Evaluation of Novel 3D Architectures Based on Knitting Technologies for Engineering Biological Tissues*

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Abstract: Textile-based technologies are considered as potential routes for the production of 3D porous architectures for tissue engineering applications. We describe the use of two polymers, namely polybutylene succinate (PBS) and silk fibroin (SF) to produce fiber-based finely tuned porous architectures by weft and warp knitting. The obtained knitted constructs are described in terms of their morphology, mechanical properties, swelling ability, degradation behaviour and cytotoxicity. Each type of polymer fibers allow for the processing of a very reproducible intra-architectural scaffold geometry, with distinct characteristics in terms of the surface physicochemistry, mechanical performance and degradation capability, which has an impact on the resulting cell behaviour at the surface of the respective biotextiles. Preliminary cytotoxicity screening shows that both materials can support cell adhesion and proliferation. Furthermore, different surface modifications were performed (acid/alkaline treatment, UV radiation and plasma) for modulating cell behavior. An increase of cell-material interactions were observed, indicating the important role of materials surface in the first hours of culturing. Human Adipose-derived Stem Cells (hASCs) became an emerging possibility for regenerative medicine and tissue replacement therapies. The potential of the recently developed silk-based biotextile structures to promote hASCs adhesion, proliferation and differentiation is also evaluated. The obtained results validate the developed constructs as viable matrices for TE applications. Given the processing efficacy and versatility of the knitting technology, and the interesting structural and surface properties of the proposed polymer fibers, it is foreseen that our developed systems can be attractive for the functional engineering of tissues such as bone, skin, ligaments or cartilage and also for develop more complex systems for further industrialization of TE products.

Key words: Textile-based technologies; Silk; PBS; Surface modifications; Human adipose-derived stem cells; Tissue engineering.

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Introduction

In the biomedical field, tissue engineering (TE) represents a specific area in which textile technologies can have an important contribution[1, 2]. Maximization of tissue attachment to materials requires a highly organized porous structure for tissue integration and a template for cell assembly, combined with structural properties analogous to those of the living tissue. Scaffolds developed for TE, need to facilitate and promote cellular proliferation and tissue regeneration. The intra-architectural scaffold geometry, as well as the scaffold material and its surface properties play an important role in this process. Many of the conventional fabrication techniques available for scaffold production do not yet enable to obtain the desired scaffold properties, as many of the processing routes still present slow reproducibility. Several methods have been developed and proposed to prepare porous scaffolds for Tissue Engineering, including gas foaming, fiber extrusion and bonding, three-dimensional printing, phase separation, emulsion freeze-drying, and porogen leaching or rapid prototyping[3, 4]. Most of these techniques have been extensively studied using different biodegradable polymers, such as polyglycolic acid (PGA), polylactic acid (PLA), polycrapolactone (PCL), starch or silk, investigated for cell transplantation and regeneration of various tissues, such as nerve, skin, ligament, bladder, cartilage, and bone[4-6]. Scaffolds processed by using fiber-based technologies represent a wide range of morphological and geometric possibilities that can be tailored for each specific tissue-engineering application[7]. In fact, fiber networks with high surface area and interconnectivity have proven to be particularly interesting in promoting cell attachment and proliferation[8-10]. Therefore, textile-based technologies are considered as potential routes for the production of complex scaffolds for TE applications, as they can present superior control over the design, manufacturing precision and reproducibility.

Being computer assisted, textile technologies have potential for the production of pre-designed architectures with highly controlled and predictable properties according to the final requirements. In this sense, textiles have found their way into a variety of medical applications, ccording to the patient need[11], such as the augmentation and reconstruction of knee[12] and shoulder[13] ligaments, tendon[14] and intervertebral disc replacement[15]. In the therapy of cardiovascular diseases, fabrics have been utilized for cardiac support devices[16], tissue regenerative vascular grafts[17] and prosthetic heart valves for percutaneous implantation[18]. In the repair and regeneration of the peripheral nerves, woven tubular shaped guides were used[19]. Skin is a very compliant tissue that constitutes one of the most obvious applications for biotextiles, as in the case of wound dressings or tissue engineering products[2]. The optimal design of such textile implants requires a multi- and interdisciplinary combination of skills[1, 2, 11].Despite the fact that some of the traditional textiles have fulfilled primary quality requirements such asbiocompatibility, flexibility or strength, there is a need for further develop new systems to meet more demanding and specialized functions. Moutos *et al.*[20] have designed a biomimetic 3D woven composite scaffold for functional tissue engineering of cartilage. Chen *et al.*[21] developed a new practical ligament scaffold based on the synergistic incorporation of a plain knitted silk structure and a collagen matrix. Liu *et al.*[22] fabricated a combined scaffold with web-like microporous silk sponges formed in the openings of a knitted silk mesh. Subsequently, Fan *et al.*[23]rolled combined silk scaffold around a braided silk cord with Mesenchymal Stem Cells (MSCs) to regenerate anterior cruciate ligament in a pig model. These few cases reveal the versatility of biotextiles and the rapid advancements in this field.

The aim of this work is to evaluate the potential of recently developed biotextile structures as scaffolds for tissue engineering[24, 25]. Polybuthylene succinate (PBS) is originally proposed as a viable extruded multifilament fiber to be processed by a textile-based technology. A comparative study is established using a SF fiber with similar linear density. Knitting technologies are used to fabricate the biodegradable textile matrices. The rational for using these technologies is that the knitted textile substrates are known to exhibit better extensibility or compliance as compared to other woven substrates, with an enhanced porosity/volume although with limited thickness[26]. To overcome this, natural silk yarns were also processed for the first time into different 3D structures using a warp-knitting technology to increase the scaffold's tridimensionality. In the latter case two knitted silk

layers are assembled and spaced by a monofilament of polyethylene terephthalate (PET). Each type of polymer fiber can allow for the generation of constructs with distinct characteristics in terms of the surface physicochemistry, mechanical performance and degradation capability, which has an impact on the resulting cell behaviour at the surface of the respective biotextiles. Preliminary cytotoxicity screening shows that both materials can support cell adhesion and proliferation after processing. Furthermore, different surface modifications were performed (acid/alkaline treatment, UV radiation and plasma) for modulating cell behavior. Human Adipose-derived Stem Cells (hASCs) became an emerging possibility for tissue replacement therapies. The potential of recently the developed silk-based biotextile structures to promote hASCs adhesion, proliferation and differentiation was also evaluated.

1 Experimental

1.1 Materials

Granulated polybutylene succinate (PBS) was obtained from Showa Highpolymer Co. Ltd., Tokyo, Japan. Silk derived from silkworm *Bombyx Mori* in the form of cocoons was obtained and spun into yarns at the sericulture of APPA-CDM (Portuguese Association of Parents and Friends of Mentally Disabled Citizens), Portugal.

PBS fibers with 36 filaments were processed and optimized in a multicomponent extruder in mono-component mode (Hills, Inc., West Melbourne, FL, USA). The Melt Flow Rate (MFR) was determined in order to obtain the adequate processing window. Modular Melt Flow equipment with automatic cutter was used according to the ASTM D1238 standard. The test was performed at 190 ºC with application of a 2.160 kg force. The results allowed to define the optimal parameters for the extrusion process: pre-drying of two hours at 60 ºC; thermal profile: 120-130 ºC; draw ratio: 2; speed: 300 to 600 m/min.

1.2 Fibers characterization

The SF and PBS fibers were characterized in terms of linear density, tenacity and elongation. The linear density (tex) is defined as the mass in grams per 1000 meters and was determined according to EN ISO 2060 standard. The tests were performed using three samples of 10 meters each in conditioned atmosphere 20 ± 2ºC and 65 ± 4% of relative humidity. The tenacity and elongation were measured according to EN ISO 2062 standard. For these tests samples with size of 250 mm were used and 50 replicates were performed. A pre-tension of 0.5 cN/tex and a velocity of 250 mm/min in conditioned atmosphere 20 ± 2ºC and 65 ± 4% of relative humidity were applied.

1.3 Development of the textile constructs

2D constructs were produced through weft knitting using PBS and raw silk fibers (Tricolab machine, Sodemat, SA, Germany). All constructs were washed in a 0.15% (w/v) natural soap aqueous solution for 2 hours and then rinsed with distilled water. Silk structures underwent a subsequent purification process; Bombyxmori silkworm fibers are composed by a core protein called fibroin that is naturally coated by sericin, which is known to be cytotoxic[27, 28]. Thus, SF constructs were boiled for 60 minutes in a 0.03 mol/L Na_2CO_3 solution and rinsed with distilled water to ensure the full extraction of sericin.

3D spacer knitted structures with different porosity, permeability and mechanical behaviour, were manufactured through the technology of warp knitting on a double needlebar high speed Raschel machine (Germany).

1.4 Surface treatments

Etching with NaOH: Constructs were immersed in 0.5 M NaOH solution for 60 min at 30 ºC.UV/O_3 treatment: the UV/O_3 treatment was performed in a commercial UV/O_3 chamber (Jelight Company, Inc., model 42) using a standard fused quartz lamp that emits a continuous radiation of 254 nm with an intensity of 28 mW/cm^2 (O_3 purity: 99.995%, total pressure of 5 mbar). *Plasma grafting*:

the substrates were subjected to a previous treatment in an atmospheric-pressure plasma equipment (by Dielectric Barrier Discharge - DBD), in the following conditions: reactive gas oxygen at 3% in a stream of Argon, speed of 10 m/min, and power of discharge of 9 kW, for 15 min. Immediately after plasma treatment the activated surfaces were immersed a solution with 10% (v/v) of vinyl sulfonic acid (VSA) for 2 h at RT, in order to introduce sulfonic groups. Solutions were previously degassed by nitrogen (N_2) bubbling. After the treatments samples were washed with distilled water for 48 h at 50 ºC and dried for 24 min at 37 ºC.

1.5 Scaffolds characterization

The surface morphology of the produced textile constructs was analysed before and after the different surface treatments using a Leica Cambridge S-360 (UK) Scanning Electron Microscope (SEM)at different magnifications (15 kV).All samples were previously sputter-coated with gold (Fisons Instruments, Sputter Coater SC502, UK). Microcomputed tomography (μ-CT, SkyScan, Belgium) was used as a nondestructive technique for a detailed analysis of the 3D morphology of the developed textile constructs. A μ-CT analyser and a μ-CT Volume Realistic 3D Visualization were used as image processing tools for both μ-CT reconstruction and to create/visualize the 3D representation.

The surface roughness was determined by Atomic Force Microscopy (AFM). The analysis was performed for three regions per sample (5x5 μm) using tapping mode (Veeco, USA) connected to a NanoScope III (Veeco, USA) with non-contacting silicon nanoprobes (about 300 kHz, set point 2-3 V) from Nanosensors (Switzerland). The surface roughness was calculated as Ra (mean absolute distance from mean flat surface).

The wettability was assessed by contact angle (θ) measurements. The static contact angle measurements were obtained by the sessile drop method using a contact angle meter OCA15+ with high performance image processing system (DataPhysics Instruments, Germany). H_2O was added by a motor-driven syringe at room temperature. Two samples of each material were used and five measurements were carried out for each sample.

X-ray Photoelectron Spectroscopy (XPS) analysis was performed to characterize the surface elemental composition of the modified and unmodified samples using a Thermo Scientific K-Alpha ESCA instrument.

1.6 Cell Culture

For the cell culture studies, the materials were cut into 16 mm diameter discs, and immobilized into the bottom of 24-well culture plates (BD Biosciences, USA) using CellCrown® inserts (Scaffdex, Finland). Tissue culture polystyrene (TCPS; Sarstedt, USA) coverslips and SF membranes were used as control.

A mouse fibroblast cell line (L929), acquired from the European Collection of Cell Cultures (ECACC UK), was used to assess the eventual cytotoxicity of the developed constructs, as described elsewere[24]. Cells were seeded at the surface of the materials at a density of 3x104 cells/sample. The seeded constructs were incubated at 37ºC, 5% CO_2 and 95% humidity, for 1, 5 and 24 hours, 3, 7 and 14 days. The textile constructs were analysed in terms of cell adhesion and proliferation through SEM analysis and DNA quantification.

hASCs cells were replated and culture after cryopreservation and then seeded after confluence on the materials at a density of 2000cell/cm^2 for 14, 21 and 28 days instandard osteogenic conditions[29]. The textile constructs were analysed in terms of cell adhesion, proliferation and differentiation potential through SEM analysis and preliminary biological assays: alkaline phosphatase (ALP), DNA and Ca^{2+} quantification.

2 Results and Discussion

2.1 Linear density, tenacity and elongation of the fibers

The applied textile processing methodology is highly demanding in terms of mechanical properties of the used fibers and filaments. Therefore, the linear density, tenacity and elongation of the produced fibers were optimized and are presented in Table 1.

Table 1 Linear density, tenacityand elongation of the PBS fibers and raw silk fibers

	PBS fibers	Raw silk fibers
Linear density/tex	77.3	77.4
Tenacity/(cN/tex)	15.2	45
Elongation/%	120	30

The linear density of PBS fibers was designed to match the value obtained for the silk fibers (77.4 tex) so that both systems could be comparable after knitting. Although PBS fibers presented a lower specific strength to rupture, both fibers presented mechanical properties in the processing window that allowed for an effective knitting of the textile matrices. The obtained elongation values for PBS fibers were considerably higher demonstrating its great capacity for deformation.

2.2 Development, modification and in vitro cytotoxicity of the knitted scaffolds

Figure 1 presents details about the morphology of the obtained PBS and SF weft knitted constructs.

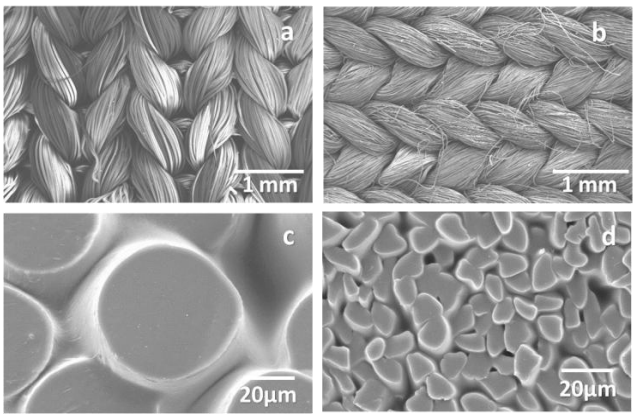


Fig. 1 SEM micrographs showing the morphology of the PBS (a) and SF (b) 2D weft knitted constructs: fibers top view (a, b) and respective cross-sections (c, d)

Both matrices present a very regular and well-controlled pattern with similar morphology. PBS fibers (Fig. 1(a)) present filaments with a circular cross-section having an average diameter of (48.9±0.3) µm. In case of silk (Fig. 1(b)) the filaments show an irregular cross-section, typical for this naturalfiber, with a lower average cross-section, (9.1 ± 2.2) µm. The number of filaments, however, is much higher for SF than for PBS. Therefore, although both fibers have the same linear density they are structurally different, as their filaments differ in number and size. In the SF matrices some filaments of the fibers are loose in the textile structure due to some physical wear during the degumming process to eliminate sericin. The measured thickness of the PBS knit was about 0.7 mm while the silk matrix was ~0.8 mm.

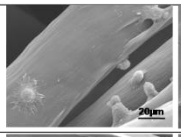
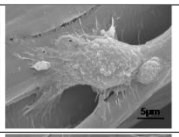
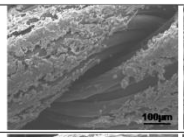
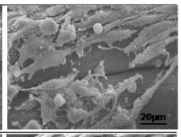
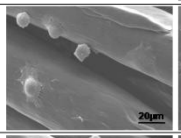
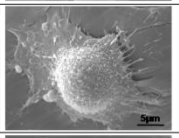
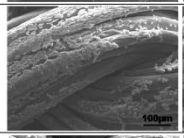
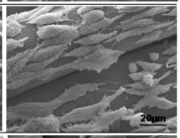
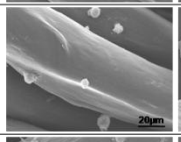
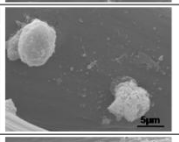
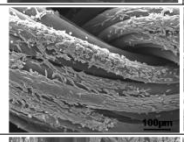
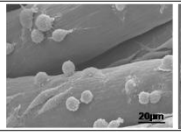
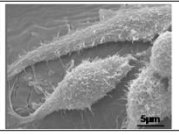
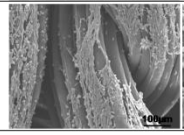
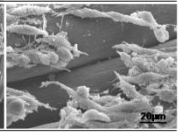
In general, both constructs (PBS and SF) present a relatively high porosity and an interconnectivity of 100%, meaning that all of the pores are interconnected. Although the same fiber linear densities and processing parameters were used for building both knitted structures, SF matrices present significantly lower porosity (68.4±3.7 for SF and 78.4±2.3 for PBS) and pore size than of those of PBS (54.5±9.4 for SF and 72.4±13.0 for PBS). These differences can be justified with the

differences on the respective filaments' thickness. The mean pore size of both SF and PBS matrices is suitable for applications in TE[30]. When considering skin regeneration and wound healing, for example, a study by O'Brien *et al.*[31]has shown that the critical range of pore size of collagen-based scaffolds was between 20 and 120 µm for allowing optimal cellular activity and simultaneously blocking of the wound contraction. In case of bone tissue engineering the minimal pore size has been considered approximately 75-100 µm[30]. This is due to cell size and migration, and nutrient/oxygen transport requirements. This pore size has been also associated with ability for vascularisation. By the present knitting technology it is possible to adjust with precision the space interloop of the textile matrices so that the final porosity (and pore size) can be tailored according with the specific need.

The mechanical properties of the obtained textile constructs were also investigated by performing quasi-static tensile tests and Dynamic Mechanical Analysis (DMA) analysis, as presented elsewhere[32]. In the dry state, when comparing both structures, the average tensile modulus of PBS matrices (7.9 MPa) is significantly lower than the one determined for SF (31.6 MPa). As expected, SF matrices presented a considerable higher strength and stiffness when compared to PBS. SF fibers are known for their extraordinary mechanical properties that rival most of the high performance synthetic fibers. This behaviour results from their unique molecular structure and protein conformation[31].In the hydrated state the mechanical properties of SF constructs have shown to be more affected than PBS. This result is related with the effect of water molecules incorporated in amorphous regions of SF fibers, which will contribute for the softening of the structure, leading to an increase in ductility[49].

As a starting surface, PBS and SF are known to be very biocompatible substrates[24]. Nevertheless we also investigate the possibility of further improving these native properties by performing different surface modifications (ex: acid/alkaline treatment, UV radiation and plasma) for increasing cell adhesion and proliferation and also for further surface immobilization of biomolecules of interest. In Table 2 the surface carbon and oxygen composition, contact angle and roughness of the PBS knitted constructs are presented after different surface treatments and its relationship with cell morphology after 24 h and 14 d of culturing.

Table 2 Surface carbon and oxygen composition, contact angle, roughness and cell morphology after 24 hours and 14 days of culturing for PBS knitted constructs after different surface treatments

Surface treatment	XPS ratio O/C (a.u)	Contact angle (θ)	Roughness (Ra)	Fibroblasts cell line (L929) after 24 hours		Fibroblasts cell line (L929) after 14 days	
Control	0.23	105.7 ± 1.8	21.82 ± 3.23				
NaOH	0.36	61.3 ± 5.8	111.78 ± 2.48				
UV/O3	0.31	93.7 ± 3.4	36.34 ± 7.02				
Plasma/VSA	0.34	116.6 ± 5.5	32.37 ± 10.06				

The differences in cell morphology were relevant in the first hours of culturing, indicating that the surface played a role on the first cell-material interactions. NaOH treatment has induced the highest changes in the surface with a great increase of oxygen, decrease of contact angle and significant increase in the average roughness. After 24 h of cell culture, extensive cell colonization can be observed for all studied fiber surfaces. Cells still presented a round morphology in case of

surfaces untreated and treated with NaOH and UV/O_3, showing a higher degree of spreading with some extended lamellipodia over the surface in the first two cases. For surfaces treated with plasma/VSA a great amount of cells presented already the typical spindle-like fibroblast morphology. This effect can be justified by the presence of sulfonated moieties in the surface that can better mimic the natural extracellular environment and modulate cell adhesion mediated through the adsorbed proteins from the culture medium[33].After 14 days of culture all proposed silk and PBS knitted constructs have shownto support cell adhesion and proliferation throughout the fibers.

2.3 Suitability of 3D warp knitted scaffolds for a bone TE approach

Natural silk yarns were processed into 3D spacer structures using warp knitting technology. The obtained complex 3D architectures are composed of two silk knitted layers assembled and spaced by a polyethylene terephthalate (PET) monofilament to increase the tri-dimensionality and robustness of the scaffold. Figure 2 presents the 3D morphology of the obtained scaffolds and calculated porosity by µ-CT analysis (a) and SEM micrographs showing hASCs morphology and attachment to the fibers (b-d).

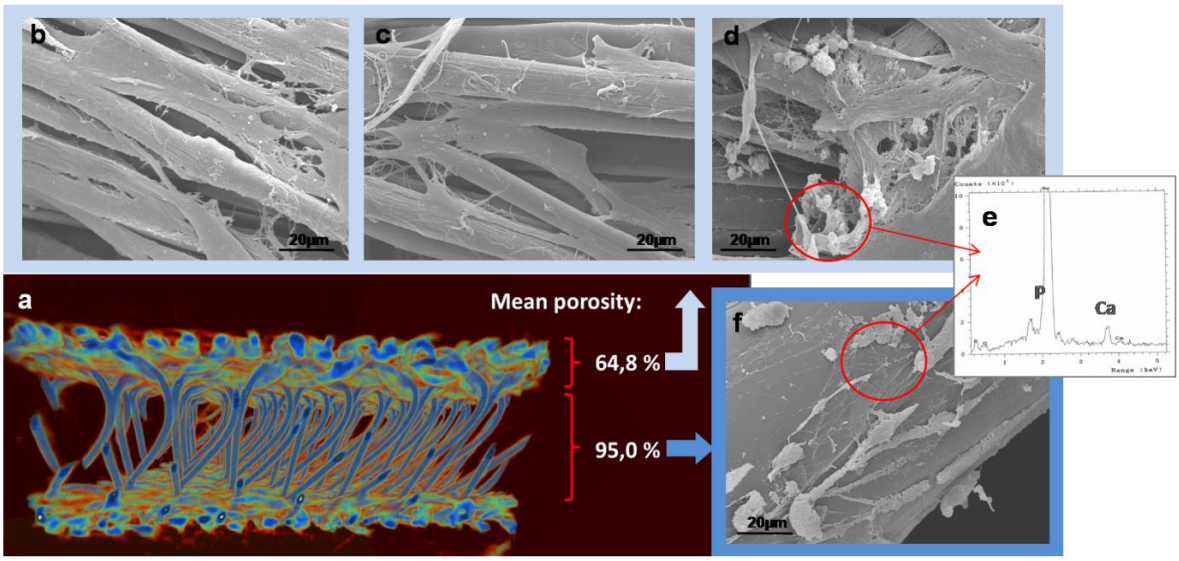


Fig. 2 Novel silk fibroin 3D spacer structures. (a) µ-CT 3D reconstruction. (b-d; f) SEM micrographs showing the hASCs behavior over the 28 days of culture.(e) EDS typical spectrum showing the chemical elements detected in the surface of the fibers after 28 days of culture.

Human Adipose-derived Stem Cells (hASCs) became an emerging possibility for tissue replacement therapies. Due to their osteogenic differentiation potential, easy isolation, expansion and in vitro proliferation, they have demonstrated promising prospects in bone regeneration. SEM analysis (Fig. 2) revealed that at an early time point hASCs adhered to the SF scaffolds presenting their typical fibroblastic morphology, with a higher degree of spreading over the constructs surface (Fig. 2(b)). After 14 days in culture, an extensive cell colonization can be observed (Fig. 2(c)). After 28 days a mineralized matrix was deposited over the textiles surface (Fig. 2(d)), as confirmed by the calcium and phosphorous peaks detected by Energy-dispersive X-ray spectroscopy (EDS) analysis (Fig. 2(e)). When analyzing the cross-section of the scaffold it is possible to observe that cells were able to penetrate deeply into the scaffold and colonize the PET monofilament (Fig. 2(f)) with great evidences of extracellular matrix (ECM) mineralization.

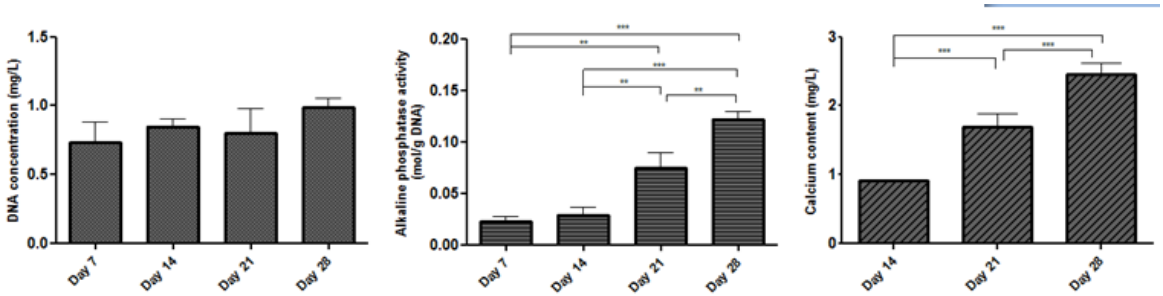


Fig. 3 Biochemical characterization of (a) DNA, (b) ALP and (c) Ca2+ for hASC cells cultured on silk fibroin textile scaffolds for 7, 14, 21 and 28 days. Data are shown as mean ± standard deviation from n=4 samples (**p<0.01, ***p< 0.001)

DNA quantification results showed that hASCs were able to proliferate on the silk fibroin scaffolds, although the DNA amount achieved after 7 days of culture was maintained until day 28 (Fig. 3(a)). ALP activity was also determined for up to 28 d of culture (Fig. 3(b)). This biochemical assay is a marker of early osteogenic differentiation and as expected a gradual and significant increase (p<0.01 and p<0.001) in ALP activity was observed over the culture period. A significant increase in calcium content (p<0.001) obtained from day 14 until day 28 of culture is observed (Fig. 3(c)), indicating that cells were able to produce and deposit mineralized matrix on the SF textile constructs, which is in agreement with previous SEM data(Figs. 2(d), (e) and (f)).These results reflect the suitability of the presented polymeric material and scaffolding strategy towards a bone TE strategy. The efficiency and high level of control of the warp-knitting technology together with the interesting structural properties of the resulting constructs makes this a very versatile system and easily adaptable to the specific bone tissue anatomy and function.

In the literature, knitted structures from synthetic or biological materials have been already proposed, either alone[34]or in a synergistic combination with other types of biomaterials/structures for the construction of functional 3D scaffolds, applicable in the repair/replacement and regeneration of tissues or organs such as the vascular[35-37], tendons and ligaments[21-23, 35, 38-40], cartilage[41-43] and skin[44]. As this is a new field of application for knitting technologies most of these applications are still in exploratory stages. Using the herein presented strategies it is possible to produce fiber-based 3D architectures that can be tuneable in terms of degradation, mechanical behaviour or chemical and surface properties maximizing the biological performance. The control can be taken to the nano-level by investigating new formulations and changing the surface properties through immobilization of bioactive agents.

3 Conclusions

Silk and PBS biodegradable polymers are promising materials for developing new biotextiles to be applied in the engineering of biological tissues. Silk fibers and yarns have been widely validated for the biomedical field. Nevertheless, this natural fiber is far from being fully explored, which makes the present silk-based biotextiles good candidates to rival with existing systems. Also, the validation of new polymeric systems, such as PBS, as a viable fiber and textile matrix constitute new opportunities with high potential in the biomedical area. The proposed silk and PBS textile matrices demonstrated to have mechanical properties and tailorable surfaces that can easily fit in different TE scenarios. Preliminary *in vitro* biological assessment has shown that the materials can support cell adhesion, proliferation and differentiation towards neotissue genesis and ECM formation. The versatility and reproducibility of knitting technologies can open room to the further industrialization of TE products.

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