmetric and symmetric stretching vibrations were observed at 2946 and 2869 cm^{-1} , respectively. Carbonyl stretching ($\text{C}=\text{O}$) appeared between 1729 cm^{-1} and 1760 cm^{-1} , which includes the ester group from the PCL, and around 1700 cm^{-1} , which includes the urethane group (NHCOO). Amide II absorption (urethane N-H bending + C-N stretching) and amide III absorption (C-C stretching) were located at 1522 cm^{-1} and 1233 cm^{-1} , respectively, while the peak at 1166 cm^{-1} was attributed to C-O-C stretching vibration in the soft segment. The peak at 1634 cm^{-1} was assigned to the urea linkages confirming the reaction of amine groups (from the glutamine) with NCO, although, the presence of water might also contribute in the other polyurethanes. Even though the bands of chain extenders usually overlapped, spectrum of SPUDExA show the characteristic band at 1668 cm^{-1} due to the carbonyl-stretching vibration of the dexamethasone A-ring.

As shown in Table 1, Mn and Mw were higher in SPUGLU while SPUDExA exhibited lower Mn and Mw than SPUBETA. In contrast, SPuASC exhibited the lowest molecular weight. In spite of these low Mn and Mw values, they were high enough for film formation and mechanical testing.

Thermal properties

All types of SPUs containing PCL as the soft segment were semi-crystalline materials as they exhibited melting peaks between 51.0°C and 53.8°C . However, the percentage of crystallinity followed the order $\text{SPUGLU/SPuASC} > \text{SPUDExA/SPUBETA}$. The presence of a crystalline phase is attributed to PCL as the rigid segment (HMDI-chain extender) of these aliphatic polyurethanes which do not tend to crystallize but tend to form ordered regions as will be discussed later from XRD results. Although the presence of a melting peak suggested that the crystalline structure of the PCL is preserved due to its relatively high molecular weight (Mn 2000), the reduction in crystallinity is caused by the high molecular weight of the chain extender (either DEXA or BETA) that impedes proper chain organization.

The $\tan \delta$ variation with temperature showed a well-defined α transition which was related to the soft segments of the SPU. For SPuASC and SPUDExA, this was higher (-48°C to -43°C) than for SPUGLU and SPUBETA (-51°C). The shift in the T_g of the amorphous

phase PCL suggests that the rigid segment content is restricting PCL chain movement as the T_g of pure PCL was found at -60°C . The T_g obtained by DSC, although generally accepted to be lower compared to DMA, followed the opposite trend i.e. lower for SPUBETA (-37°C) than for SPUDExA (-20.0°C), SPuASC (-21.1°C) and SPUGLU (-27°C).

Thermogravimetric analysis of the SPUs showed a main decomposition temperature in the 365°C to 398°C range (denoted as T_{d2}) which was attributed to the decomposition of the flexible segment (PCL). However, small decomposition peaks were also detected at lower temperatures for SPuASC only ($T_{d1}=203^\circ\text{C}$) and at higher temperatures ($T_{d3}=465\text{--}478^\circ\text{C}$) being the later assigned to the rigid segments. Table 2 summarizes the thermal properties of SPUs.

Microstructure by XRD and SEM

Figure 3 shows the XRD patterns of SPUs. The semi-crystalline nature of these SPUs can be confirmed from the presence of reflections at $2\theta = 21.3^\circ$, 22.0° and 23.4° due to the PCL soft segments. The broad reflection with a maximum at 19.3° can be related to ordered regions from the rigid segment formed. As seen in figure 3, there is a higher content of rigid segments (as observed from the higher intensity/area) in SPUGLU and SPuASC explaining their better mechanical performance.

Figure 4 shows the SEM images for SPU samples. The formation of agglomerates (spherulites) in the neat polyurethanes is evident only in SPuASC and SPUGLU, with the size of the spherulites being smaller in glutamine containing polymers. The absence of these structures in SPUBETA and SPUDExA can be related to its poor crystallinity as shown by DSC and XRD. SPUDExA also showed the presence of pores on its surface.

Mechanical properties

Analysis of the mechanical properties of SPUs showed an initial elastic behavior (less than 1% as proved by hysteresis analysis) and elastomer-like behavior in the sense that it sustained large deformations (see Figure 5). SPUGLU and SPuASC exhibited higher moduli than the other formulations, but deformation was higher in SPUGLU. SPUBETA exhibited the lowest modulus, but a large strain to failure suggested lower crosslinking than SPUDExA. Table 3 summarizes the tensile mechanical properties of the SPUs.

Cell adhesion and proliferation hMSC adhesion and growth on SPU polymers were examined by quantifying cell numbers at days 1 and 7 (See Fig. 6). Equivalent cell numbers were observed among SPU polymers as well as the tissue culture polystyrene (TCP) reference at day 1. Over 7 days in culture, cell numbers increased for SPUBETA, indicating that this polymer supports cell proliferation. In contrast, there were no differences in cell numbers between days 1 and 7 for SPuASC and SPUGLU. The number of cells significantly decreased with time in culture for SPUDExA, suggesting that although this polymer supports short term adhesion, it does not support long term cell adhesion or growth.

Osteogenic differentiation

Alkaline phosphatase is an early indicator of osteogenic differentiation due to its ability to catalyze the hydrolysis of phosphate esters leading to calcification. ALP enzymatic activity, assayed at day 9, indicated that SPUBETA supported osteogenic differentiation when compared to cells cultured in growth media on tissue culture polystyrene (Figure 7A). Alizarin red staining for calcium deposits performed at day 21 shows high calcification on both SPUBETA and SPUGLU compared to cells grown in growth media on tissue culture polystyrene (Figure 7B).

DISCUSSION

In order for biodegradable SPUs to be used as a biomaterial within an implant, the ability for these SPUs to promote cellular adhesion and growth must be evaluated. As our interest is specifically for the use as an orthopedic implant, this material should also support osteogenic differentiation of hMSCs [24, 25]. In the present study, we synthesized various SPUs incorporating osteogenic compounds such as ascorbic acid, β -glycerol phosphate and dexamethasone as chain extenders. It is known that L-Ascorbic acid enhances collagen synthesis and up-regulates adenosine triphosphatase and alkaline phosphatase activity [26], but has also been shown to decrease cellular proliferation, possibly acting as one of the signals theorized as necessary for the progression from proliferation to the matrix maturation phase [27]. β -glycerol phosphate serves primarily as a source of inorganic phosphate ions, and in combination with L-Ascorbic acid, allows the formation and mineralization of the extracellular matrix in culture [26]. Dexamethasone is a synthetic glucocorticoid that exerts various inhibitory effects on the inflammatory processes and is known to play a key role in controlling the regulation of genes and cellular processes essential for cellular growth and division [28]. On the other hand, dexamethasone exerts a powerful effect on the osteogenic differentiation of MSCs. Continual exposure to dexamethasone, beginning shortly after cell harvest, is required to drive and maintain the osteoblastic phenotype of marrow-derived progenitor cells. Although maintenance of the osteoblastic phenotype is desirable for long-term cultures, dexamethasone has been known to drive MSCs down the osteogenic pathway so quickly that they do not proliferate well [26].

Physicochemical properties of SPUs with biologically active chain extenders

FTIR showed that the synthesized polymers contained urethane, urea or amide linkages depending on the chain extender used. Solubility tests (in deuterated chloroform or DMF) suggested that linear or slightly crosslinked polymers (SPUASC, SPUGLU, SPUBETA, SPUDExA) were obtained in spite of the multifunctional nature of the chain extender. This can be attributed to the lower reactivity of carboxylic acids and secondary hydroxyl groups in glutamine and ascorbic acid/dexamethasone respectively [29, 30].

DSC and XRD showed that semicrystalline polyurethanes were obtained where crystallinity tended to decrease as the molecular weight of the chain extender increased. This can be explained as the formation of an ordered structure was hindered by a bulky molecule (dexamethasone) or due to the presence of phosphate groups (β -glycerol phosphate). It is also possible that the remaining hydroxyl groups formed intermolecular hydrogen bonds

between PCL chains and the rigid segment competing with intramolecular PCL hydrogen bonds.

In the temperature interval of our dynamic mechanic experiments, the poly (ϵ -caprolactone) showed only the main relaxation associated with the co-operative rearrangements of the polymer chain in the amorphous phase. However, the T_g of PCL tend to increase due to chain movement restrictions, i.e. due to the presence of the bulky rigid segments (approximately 25%) or even due to crosslinking. Therefore, SPUASC and SPUDExA showed a higher T_g than SPUGLU and SPUBETA. The different trend observed in terms of the T_g measured by DSC can be explained by further crosslinking reactions during the first heating cycle. In the case of SPUASC, these crosslinking reactions also account for the slightly higher thermal stability i.e. higher T_{d2} and T_{d3} .

In general, the mechanical behaviour of these materials can be attributed mainly to the PCL in the SPU matrix, as once unfolded and aligned, the PCL chains tend to sustain the load and to show an increase in resistance. However, M_n and M_w were higher in SPUGLU, which likely explains their higher Young's modulus. SPUDExA also exhibited high modulus, but in spite of exhibiting a lower M_n and M_w than SPUBETA, the crosslinking reaction might explain their mechanical performance. In contrast, SPUASC exhibited the lowest molecular weight but the highest modulus and tensile strength possibly due to crosslinking reactions as observed in the gel fraction of this polyurethane. SPUASC exhibited the highest Young's modulus (26.7 MPa) of the synthesized polyurethanes. This value is still far from the cortical bone (12–18 GPa) and spongy bone (100–500 MPa) or even foamed polyurethanes used to mimic trabecular bone[20, 31]. However, it is clear from previous reports that our polyurethanes can be improved by increasing molecular weight of PCL [3], by increasing rigid segment content [4] and by the addition of bioactive fillers [8, 9]. It is convenient to remember that for tissue engineering, as the tissue grows, the polymer degrades. In this sense, we expect that tissue formation will be accelerated by the presence of osteogenic molecules which will then compensate for their low mechanical properties.

Biological performance of SPU with biologically active chain extenders

To evaluate cell growth and number, hMSCs were cultured on the various SPUs. Resulting cell numbers showed all SPUs allowed for initial cell adhesion at day 1 at levels equivalent to the reference material. By day 7, cell number did not increase on SPUGLU and SPUASC suggesting cell death or the lack of support for long-term cell adhesion. Even when the amount of glutamine released from the polyurethanes (SPUs) was not quantified in the culture media, the decrease in cell number may be due to the degradation of L-glutamine to ammonia or the high concentration of L-glutamine used in this study compared to levels found in culture media (2 – 4 mM). In addition, the absence of alkaline nitrogen atoms in the lateral chain but the presence of an amide as the functional group in the lateral chain reduced cell adhesion. In the case of ascorbic acid, this was also used in higher amounts (approximately 3 wt. % of ascorbic acid for SPU synthesis) compared to the recommended for osteoblast cell culture (50 μ g/ml or 150 mg/l). In low concentrations, ascorbic acid or L-ascorbate-2-phosphate, a long-acting ascorbic acid derivative, is essential for the expression

of osteoblastic markers (collagen synthesis and alkaline phosphatase activity) and mineralization[31, 32].

SPUDEXA showed reduced cell numbers, while SPUBETA showed a significant increase in cell number and all other SPUs at least sustained the number of cells that initially adhered. These results support the use of SPUBETA as a biomaterial as this showed significant cell growth over 7 days in culture. Therefore, we can speculate that degradation of SPUBETA by hydrolysis is occurring.

A typical complete media also contains 10 mM Na β -glycerophosphate, and 10 nM dexamethasone. Once again, even when β -glycerophosphate and dexamethasone were used in higher concentration for the SPU synthesis its initial effect was to allow cell adhesion. Not only should a biomaterial allow for cell proliferation, as accessed by the cell number assays, it should also promote osteogenic differentiation of hMSCs if intended for orthopedic use. Increased ALP activity was observed for SPUBETA. Alizarin red staining, performed at day 21, stains for calcium and can be used as a measure of mineralization of hMSCs. SPUBETA had an increase in mineralization when compared to a non-induced hMSC control. This increase in mineralization is in agreement with the enhanced cell numbers and ALP activity. SPUGLU also showed increased Alizarin red staining when compared to the non-differentiated cells, but it did not support increases in ALP activity or cell numbers. Dexamethasone has been reported to have an effect on mesenchymal cell morphology, expression of ALP, osteocalcin production and matrix mineralization [27]. However, in our study, SPUDEXA induced low levels of ALP and mineralization. It is possible that transient exposure of stem cells to dexamethasone may be more effective in inducing and maintaining the osteoblastic phenotype. Further, in addition to medium components and their concentrations, the temporal presentation in which osteogenic supplements are added can affect both proliferative potential of the cells and their differentiated function.

CONCLUSION

In this study, we created segmented polyurethanes that were synthesized from poly(ϵ -caprolactone)diol and 4,4'-methylene bis(cyclohexyl isocyanate) (HMDI) using osteogenic compounds such as ascorbic acid, L-glutamine, β -glycerol phosphate and dexamethasone as chain extenders. These SPUs have significant potential for the use in bone tissue regeneration due to their variable mechanical properties and high bio tolerance. From these SPUs, it was determined that SPUBETA allows hMSC adhesion and proliferation, high levels of ALP expression and high mineralization potential. Therefore, SPUBETA is a very promising material that has been distinguished for further investigation through *in vivo* studies and other mechanisms of characterization for bone tissue regeneration.

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Figure 1.

A) Suggested chain extension mechanism reaction of prepolymer with biologically active compounds. B) Possible crosslinking reactions between isocyanate and chain extenders.

Figure 2. FTIR spectra of SPUs prepared with biologically active chain extenders. SPUs with ascorbic acid (SPUASC), glutamine (SPUGLU), β -glycerol phosphate (SPUBETA) and dexamethasone (SPUDEXA).

Figure 3.

XRD patterns of SPUs prepared with biologically active chain extenders. SPUs with ascorbic acid (SPUASC), glutamine (SPUGLU), β -glycerol phosphate (SPUBETA) and dexamethasone (SPUDEXA).

Figure 4.

SEM micrographs of SPUs prepared with biologically active chain extenders. SPUs with ascorbic acid (SPUASC), glutamine (SPUGLU), β -glycerol phosphate (SPUBETA) and dexamethasone (SPUDEXA). SPUASC and SPUGLU show formation of agglomerates (spherulites) marked by double arrows. Pores in the surface of SPUDEXA were observed and marked by a double arrow.

Figure 5. Tensile stress-strain curve for SPUs prepared with biologically active chain extenders. SPUs with ascorbic acid (SPUASC), glutamine (SPUGLU), β -glycerol phosphate (SPUBETA) and dexamethasone (SPUDEXA).

Figure 6. hMSC number for cells cultured on SPUs. Cell number was quantified at days 1 and 7 by DNA content. Results were analyzed using a one-way ANOVA followed by a Tukey's multiple comparison test with an alpha = 0.05. * denotes p-value < 0.05, ** denotes p-value < 0.005.

Figure 7.

ALP activity at day 9. (A) SPUBETA show a significant increase in ALP over non-induced hMSCs. (B) Mineralization of hMSCs quantified by Alizarin red staining after 21 days. SPUBETA and SPUGLU show significantly increased calcification over non-induced hMSCs. A one-way ANOVA followed by a Tukey's multiple comparison test with and alpha = 0.05. * denotes a p-value of 0.05, ** denotes a p-value of 0.001.

Table 1

Identification of synthesized polyurethanes, content of soft and rigid segments and their molecular weight.

SPU	SS (%)	SR (%)	Mn (g/mol)	Mw (g/mol)	PI
SPUASC	72.8	27.2	26,681	33,688	1.25
SPUGLU	73.7	26.3	49,737	86,871	1.74
SPUBETA	69.3	30.7	43,441	64,064	1.47
SPUDEXA	67.0	33.0	31,810	54,026	1.69

Table 2

Thermal properties of SPUs prepared with biologically active chain extenders

SPU	T_g^1 (°C)	T_m^2 (°C)	X_c^3 %	T_d^3 (°C)		
				T_{d1}	T_{d2}	T_{d3}
SPUASC	-48.4	53.8	38.0	203	398	478
SPUGLU	-51.2	51.4	40.0	-	370	472
SPUBETA	-51.5	51.0	20.0	-	365	465
SPUDEXA	-43.19	51.3	21.0	-	368	467

Measured by

$^1_{DMA}$,

$^2_{DSC}$,

$^3_{TGA}$.

Table 3

Tensile mechanical properties of SPUs prepared with biologically active chain extenders

SPU	E_{100} (MPa)	σ (MPa)	ϵ (%)
SPUASC	26.7 ± 25.6	47.75 ± 16.7	1298 ± 286
SPUGLU	12.0 ± 2.2	19.6 ± 11.2	1879 ± 302.4
SPUBETA	1.96 ± 0.19	19.00 ± 1.76	1141 ± 110
SPUDEXA	6.25 ± 0.38	10.77 ± 1.85	520 ± 70