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ORIGINAL ARTICLE

TERM

The Effect of Polyurethane Scaffold Surface Treatments on the Adhesion of Chondrocytes Subjected to Interstitial Perfusion Culture

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The purpose of this study was to measure chondrocytes detachment from cellularized constructs cultured in a perfusion bioreactor, and to evaluate the effect of different scaffold coatings on cell adhesion under a fixed flow rate. The scaffolds were polyurethane foams, treated to promote cell attachment and seeded with human chondrocytes. In a preliminary static culture experiment, the scaffolds were imbibed with fetal bovine serum (FBS) and then cultured for 4 weeks. To quantify cell detachment, the number of detached cells from the scaffold treated with FBS was estimated under different interstitial perfusion flow rates and shear stress levels (0.005 mL/min equivalent to 0.05 mPa, 0.023 mL/min equivalent to 0.23 mPa, and 0.045 mL/min equivalent to 0.45 mPa). Finally, groups of scaffolds differently treated (FBS, plasma plus FBS, plasma plus collagen type I) were cultured under a fixed perfusion rate of 0.009 mL/min, equivalent to a shear stress of 0.09 mPa, and the detached cells were counted. Static cultivation showed that cell proliferation increased with time and matrix biosynthesis decreased after the first week of culture. Perfused culture showed that the number of detached cells increased with the perfusion rate on FBS-treated constructs. The plasma-treated/collagen-coated scaffolds showed the highest resistance to cell detachment. To minimize cell detachment, the perfusion rate must be maintained in the order of 0.02 mL/min, giving a shear stress of 0.2 mPa. Our set-up allowed estimating the resistance to cell detachment under interstitial perfusion in a repeatable manner, to test other scaffold coatings and cell types.
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Key Words: Tissue engineering; Perfusion; Bioreactor; Scaffold; Polyurethane; Plasma coating

INTRODUCTION

Cartilage tissue engineering could be a good alternative to the direct *in vivo* implantation of autologous or allotropic cells, which has shown severe limitations in providing the correct shape to the regenerated tissue [1]. In engineered cartilage, three-dimensional (3D) scaffolds provide support to the cells in the early stages of tissue culture and then gradually degrade while the tissue starts to produce a physiological extra-cellular matrix (ECM) [2]. However, in static culture, a non-uniform diffusion of nutrients and waste products in the core of the construct may result [3]. Thus the application of dynamic culture, in which the culture medium is perfused through the construct by means of perfused culture systems, offers a solution [4], by enhancing the transport of nutrients to cells, of catabolites away from cells, and a good control over culture conditions [5]. Up to now several perfusion bioreactors for the culture of cartilage have been developed [6,7], showing that an optimised diffusion of nutrients and a good control over the seeding conditions result in better cell proliferation throughout the scaffold [8]. On the other side, interstitial perfusion causes fluid-induced shear stimulation on cells, which can cause cell damage or detachment from the scaffold [9-11].

Cell adhesion to the scaffold is influenced by several factors including material surface properties, architectural structure and fluid-dynamics of the perfused medium [12]. Most tissue-engineering scaffolds are made of polymers. Polyurethane (PU) is a polymeric scaffold that has been widely used in tissue engineering applications. In particular, several studies report the use of PU as scaffold for the regeneration of cartilage, also in

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clinical applications. A PU scaffold has been recently used in a clinical trial, proving to be safe and potentially effective for the treatment of partial meniscal loss with encouraging results at short-term follow-up [13]. Moreover, PU scaffolds, compared to conventional poly(d,l-lactide) (PLA) scaffolds, showed higher chondrocytes proliferation and sulphated glycosaminoglycans content [14]. Intermediate values of wettability are required to promote protein adsorption and cell adhesion and to increase hydrophobic scaffold wettability different strategies have been proposed, amongst which surface activation with plasma, showing an improvement of the material biological performance [15]. Mechanical properties, such as shear modulus, stiffness and compliance also play an important role in promoting cell attachment. In addition, the architectural features of the 3D structure influences cell attachment. Small size of pores, below 85 μm, restricts cell migration generating a non-uniform distribution of the cells throughout the scaffold, while larger pores, exceeding 325 μm, reduce the available area for cell adhesion [16]. In perfused culture, cell attachment is influenced not only by scaffold properties, but also by shear stresses generated by the flow of medium in contact with the cells [9]. High values of shear stress, exceeding 170−190 mPa, can cause anoikis, i.e., not programmed apoptosis induced by cell detachment. The exposure time and shear intensity are directly connected to cell detachment.

To increase cell adhesion on tissue-engineering scaffolds, different surface treatments have been studied. The aim is to create a surface layer able to mimic the physiological composition of ECM and to promote the adsorption of adhesive proteins, such as integrins, which play a key role in cell attachment to a substrate [17]. Recently, different natural compounds and proteins have been proposed to this end, for example platelet rich plasma [18] which has been used also in clinical practice, showing improvements in the quality of regenerated cartilage over control treatments. Also, scaffold coating with serum proteins [19,20] and collagen [21,22] resulted in increased cell adhesion and enhanced proliferation as compared to bare scaffolds, both for chondrocytes [21] and for mesenchymal stem cells [20]. As for the surface treatments for PU, fetal bovine serum (FBS) coating is a simple procedure based on components available in every laboratory. Serum components absorbed on scaffolds allowed to increase the adhesion of cells to polymeric microspheres [19] and enhanced the adhesion and proliferation of mesenchymal stem cells [20]. Moreover, treatment of PU scaffolds with plasma combined with coating showed an improvement of the material biological performance [15]. Regarding collagen I coating, it has been shown that chondrocytes seeded on collagen I produced more matrix [21] and cartilage had better histological and mechanical properties as compared to controls [22].

The aim of this study is thus to analyse the effect of different surface treatments to prevent cell detachment caused by fluid shear stress in 3D cartilaginous cellular constructs cultured under interstitial perfusion. Three different treatments were tested: 1) FBS adsorption, 2) plasma treatment followed by FBS adsorption, and 3) plasma treatment followed by coating with collagen type I. An experimental set-up was developed to subject cells to controlled flows of culture medium and the set-up was used to collect biological data from the cellularized constructs.

MATERIALS AND METHODS

Scaffold preparation and characterization

The PU foam used as scaffold was synthesized with a previously described one-step bulk polymerization method [23,24], using water as expanding agent and iron-acetylacetonate (FeAA) as the catalyst. Briefly, to a polyether-polyol mixture (OH=4.0998 mmol×g^{-1}), FeAA (0.001% w/w_{polyol}) and water (2% w/w_{polyol}) were added and mixed with a mechanical stirrer. The necessary amount of methylene diphenyl diisocyanate prepolymer (NCO=5.24 mmol×g^{-1}) was then added to obtain the stoichiometric ratio of OH/NCO=100/73. The reaction mixture was stirred for 60 seconds and then poured into a custom-made poly(methylmethacrylate) mold (V=500 cm^3). The mold was firmly closed and the expanding reaction was allowed to take place at room temperature (RT). The foam was extracted from the mold after 72 hours and the superficial compact skin was removed to obtain a homogeneous porous structure. Finally, the foam was post-cured at RT for 7 days. The synthesized PU foam was purified by a 48 hours immersion in absolute ethanol at RT, and subsequently carefully dried in air at RT before following characterization.

Density analyses were performed on cylindrical specimens (n=5; Ø=15 mm, h=10 mm). Foam density was evaluated according to EN ISO 845 standard practice, by weighing and measuring the specimens after conditioning for 24 hours at 25°C.

Porosity, average pores size and pores size distribution were evaluated by micro CT analysis using a 1172 microCT imaging system (Skyscan, Aartselaar, Belgium) at 5.6 μm voxel resolution, 204 μA X-ray tube current, and 33 kV voltage without any filters. The specimens were rotated through 180° around the long axis of the sample, with a rotation of 0.4°. The projection radiographs of the sample were reconstructed to serial coronal-oriented tomograms using a 3D cone beam reconstruction algorithm, setting the beam hardening to 20% and the ring artifact reduction to 12. Tridimensional reconstruction of the internal pore morphology was carried out using axial bitmap images and analyzed by CTan software (Skyscan, Aartselaar,

Belgium). The grey scale threshold was set between 60 and 255, removing all objects smaller than 400 voxels and not connected to the 3D model. In order to eliminate potential edge effects, the cylindrical volume of interest (VOI) was selected in the center of a scaffold (Ø=2.5 mm, h=2 mm). Scaffold porosity was then calculated as:

Porosity=100%-vol% of binarised object (scaffold materials) in VOI (1)

All images underwent a 3D analysis, following by a shrink-wrap function, which allowed measuring the fraction of the pore volume accessible from the outside through openings of a certain minimum size. The shrink-wrap process was performed between two 3D measurements to shrink the outside boundary of the VOI in a sample through any opening whose size is equal to or larger than a threshold value (the range 0−175 μm was used in this study). Interconnectivity was calculated as follows:

Interconnectivity=100 (V-$V_{shrink\text{-}wrap}$) (V-V_m)-1 (2)

where V is the total volume of the VOI, $V_{shrink\text{-}wrap}$ is the VOI volume after shrink-wrap processing, and V_m is the volume of the material.

The interconnectivity pore size is hereby called cut-off pore diameter.

Scaffold surface treatments

The three types of surface treatments performed on the PU scaffolds in this work were: 1) FBS adsorption, 2) surface treatment with oxygen plasma plus coating with FBS, 3) surface treatment with oxygen plasma plus coating with collagen type I. For FBS adsorption, PU scaffolds were immersed in pure FBS (DE14-801F, Lonza). The scaffold was left vacuum-sealed for 1 h in order to extract air and facilitate the liquid infiltration inside the porosity, while increasing the adsorption of cell adhesive factors such as fibronectin and vitronectin. The treatment with oxygen plasma aimed at increasing the hydrophilicity of the PU scaffold surface in order to facilitate the adsorption of adhesive proteins during the following coating stage. The surface activation was performed in a PDC002 system (Harrick Plasma, Ithaca, NY, USA). After the surface activation, the scaffold was treated either with FBS as previously described, or with collagen type I. For this treatment, a solution of collagen type I (50 μg/mL) was prepared starting from a solution 4.46 mg/mL of collagen Type I (BD Biosciences, Franklin Lakes, NT, USA) diluted with acetic acid 0.02 N. The collagen solution was then poured on the scaffold, which was then incubated for 1 hour under vacuum. The scaffold was then washed with phosphate buffered saline (PBS) in order to remove traces of the acetic acid and finally dried under sterile conditions for 36 hours. The scaffolds were washed repeatedly with deionized water, dried and UV-sterilized.

Cell preparation and scaffold seeding

The reagents used were from Sigma-Aldrich unless differently specified. Isolation of the cells was obtained, following patient's informed consent, by enzymatic-mechanical digestion of waste cartilage fragments obtained from the femoral head removed from patients undergoing arthroplasty as previously described [25]. Briefly, under sterile conditions, the samples were washed with PBS added with antibiotics (10 mg streptomycin, 10000 U penicillin), and 20 mM L-Glutammine in 0.9% NaCl (PSG). After several washings, the cartilage samples were fragmented with a scalpel. Cells were isolated from the surrounding matrix by enzymatic digestion in a solution of collagenase type II at 0.15% w/v in culture medium. The suspension was placed in a mechanical oscillator to enhance the enzymatic process and incubated at 37°C, 5% CO_2 for 22 hours. The employed culture medium formulation was Dulbecco's Modified Eagle's Medium (DMEM, DE14-801F, Lonza, Basel, Switzerland), 1% HEPES 1M, 1% Sodium-Pyruvate, 1% PSG. 10% FBS was added to obtain the complete culture medium and to inactivate the collagenase to stop the digestion process. The suspension was filtered in order to eliminate the undigested fragments, centrifuged, re-suspended in the culture medium, and counted with trypan blue dye exclusion in a cytometer. Once isolated, cells were seeded at a density of 10 kcells/cm^2 in culture flasks. The culture medium used for expansion was the DMEM complete medium added with the transforming beta 1 growth factor (TGF-ß1) at 1 ng/mL, and the fibroblastic growth factor at 5 ng/mL. Cells were incubated until 70% confluence, trypsinized with trypsin/EDTA, passaged again and cryo conserved at -80°C in 90% FBS, 10% dimethyl sulfoxide until use.

For cell seeding, cells were thawed and pipetted on the PU scaffolds, of diameter 6 mm and thickness 3 mm, manually at a density 6.4×10^6 cells/mL in a suspension of 60 μL. Cells were allowed to adhere for 4 h and then complete DMEM was added to the wells. FBS-treated samples were incubated for 4 weeks for the static culture experiments, with medium completely replaced every three days. For the perfusion experiments, PU-treated samples were incubated for 48 hours before being used. All samples were used for the quantitative experiments of cell detachment, while only FBS-treated PU samples were used for qualitative experiments.

Experiments of cell detachment

Two different set-ups were used to evaluate cell detachment. One was used to quantify cell detachment from FBS-treated samples in function of the culture medium flow rate. This set-up was conceived to be experimentally accessible to light microscopy, thus was placed on the microscope to analyze one sample at the time. The other set-up was used to quantify cell detachment

under three different perfusion flow rates and shear stresses.

The first set-up consisted of the following components (Fig. 1): a syringe pump to control the medium flow perfused through the cellularized construct, an oxygenator tube placed over a heating plate at 37°C, which allows the heated medium to reach the perfusion chamber. This chamber was made of polydimethylsiloxane (PDMS) and was assembled vertically inside a conical tube, in order to channel the cell-enriched medium perfused interstitially through the scaffold towards another optically-accessible chamber. The second chamber (arrow in Fig. 1A) allowed visualization and image acquisition of the cells detached from the scaffold. The visualization chamber was realized in PDMS plasma-bonded to a microscope glass slide. The visualization chamber was laid on an inverted microscope for image and video acquisition in phase contrast. This set-up was used to quantify cell detachment on the FBS-treated samples as follows. The set-up was disinfected in 70% ethanol, also eliminating air bubbles in the circuit and then rinsed in bi-distilled water. Cell detachment was evaluated by counting the cells suspended in the outlet flow, on phase contrast images extracted from videos of the visualization chamber (Fig. 1A), under three different flow rates and shear stresses adopted in independent experiments: 0.005 mL/min (shear 0.05 mPa), 0.023 mL/min (shear 0.23 mPa), and 0.045 mL/min (shear 0.45 mPa). The videos obtained showed the medium and detached cells flow over the glass slide. Nine frames for each flow rate were analysed in order to evaluate cell detachment at the various flow rates. Areas of 100×100 μm^2 square were created on the frame and cells were counted on each area. The counts were divided by the counting area and by the average cell crossing time through the counting area.

The second set-up was used to quantify cell detachment in all the differentially-treated samples (Fig. 2). It was composed of 9 parallel perfusion systems, each one containing a culture chamber, a tube, an oxygenator and a collection system. A syringe pump allowed to perfuse the 9 systems simultaneously. Each culture chamber contained a scaffold holder in PDMS, which linked the cellular construct to the chamber, and a cylindrical element made of Derlin, which sealed the external circular area of the construct in order to force the culture medium to flow through the pores of the scaffold. The perfused surface of the construct was 6 mm in diameter. The setting up operation was carried out under sterile conditions with all the single components sterilized by oxygen peroxide plasma. The experiment was performed on three groups of constructs (FBS, plasma+

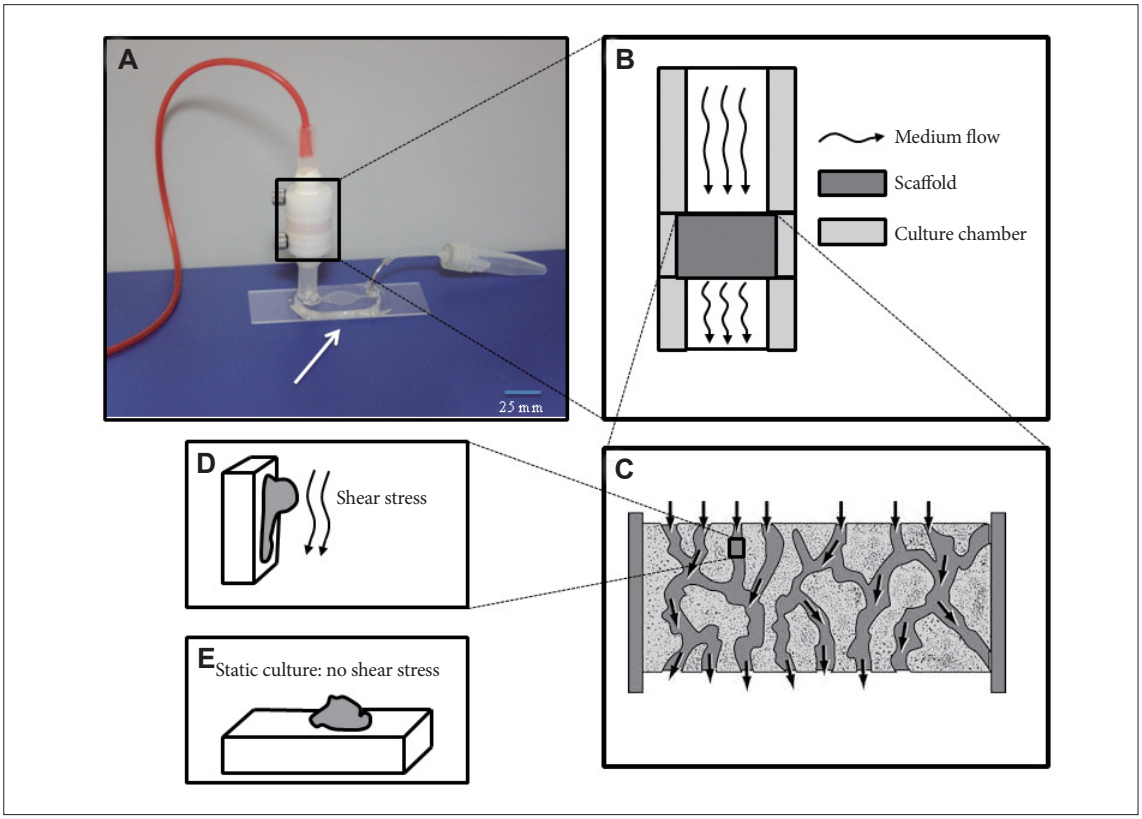

Figure 1. (A) Image of the experimental setup used to visualize and quantify cell detachment from single FBS-treated samples. Scheme of the perfusion chamber, showing culture medium flowing in the chamber (B) and through the scaffold porosity (C). (D) Medium flow generates a shear stress acting on cells attached to the scaffold pores. (E) Absence of extra-cellular stresses in static culture.

FBS, and plasma+collagen type I) composed of three scaffolds each. Other three constructs for each treatment were cultivated in static condition as control. Prior to construct insertion, the circuits were perfused with culture medium to prevent the presence of air bubbles. Then, one construct was arranged in the culture chamber of each perfusion system and secured in the holder. The culture chamber was then closed and the syringe-pump was activated with a flow rate of 0.009 mL/min, equivalent to a shear level of 0.09 mPa, maintained for a perfusion time of 15 hours. This flow rate value was in the order of the 0.005 mL/min value, equivalent to a shear level of 0.05 mPa, that showed the least cell detachment in the preliminary experiments. The constructs were extracted from the chambers and prepared for the biological assays. The tubes in which the perfused medium was collected were centrifuged to collect the detached cells.

Biological assays

Metabolic activity

AlamarBlue (Invitrogen, Waltham, MA, USA) assay was used to observe the cell metabolic activity. Each sample was immersed in a solution of medium and AlamarBlue at 10%, and then placed in an incubator at 37°C for 150 minutes. Then, the culture medium containing the AlmarBlue colorant was extracted and transferred to a black bottom plate in triplicate. Fluorescence analysis was performed via spectrophotometer (λ_{ecc}=530 nm, λ_{ecc}=590 nm). The scaffolds were then washed in order to remove the AlamarBlue residuals and were prepared for the other analyses: each scaffold was weighted, cut in two parts with a surgical blade and then each part of the sample was re-weighted. One half of the scaffold was used for biochemical analyses, the other for histologic assays.

DNA content

A CyQuant kit was used to quantify the DNA content. Each construct was immersed in a lysing solution of papaine (1:100) added to a buffer solution and cysteine and then maintained at the temperature of 60°C for 16 hours. Each obtained sample was diluted in bi-distilled water (H20 RNA-ase, DNA-ase free, GIBCO, Waltham, MA, USA) and a 600 µL solution of CyQuant GR dye (0.25%), cell-lysis buffer (5%) and bi-distilled water (H20 RNA-ase, DNA-ase free, GIBCO, Waltham, MA, USA) was prepared for all the samples. A standard reference curve was employed to convert absorbance level in DNA concentration values and the samples were diluted 10 times to obtain results in the right range of values. 5 µL of the first solution were added to 195 µL of the second solution and transferred to a black bottom plate for the fluorescence analysis (λ_{ecc}=480 nm, λ_{em}=520 nm).

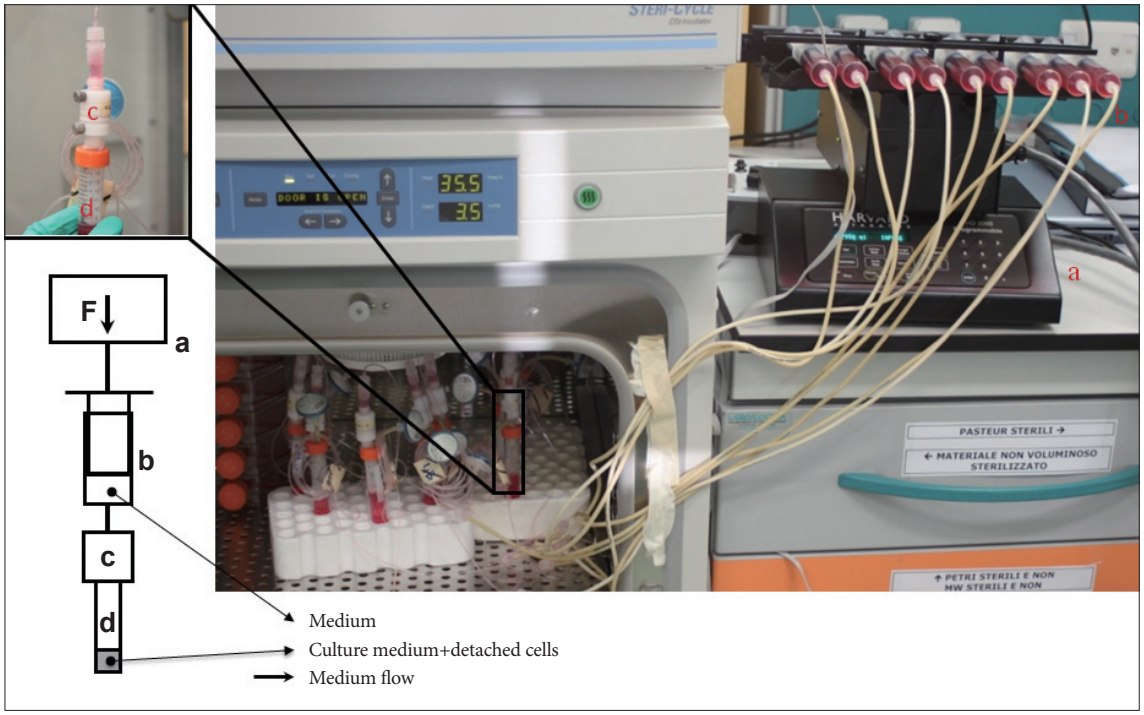

Figure 2. Image and schematic representation of the experimental set-up used to quantify simultaneously cell detachment from 9 samples (n=3 coated with FBS, n=3 treated with plasma and coated with FBS, n=3 treated with plasma and coated with collagen I). F: force generating the flow of culture medium, a: syringe pump, b: syringes, c: perfusion chamber, d: falcon tube. FBS: fetal bovine serum.

Sulphated glycosaminoglycan content

The sulphated glycosaminoglycan (sGAG) was marked through the di-methyl-methylene blue (DMMB) assay and the sGAG concentration was evaluated by a spectrophotometer. A standard reference curve was fulfilled to convert absorbance level in sGAG concentration values. A 200 μL solution of 50 μL of lysing solution and 150 μL of DMMB on a transparent bottom plate was used for the absorbance reading (λ=520 nm).

Histology

The sample halves used in histologic analyses were washed by PBS and then fixed through a 4% formalin solution at the temperature of 4°C. Formalin was then substituted by PBS after 24 hours and the samples were conserved at the temperature of 4°C. The samples were then included in a cryo-hardener gel (Killik, Bio-Optica, Milan, Italy) and were sectioned through a cryostat (Microm HM500, Thermofisher, Waltham, MA, USA) obtaining sections of 30 μm thickness. The sections were then coloured by Haematoxylin to visualize cell nuclei, counter-coloured by Fastgreen and by Safranin O to visualize sGAG. Each section was included between two transparent slides and a resin drop was added. Histologic analysis was realized in bright light through a transmission optical microscope (Olympus IX71, Olympus Italy, Segrate, Milan, Italy).

Table 1. Physico-morphological properties of PU foam

Density (g/cm³)	Open porosity (%)	Average pore size (μm)
0.113±0.035	98.1±0.2	530±62

PU: polyurethane

Statistical analyses

The FBS-treated constructs cultured statically were subdivided into five groups depending on their culture time: 2 days (time zero), 1, 2, 3 and 4 weeks of culture. Mean and standard deviation were calculated for each quantitative assay on these samples. AlmarBlue results were normalized with the total cell

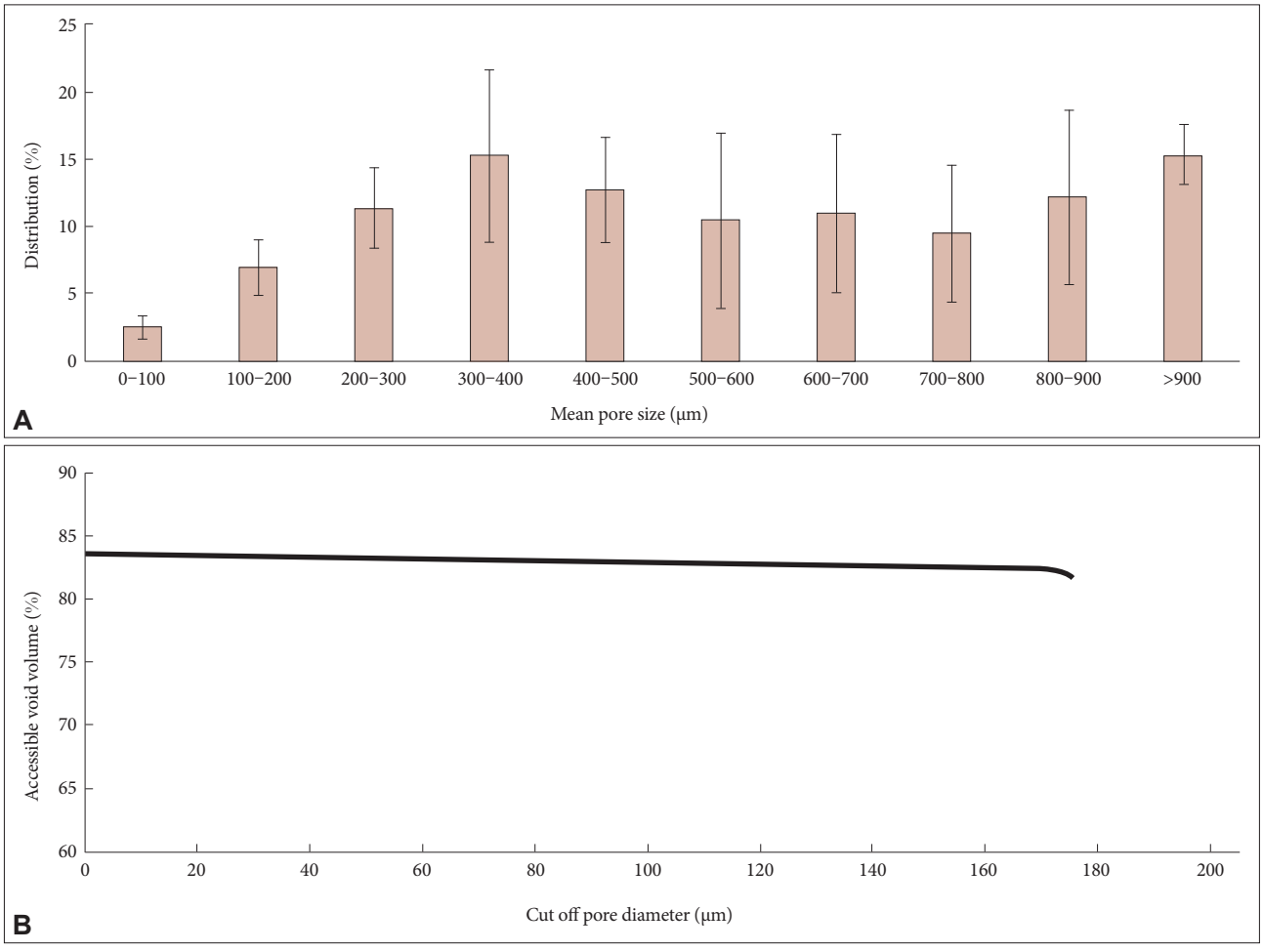

Figure 3. Results of micro CT analysis. (A) distribution of pore sizes, showing an homogeneous pore size in the range 200–900 um. (B) Quantification of accessible void volume as a function of pore diameter, defined as the volume accessible from the outside of the scaffold by pores with diameter less than 175 μm.

number through DNA content in the construct. CyQuant results were normalized with the digested sample weight in order to compare different samples. DMMB results were normalised with DNA content. The cell counts from the experiment of perfusion with FBS-coated samples were subdivided into three groups based on the experimental flow rate used. The samples from the experiment of perfusion with different treatments were subdivided into 6 groups, 3 static and 3 dynamic: 1) FBS, 2) oxygen plasma and FBS, and 3) oxygen plasma and collagen type I. CyQuant results were elaborated in order to obtain average and standard deviation for each group. The results of the dynamic experimental groups were normalized with respect to the results obtained for the static groups with paired coating. The mean and standard deviation were calculated for all experimental groups. For each experiment, statistical differences between groups were assessed through ANOVA. Differences were considered significant with $p<0.05$.

RESULTS

PU foams scaffold morphological characterization

We report in Table 1 the density value and the morphological properties, as obtained by microCT, of the PU foam. The open porosity value (up to 98%) was measured with an average pore size of about 530 micron. The pore size distribution trend (Fig. 3A) presented a wide range with pores from 100 to 900 micron. Quantitative assessment of pore dimension distribution and interconnection, in terms of accessible void volume at different connection size (cut-off pore diameter), was also performed by microCT analysis. Figure 3B shows the trend of accessible void volume as a function of the cut-off pore diameter for the synthesized PU foam. Accessible void volume did not decrease by increasing cut-off pore diameter, thus indicating that pore interconnection was not influenced by the connection size. In addition, the mean pore size and accessible void volume were stable after the treatments.

Static culture

Alamar blue assay results did not show any significant variation of the metabolic activity through weeks, probably due to the scarce diffusion of the nutrients throughout the construct in the static culture condition (Fig. 4A). The CyQuant test displayed a growing trend of cell proliferation, evaluated by the increasing of DNA content, after the first week of culture (Fig. 4B). On the other hand, the sGAG production decreased with culture time as showed by the DMMB assay (Fig. 4C). The comparison between the graphs reported in Figure 4B and C suggests a cell tendency to proliferate rather than to differentiate in

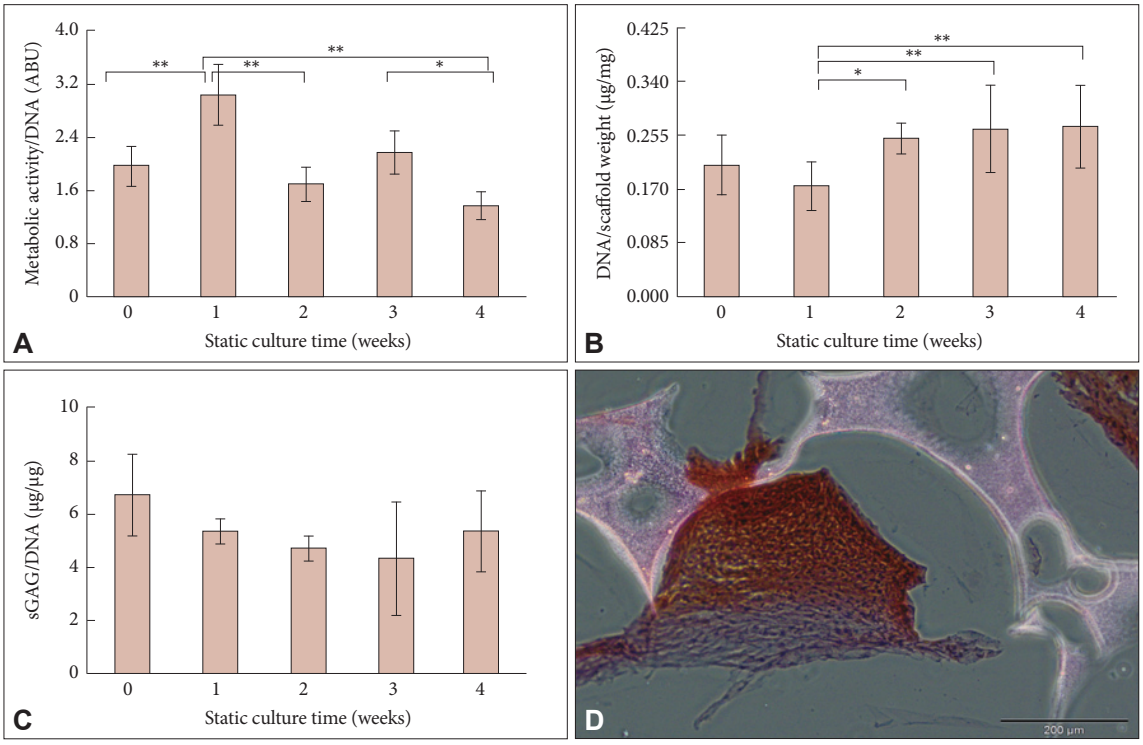

Figure 4. Results of the static culture experiment performed on FBS-coated PU foams. (A) Alamar Blue. Normalized CyQuant (B) and normalized DMMB (C) results are reported. Bars represent average and standard deviation of 3 samples, with *$p<0.005$ and **$p<0.001$. (D) Histological image taken at four culture weeks, in which orange staining evidences sGAG presence (bar dimension 200 microns). FBS: fetal bovine serum, sGAG: sulphated glycosaminoglycan.

culture. Histological assay (Fig. 4D) allowed the visualisation of the distribution of cells and matrix within the scaffold, confirming the presence of secreted GAG.

Cell detachment from FBS-treated samples

The first perfusion experiment demonstrated the existence of a correlation between cell detachment and medium flow rate. In particular, a statistically significant (p<0.001) increase in cell detachment was induced by the increase in the flow rate (Fig. 5).

Cell detachment from differentially treated PU foam

The considered treatments (FBS, plasma+FBS, plasma+collagen I) exhibited different effects on the adhesion of chondrocytes. Both the experimental groups showed a better behaviour when the PU foams were previously treated by oxygen plasma (Fig. 6). In fact, this surface treatment increased the wettability of the surface of the PU scaffold allowing a higher absorption of adhesive proteins. A far better solution was achieved by combining plasma modification with collagen type I coating. PU scaffolds treated with FBS after plasma treatment showed, in fact, an 11% higher number of cells detached in the falcon tube and a 31% lower number of cells on the scaffold compared to the one coated with collagen.

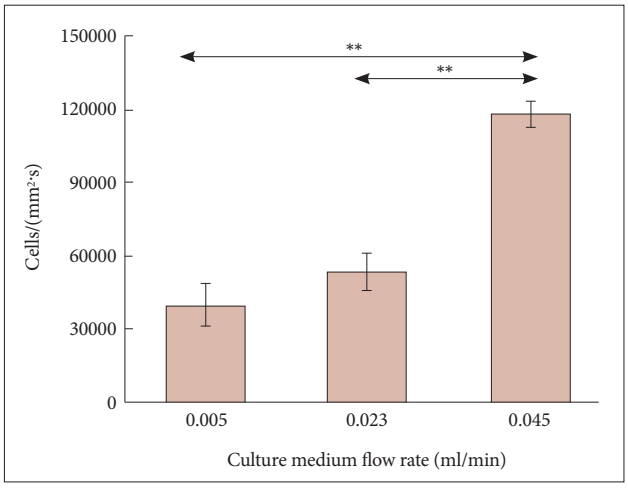

Figure 5. Quantification of cell detachment from FBS-treated PU foam samples as a function of applied flow rate. Data represent the average and standard deviation of n=9 measurements, with **p<0.001. FBS: fetal bovine serum, PU: polyurethane.

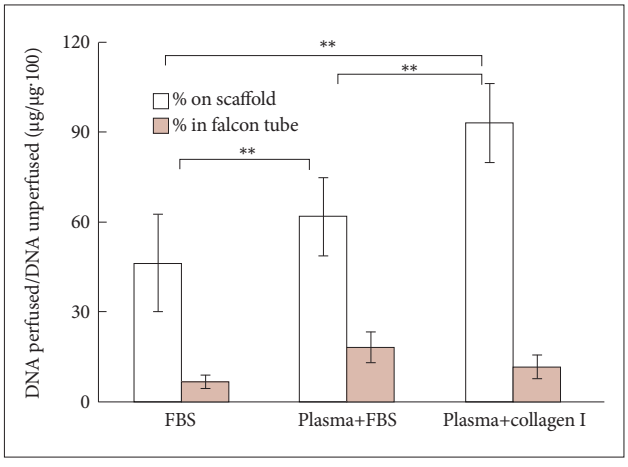

Figure 6. Comparison of detached and adherent cell between the three experimental conditions (FBS coated, plasma treated and FBS-coated, plasma-treated and collage-coated PU scaffolds) after 15 hours of culture at a flow rate of 0.009 mL/min. Data represent the average and standard deviation of n=3 samples for each group, with **p<0.001. FBS: fetal bovine serum, PU: polyurethane.

DISCUSSION

In recent years, the use of PU for tissue engineering applications raised increasing interest, in particular for the production of porous scaffolds (i.e., foams). This is mainly due to their versatility, related to their unique chemical structure that allows obtaining materials with tailored physical and mechanical properties, so to be used in different areas of tissue engineering. Furthermore, by varying the base reagents used for the PU synthesis and the ratio between them, the PU mechanical and physical properties can be tuned to the target values for specific clinical applications. Another important advantage in the use of PU for scaffold production is their easy processability to obtain 3D porous scaffolds with different fabrication techniques [26-28]. In particular, the PU foams used in this research work are obtained using a gas foaming process previously developed by our group [24], and this method allows producing scaffolds with high open porosity value and with interconnected pores. The citocompatibility and biocompatibility of the considered PU scaffolds was already demonstrated by *in vitro* tests with cell lines and primary cells [24,29,30] and *in vivo* by implantation in subcutaneous tissue in the rat animal model [30].

However, polymeric foam scaffolds may not offer a suitable anchorage site for cells. In order to increase the ability of the substrate to adsorb proteins representing stable anchoring sites for cells, the material can be treated with different coatings. The surface treatments considered in this research were all chosen to obtain a biomimetic surface, so to increase cell adhesion and scaffold colonization. In particular, the PU surface was made able to react with FBS and type 1 collagen, used for the surface functionalization, thanks to the oxygen plasma treatment, that exposes on the surface reactive chemical groups (i.e., carboxylic or hydroxyl groups), that can possibly bind to the collagen functional groups [31]. FBS adsorption could represent a good solution due to the presence of adhesive proteins in it. In fact, the results of the static experiment in this work have shown the tendency of chondrocytes to proliferate once

seeded on the FBS-treated scaffold. However, this behaviour could have been induced by the growth factors used during expansion of the cells and maintained in 3D despite a limited interaction between cells and ECM.

The experiment of cell detachment from FBS-treated samples aimed at finding a correlation between medium flow rate and cell detachment. This study demonstrated the existence of a relation between the two parameters. In particular, a 9-fold increase of flow rate value caused a 3-fold increase in the number of detached cells, probably due to the increase in shear stress acting on cells. This result was in accordance with previous data, which showed that a 20-fold increase of flow rate (from 0.05 mL/min to 1 mL/min, equivalent to shear stress levels from 0.5 mPa to 10 mPa) caused a 7-fold increase in the number of cells detached from a scaffold with a 325 μm average pore size [9]. Also, a 2-fold increase of flow rate (from 2.3 mL/min to 4.9 mL/min, equivalent to shear stress levels from 23 to 49 mPa) produced a 3-fold increase in cell detachment [11]. Our experiment also shown trends of decreasing cell detachment with perfusion time (data not shown). This could be related to the increase of the perfused section, caused by cell detachment, which brings to a reduction of shear stress acting on attached cells. This experiment also demonstrated that the optimal flow rate range was 0.005−0.023 mL/min, equivalent to shear stress levels of 0.05−0.23 mPa, in order to maximize the perfusion of nutrients and to minimize cell detachment from the PU scaffold. The means shear stress acting on cell expressed in mPa, could be estimated, having the scaffold a mean pore size of 530 μm and a perfused section of 6 mm, as 10 times the flow rate value expressed in mL/min, as already demonstrated by our group [32]. Previous data showed that, in the range 0.05−0.23 mPa, cell detachment can be estimated as 10−20% of the total number of cells before perfusion. On the other hand, a 0.45 mPa shear stress was estimated to cause cell detachment in the order of 20−30% [9]. The 0.009 mL/min flow rate value chosen for the experiment of cell detachment from differentially-treated samples, equivalent to a shear stress level of 0.09 mPa, represents thus the best compromise between increase of convective nutrient delivery and reduction of wall shear stresses, as also suggested by the experiment of cell detachment performed with the FBS-treated samples.

The experiment of cell detachment from differentially-treated samples was aimed at quantifying cell detachment under interstitial perfusion by estimating, after dynamic culture, both the number of cells left on the construct and the number of cells detached from the construct. The three different considered treatments were also compared in order to evaluate which one could provide a better adhesion of cells to the scaffold in order to resist detachment. The experimental results showed that a significantly greater resistance to perfusion characterized the PU-FBS samples treated with plasma. This result may be explained by the ability of this surface modification to increase the wettability of the scaffold, which allowed a better absorption of adhesive proteins [12]. In our study, a 31% greater resistance of the cells to detachment, compared to what measured on the plasma/FBS-treated scaffolds, could be achieved by coating the plasma-treated scaffolds with collagen type I. The collagen-coated scaffolds resulted in the lowest cell loss (8% cell loss compared to static controls) compared to the other treatments. These results are consistent with previous observations [17], demonstrating that a collagen coating promoted a 40% increase in cell number on tissue-engineered bone constructs, compared to the uncoated scaffolds, although these experiments were on a different cell population and in static culture. To the best of our knowledge, only Chlupáč et al. [33] reported cell detachment under medium flow using coated scaffolds. In their study, resistance to flow perfusion was studied on endothelial cells adhered to a PET scaffold. They demonstrated that a collagen coating caused a 39% loss of cells after 2 hours of dynamic culture, compared to static culture. This loss is 5-fold higher than what measured in our study at 15 hours of culture, likely due to the higher shears (in the order of 150 mPa) acting on the endothelial cells in the mentioned previous study, compared to the shear stress levels in our experiment (0.1 mPa).

The cell type used in our study, articular chondrocytes, has a low tendency to adhere on a substrate. Other adherent cell types, such as mesenchymal stem cells or human embryonic stem cells, are known to better resist flow perfusion [1,2]. Despite all the limitations of our study, we were able to define the optimal combination of scaffold coating (plasma+collagen type I) and flow rate range (lower than 0.02 mL/min, corresponding to a shear stress level of 0.2 mPa) allowing minimizing the loss of this type of cells under interstitial perfusion in the cellularized constructs.

Conclusion

At the state of the art, the success in the engineering *in vitro* of high-density 3D tissue constructs resides primarily in the ability to provide an adequate transport of solutes to cells by culture medium perfusion, without causing damage or detachment to cells subjected to the fluid-dynamic shear stresses caused by the medium flow. Our main finding resides in suggesting the combination of a biomaterial property, i.e., the scaffold coating, with a parameter of bioreactor culture, i.e., the medium flow rate, as a new design specification for engineering cartilaginous tissue in bioreactors. The methodology developed in this study, consisting in a multi-chamber perfusion bio-

reactor and relevant method to compare cellularized constructs based on differentially-coated scaffolds, could serve as the reference method to select the optimal combination of scaffold coating-flow rate to successfully engineer cartilaginous tissues, and other tissues as well.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

In this study, we used cells from anonymized waste cartilage fragments harvested from the femoral head removed from patients undergoing hip arthroplasty, following Institutional approved protocols and SOPs (PQ7.5.125) and with the patient's informed consent (M-SPER-001-v2).

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