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Silk fibroin–polyurethane blends: Physical properties and effect of silk fibroin content on viscoelasticity, biocompatibility and myoblast differentiation

Hyung-seok Park [a,b], Myoung-Seon Gong [a], Jeong-Hui Park [a], Sung-il Moon [b], Ivan B. Wall [a,d], Hae-Won Kim [a,e], Jae Ho Lee [a,*], Jonathan C. Knowles [a,c,*]

[a] Department of Nanobiomedical Science & WCU Research Center, Dankook University Graduate School, Cheonan 330-714, South Korea
[b] Development Group 2, Uiwang R&D Center, Samsung Cheil Industries Inc., Gyeonggi-do 437-801, South Korea
[c] Division of Biomaterials and Tissue Engineering, UCL Eastman Dental Institute, 256 Gray's Inn Road, London WC1X 8LD, UK
[d] Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK
[e] Institute of Tissue Regeneration Engineering (ITREN), Dankook University Graduate School, Cheonan 330-714, South Korea

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ABSTRACT

As a way to modify both the physical and biological properties of a highly elastic and degradable polyurethane (PU), silk fibroin (SF) was blended with the PU at differing ratios. With increasing SF content, the tensile strength decreased as did the strain at break; the stiffness increased to around 35 MPa for the highest silk content. C2C12 (a mouse myoblast cell line) cells were used for in vitro experiments and showed significantly improved cell responses with increasing SF content. With increasing SF content the number of non-adherent cells was reduced at both 4 and 8 h compared to the sample with the lowest SF content. In addition, muscle marker genes were upregulated compared to the sample containing no SF, and in particular sarcomeric actin and α-actin.

1. Introduction

Tissue engineering methods are being developed as a means to replace damaged or diseased organs. This approach uses tissue-specific cells, which are grown on a scaffold material with the purpose of creating a functional tissue or organ. Many different compounds are being studied for use as scaffolds in tissue engineering. These include both synthetic and natural materials [1].

Recently natural polymers have been studied as resources due to their unique properties including non-toxicity, biodegradability and biocompatibility. However, natural homopolymers by themselves are inadequate to meet the diversity of demands for biomaterials and do not offer the opportunity for property-tuning via modulation of the chemistry. In order to improve the performance of individual natural polymers, many blends have been developed, such as silk fibroin (SF) with calcium phosphate cements [2] and blends of two or more degradable polymers [3]; chitosan [4], hyaluranon [5] and poly(vinyl alcohol) [6] blends, amongst others, have been prepared using solution blending methods.

Synthetic biocompatible/biodegradable polymers have good potential for clinical use as control over their degradation rate and mechanical properties may be accomplished for a particular application [7]. These polymers are especially useful in biomedical approaches due to their potential ability to enable migration, cell adhesion, differentiation and proliferation [8,9], as well as their temporary nature. These polymers could also be implanted in the human body by injection, having potential applications in the areas of gene delivery, sustained drug delivery and tissue engineering [10].

Polyurethanes (PUs) can be fabricated via reaction between dialcohols and diisocyanates forming urethane linkages [11]. There are many methods for manufacturing PUs, either with or without the use of organic solvents. The most widely used approach the one-shot process, where direct mixing of monomers and catalysts and other additives is carried out [12]. Recently, many papers on biodegradable PUs have appeared [13]; PUs are a class of biodegradable polymers that have been applied as tissue-engineering scaffolds as they show low cytotoxicity in vitro and in vivo [14–18].

* Corresponding authors at: Department of Nanobiomedical Science & WCU Research Center, Dankook University Graduate School, Cheonan 330-714, South Korea and Division of Biomaterials and Tissue Engineering, UCL Eastman Dental Institute, 256 Gray's Inn Road, London WC1X 8LD, UK. Tel.: +44 0203456 1189.
E-mail address: j.knowles@ucl.ac.uk (J.C. Knowles).

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Of the PUs investigated, the overwhelming majority are biocompatible, and designed to be in service for long periods of time [19]. One of the intended uses of these polymers is for tissue regeneration purposes and they are therefore required to be stable for extended periods; therefore great care has to be taken in the choice of their building blocks. Their degradation products have to be biocompatible, non-toxic and metabolized or reduced by the host organism. Among the different polyols, polycaprolactone diol (PCL diol) is known to be biocompatible and slowly, hydrolytically and enzymatically degradable [20]. The degradation product of PCL, 6-hydroxyhexanoic acid, is transformed by microsomal ω-oxidation to adipic acid, which is a naturally occurring metabolite [21]. The diisocyanate typically used in the hard segment of conventional PUs is not biocompatible. A common example of this structural component is toluene diisocyanate or diphenylmethane diisocyanate. The degradation products of these diisocyanates are toxic, and carcinogenic compounds such as aromatic diamines can arise, making them unsuitable for use in vivo. Aliphatic diisocyanates, on the other hand, are non-toxic but have low polymerization activity, and hence a catalyst is required. Organometallic compounds, such as stannous octoate and dibutyl tin dilaurate, and tertiary amines may be used to catalyze reactions leading to PUs, and catalysts of either type remain as a leachable residue in the final product; both types of catalysts are highly toxic and require removal following polymerization. To design biocompatible and biodegradable PUs, aliphatic diisocyanates are generally chosen, because their degradation products are non-toxic. Polyester diols such as PCL diol are also chosen as they can be hydrolytically degraded to provide caproic acid. We chose isosorbide here as the dialcohol monomer because of its chirality, rigidity and non-toxicity [22]. Previous work has established the feasibility of synthesis of biocompatible and biodegradable PUs synthesized via a simple catalyst-free, one-shot polymerization [12] of hexamethylene diisocyanate (HDI), PCL diol (M_w 2000) and isosorbide. However, the polymers synthesized, whilst possessing excellent biocompatibility due to the absence of any catalyst and the biocompatibility of the monomers, possess limited mechanical properties. For example, these polymers have very high strain at break—in some cases over 1000%, which may be useful in certain limited applications, such as cardiac heart patches for stem cell delivery. However, in order to offer a range of materials, a second phase, such as silk, can be incorporated. Silk was chosen as it has a number of features of interest: (i) it is biocompatible when properly prepared; and (ii) like PU, it is slowly degradable in vivo and avoids additional bioburdens sometimes associated with materials derived from mammalian tissues or cells [23]. Furthermore, SF also offers the possibility to have RGD sequences included within the structure, or these may be functionalized onto the surface at a later stage [24], and this has important consequences for cell attachment. Another important parameter is the miscibility of the two solutions being mixed as this is necessary to prepare a well-blended and homogeneous end product. Finally, silk was chosen as it is relatively stiff compared to the extremely elastic PU polymer, and so will give a range of composites of increasing stiffness.

Natural silk from the silkworm *Bombyx mori* has long been used as a fabric material in the textile industry and as surgical sutures in clinical applications [25–30]. Silkworm silk consists of a core structural protein called SF, which is coated with a gum-like protein called sericin. Since the sericin contamination was identified to be the main source of problems, such as unwanted immunological reactions in vivo [31,32], purified SF has become a novel, promising biomaterial and has found increasing numbers of applications in clinical fields including tissue engineering of cartilage, bone, muscle, ligament and tendon tissues [23,33–36].

This study was aimed at determining the chemical and mechanical properties of blend films composed of *B. mori* SF and PUs and, in light of these modified mechanical properties, to assess their capacity to support myoblast differentiation. Mixtures of *B. mori* SF solution and PU solutions in various blend ratios were prepared to form blend films. The structural characteristics, thermal properties and morphology of the blend films were examined by Fourier transform infrared (FT-IR) spectroscopy, differential scanning calorimetry (DSC) and scanning electron microscopy (SEM). We also investigated in vitro adhesion, proliferation and myoblast differentiation using the C2C12 cell line.

2. Experimental

2.1. Materials and instruments

B. mori silkworm silk was purchased from Bo-eun Silk Factory, Korea. Isosorbide (98%) and (HDI (99%) were purchased from Sigma–Aldrich (St Louis, MO, USA). Polycaprolactonediol 2000 (CAPA2201A = M_w 2000) was purchased from Kangnam Chemical Company (Ansan, South Korea) and phosphate-buffered saline (pH ~7.3) was obtained from Oxoid Ltd. (Basingstoke, UK). All chemicals were used without further purification.

2.2. Preparation of polyurethane

The PU was synthesized as described previously [11] using the one-shot method shown in Scheme 1. The ingredients were all mixed together and allowed to cure [37,36]. Briefly, synthesis was carried out in a 100 ml round-bottomed four-neck flask with a reactant stoichiometry of 1:1 of diisocyanate:di-alcohol (PCL diol and isosorbide) under a dry nitrogen atmosphere. A nitrogen-flushed four-neck flask equipped with a mechanical stirrer, thermometer and condenser was filled with 27.37 mmol PCL diol (54.74 g) and 27.37 mmol isosorbide (4.0 g). The flask was maintained at 80 °C and stirred under a nitrogen atmosphere. After the solid contents were melted completely, 54.74 mmol HDI (9.21 g) was added to the reaction system and stirred for 2 min. The sample was allowed to stand in a Teflon dish to cure for 12 h at 150 °C. The synthesized PUs were dissolved in *N,N*-dimethylformamide and the solution was precipitated into a large amount of isopropyl alcohol and washed with more isopropyl alcohol. The product was dried at 40 °C for 72 h under vacuum and stored in desiccators.

2.3. Preparation of silk fibroin

Silk cocoons of *B. mori* silkworms were degummed using boiling water and Na_2CO_3 aqueous solutions to remove sericin. The alkaline solution was then drained and the degummed silk was rinsed several times with water. Finally, residual sericin was removed completely by rinsing the silk in deionized water, then drying the silk. The SF (12%, w/v) was dissolved in a solution of $CaCl_2$/C_2H_5OH/H_2O (1.0/2.5/8.0) at 80 °C for 1 h by continuous stirring. The solution was cooled and then dialyzed against deionized water using a 12,000–14,000 M_W cut-off membrane for a week. The water was changed every 24 h. After dialysis, the SF slurry was filtered using glass-sintered filter with 300 μm pore size and lyophilized to obtain sponges of regenerated SF.

2.4. Preparation of blended SF/PU films

The films of PU as a control and blended SF/PU for mechanical testing, contact angle measurement and cell testing were prepared by solvent casting in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). PU and SF/PU blends were dissolved in HFIP and stirred at room temperature for 12 h. SF/PU blend solutions were prepared as follows.