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Selective Cell Recruitment and Spatially Controlled Cell Attachment on Instructive Chitosan Surfaces Functionalized with Antibodies

C. A. Custódio · A. M. Frias · A. del Campo ·
R. L. Reis · J. F. Mano

Abstract Bioactive constructs to guide cellular mobilization and function have been proposed as an approach for a new generation of biomaterials in functional tissue engineering. Adult mesenchymal stem cells have been widely used as a source for cell based therapeutic strategies, namely tissue engineering. This is a heterogeneous cell population containing many subpopulations with distinct regenerative capacity. Thus, one of the issues for the effective clinical use of stem cells in tissue engineering is the isolation of a highly purified, expandable specific subpopulation of stem cells. Antibody functionalized biomaterials could be promising candidates to isolate and recruit specific cell types. Here we propose a new concept of instructive biomaterials that are able to recruit and purify specific cell types from a mixed cell population. This biomimetic concept uses a target-specific chitosan substrate to capture specific adipose derived stem cells. Specific antibodies were covalently immobilized onto chitosan membranes using bis[sulfosuccinimidyl] suberate (BS3). Quartz crystal microbalance (QCM) was used to monitor antibody immobilization/adsorption onto the chitosan films. Specific antibodies covalently immobilized, kept their bioactivity and captured specific cell types from a mixed cell population. Microcontact printing allowed to covalently immobilize antibodies in patterns and simultaneously a spatial control in cell attachment.

1 Introduction

Surface functionalization with extracellular matrix (ECM) molecules or synthetic cell adhesive peptides has been used to control cell performance on different surfaces [1, 2]. Published works report the coating or tailoring of biomaterials with bioactive molecules, such as fibronectin [3], laminin [4], growth factors [5, 6] or their integrin-binding epitopes including RGD [7, 8] and GFOGER [9]. These studies report the influence of such biomolecules in cell adhesion, differentiation or proliferation. However, concerning specific and selective cell recruitment few studies are found in the literature.

The difficulty of growing specific cell types as a purified population and in large quantities is one of the major limitations in stem cell based therapies. Cell sorting according to their size has been proposed as an effective and rapid method to separate cells [10, 11]. Dielectrophoresis separates cells according to their electric properties and/or size [12]. Fluorescence-activated cell sorting (FACS) is a method for cell separation based in the specific light scattering and fluorescent characteristics of each cell that requires prior labelling of cells with fluorescent markers and is often time-consuming. The use of microfluidic devices in adhesion-based separation of cells is also a promising method that as been reported in the literature [13, 14].

Currently the most efficient technique to separate cells it is to recognize its specific surface antigens. The most common method for cell sorting and enrichment is based

C. A. Custódio · A. M. Frias · R. L. Reis · J. F. Mano (✉)
3B's Research Group-Biomaterials, Biodegradables and Biomimetics, University of Minho, AvePark, Zona Industrial da Gandra, S. Cláudio do Barco Guimarães,
Caldas das Taipas, 4806-909 Guimarães, Portugal
e-mail: jmano@dep.uminho.pt

C. A. Custódio · A. M. Frias · R. L. Reis · J. F. Mano
ICVS/3B's, PT Government Associated Laboratory,
Braga/Guimarães, Portugal

C. A. Custódio · A. del Campo
Max-Planck-Institut für Polymerforschung,
Ackermannweg 10, 55128 Mainz, Germany

on magnetic cell separation. Magnetic beads utilize a capturing surface or matrix coated with an antibody that has high affinity for a specific cell-surface marker binding target cells [15, 16]. To date, few studies reported the use of a biomaterial surfaces functionalized with antibodies for cell capture. Among them, most reported surfaces with antibodies to recruit endothelial cells improving hemo-compatibility and endothelialization [17–19]. Kutryk and co-workers were the first presenting a strategy of a stent coated with an anti-human CD34 antibody for capturing endothelial progenitor cells (EPC) from the blood stream [20]. Levy and co-workers recently presented a study were the immobilization of anti-human CD47 antibodies is effective in the reduction of acute and chronic inflammatory response to polymeric biomaterials [21].

Adipose tissue is a promising source of adult stem cells that can be harvested by a minimally invasive procedure and low morbidity. Though only a small amount of the heterogeneous cell population that is harvested from adipose tissue are stem cells [22]. Moreover within stem cells there are many subpopulations with different capabilities to differentiate through different lineages and thus with distinct therapeutic potential [23]. For further clinical research and commercial advances in this area, appropriate methods for stem cell isolation and purification are needed.

Chitosan is a linear copolymer of glucosamine and N-acetyl glucosamine obtained by N-deacetylation of chitin. It has been widely investigated for biomedical applications due to its remarkable properties, namely biocompatibility, biodegradability and nontoxicity [24, 25]. Herein we develop chitosan surfaces able to capture a specific cell types based in the concept of antibody coating for cell sorting. Our goal was to create new biomaterial surfaces capable of recruit a specific cell population within a mixture, reducing cell manipulation and time consuming. Chitosan membranes were functionalized with different antibodies and their ability for cell capture was accessed. To effectively immobilize the antibody we used BS3 homobifunctional (di-succinimide ester) cross-linking reagent that targets amine functional groups and forms stable amide linkages. By microcontact printing (μCP) of antibodies we were able to spatially control cell attachment. For the proof-of-concept we performed in vitro biological tests using human adipose stem cells (ASCs) and Osteoblast-like cells (SaOs-2).

2 Materials and Methods

2.1 Chitosan Film Preparation

1 % (w/v) chitosan (Sigma-Aldrich) solution in 2 % (v/v) acetic acid was dropped over 1.3 cm^2 glass coverslips

(Agar Scientific). The solvent was evaporated over 48 h. Films were then treated with 0.1 M NaOH and extensively washed with distilled water until neutral pH. The samples were sterilized with ethylene oxide before use.

2.2 Surface Functionalization of Chitosan Membranes and Characterization

Anti-human CD3, anti-human CD29 and anti-human CD105 (eBioscience), were diluted in 0.35 mM BS3 (Sigma-Aldrich) at a 4 μg/ml concentration. Chitosan films were incubated with this solution overnight at room temperature. The modified films were then washed in PBS to remove non-attached antibody.

2.2.1 XPS Analysis

Analysis of the samples was performed using a Thermo Scientific K-Alpha ESCA instrument equipped with aluminium Ka1, 2 monochromatized radiation at 1486.6 eV X-ray source. Due the non conductor nature of samples was necessary to use an electron flood gun to minimize surface charging. Neutralization of the surface charge was performed by using both a low energy flood gun (electrons in the range 0 to 14 eV) and a low energy Argon ions gun. The XPS measurements were carried out using monochromatic Al–K radiation (hν = 1486.6 eV). Photoelectrons were collected from a take off angle of 90° relative to the sample surface. The measurement was done in a Constant Analyser Energy mode (CAE) with a 100 eV pass energy for survey spectra and 20 eV pass energy for high resolution spectra. Charge referencing was done by setting the lower binding energy C1s photo peak at 285.0 eV C1s hydrocarbon peak. Surface elemental composition was determined using the standard Scofield photoemission cross sections.

2.2.2 Antibody Coupling Followed by QCM

Quartz crystal microbalance (QCM) measurements were carried out on an E1 Microbalance (Q-Sense) at a temperature of 20 ± 0.1 °C. Silica crystals were spun coated with a chitosan solution 0.5 % (w/v) in acetic acid 2 % (v/v) at 4000 rpm during 2 min. Substrates were dried over 48 h and neutralized by immersion in a 0.1 M NaOH solution. The thickness of chitosan films in the dried state was measured using the P-16 + surface profiler (KLA Tencor, USA). For the QCM experiments a flow rate of 50 μL/min was used. The films were first washed with PBS buffer until equilibration, followed by a solution of anti-human CD29 antibody (10 μg/ml) in PBS in order to evaluate non-specific adsorption on the chitosan film. After washing with PBS, a solution of the linker BS3 (0.35 mM)

was injected followed by a solution of the antibody (4 µg/ml) in BS3 (0.35 mM). The changes in the dissipation (ΔD) and frequency (ΔF) presented in this work correspond to values obtained from the fifth overtone ($n = 5$).

2.3 Cell Culture

The ability of functionalized membranes to capture specific cell types was tested using cell cultures of ASCs isolated from liposuction samples (Hospital da Prelada, Porto) and osteoblast like cells (SaOs-2) (European Collection of Cell Cultures (ECCC)).

ASCs were enzymatically isolated from subcutaneous adipose tissue as previously described [26]. Briefly lipoaspirate samples were digested with 0.2 % collagenase type II (Sigma Aldrich) in PBS for 90 min at 37 °C in gentle stirring. The digested tissue was filtered using a 100 µm filter mesh (Sigma Aldrich) and centrifuged at 1,250 rpm for 10 min to remove the floating adipocytes. The cell pellet was resuspended in lysis buffer for 10 min to disrupt the erythrocytes. After a centrifugation at 800 rpm for 10 min, cells were resuspended in culture medium and placed in culture flasks.

Both cell types were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma), supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Biochrom AG) and 1 % antibiotic antimicotic (Gibco). Cells were incubated at 37 °C in 5 % CO_2. ASCs were used until passage fourth. DMEM without FBS was used for cell capture experiments. After culturing the cells for different time points in the modified surfaces, samples were washed with PBS and imaged using fluorescence microscopy (Zeiss Z1M). DAPI (Sigma) and anti-human CD105-FITC (AbD Serotec) fluorescent markers were used to identify nuclei and CD105 cell surface antigens, respectively. Briefly membranes were incubated with DAPI (1:1000 dilution, in $1 \times$ PBS) for 5 min and washed with PBS. Samples were then incubated with anti-human CD105-FITC diluted in PBS (5 µl/ml) and washed with PBS.

2.4 Bioactivity and Capture Specificity of the Antibody-Chitosan Surface

SaOs-2 cell line is very well documented and characterized thus was used to test the suitability of the developed method. Bioactivity of the immobilized antibody and capture specificity of the antibody-chitosan surface were assessed by monitoring and comparing cell attachment in different antibody modified membranes. SaOs-2 (5×10^4 cells/cm^2) were incubated on anti-human CD3 and anti-human CD29 antibody modified chitosan membranes. Polystyrene coverslips and non-modified chitosan membranes were used as controls.

2.5 Microcontact Printing (µCP) of Antibodies

2.5.1 PDMS Stamp Preparation

The master for stamp preparation was fabricated by photolithography using SU-8 photolack [27]. The PDMS stamp was prepared by casting a 10:1 mixture of Sylgard 184 (Dow Corning) pre-polymer and cross-linker onto the master and curing at 90 °C for 3 h in a vacuum oven. After cooling, the PDMS was peeled off from the lithographic template and cut to suitable sizes. Stamps were sonicated in ethanol and distilled water for 10 min and dried before use.

2.5.2 Ink Preparation and Fabrication of Antibody Patterns by µCP

The antibody was dissolved in a solution of BS3 (0.35 mM) in PBS at a 4 µg/ml concentration. This ink solution was dropped onto the cleaned PDMS stamp and incubated for 3 min and the excess of ink was removed by carefully tilting the substrate. The stamp was placed onto the chitosan surface applying constant force for 3 min and then gently peeled off. After 12 h incubation, membranes were washed with PBS and SaOs-2 cells (1×10^5/cm^2) seeded on the patterned surfaces. The process is schematically shown in Fig. 1.

Fig. 1 **a** Microcontact printing of antibodies. (*1*) Inking the stamp with the protein in BS3, (*2*) removing the excess of solution, (*3*) contact between the inked PDMS stamp and chitosan film, (*4*) protein molecules are transferred on the chitosan along patterns that correspond to the relief structures of the PDMS stamp. **b** BSA-FITC patterned on the chitosan film
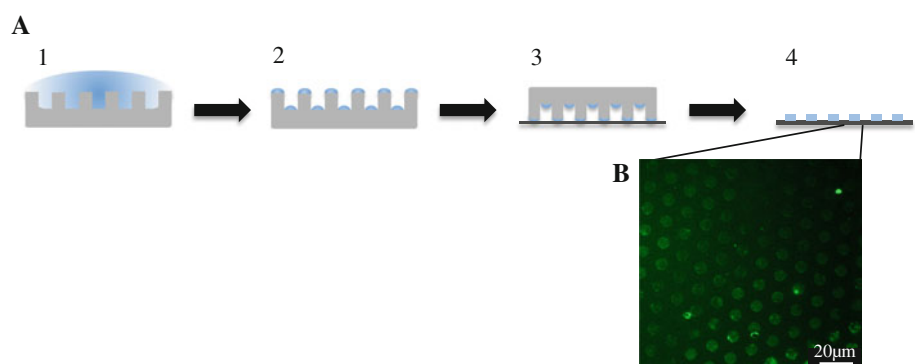
2.6 Cell Capture Selectivity

To determine the effect of the immobilized antibody in cell separation a co-culture of cells was seeded on CD105 modified chitosan films. SaOs-2 and ASCs cells were seeded in the modified substrates at a density of $1 \times 10^5/cm^2$ cells (5×10^4 each type of cell). Polystyrene coverslips were used as a control.

2.6.1 Flow Cytometry

SaOs-2, ASCs and the mixture after cell separation by the modified membranes were analysed by flow cytometry for cell surface marker expression. Markers used were chosen based on the minimal criteria for defining multipotent mesenchymal stromal cells, defined by the International Society for Cellular Therapy [28]. To evaluate cell-surface marker expression, cell suspensions were incubated at room temperature for 15 min with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies specific for human markers associated with mesenchymal lineages. Samples were analysed on a FACSCalibur cytometer and the resulting data were processed using CellQuest software (BD Biosciences).

3 Results and Discussion

3.1 Functionalization of Chitosan Membranes with Antibodies

Covalent linking of antibodies to chitosan membranes was mediated by the BS3 linker. The NHS esters react with to form stable amide bonds (Fig. 2a). The flexible alkyl spacer chain of BS3 may prevent sterically hindered effects, promoting some mobility of the antibody molecules. Therefore such elements are expected to be more available for the antigens in the cell surface when compared to other immobilization methods. The immobilized antibody provides a biding site for subsequent recruitment of cells.

The attachment of the antibody to the chitosan surface was checked by XPS. The shape of the C1s signal changes upon reaction with BS3 and the antibody (Fig. 2). This signal can be deconvoluted in different components [29]. The peak at 285.0 eV was assigned to C–H/C–C and C–NH$_2$ bonds. The peak at 286.7 eV was assigned to C–OH, C–O and C–N bonds. The C=O and O–C–O/N–C=O groups from the acetylated rings appeared at 288.2 eV. The same components were present in the C1s high resolution spectra of the non-modified and modified chitosan membranes, but changes in the relative intensities of all peaks were observed (Table 1). The intensity of C1s peak

correspondent to N–C=O increased significantly upon reaction with BS3, indicating that the NHS ester group was added to the surface. The C–O–H and C–O-C content also increased due to the presence of the BS3. The relative intensity of both peaks decreased after coupling of the antibody, indicating that the NHS group was no longer present and the formation of the amide bond.

The adsorption and covalent binding of the antibodies to the chitosan film was further quantified by QCM. Figure 3 shows the frequency and dissipation changes as a function of time. Injection of antibody (A) did not cause significant change in the QCM signal, indicating negligible non-specific physisorption of the antibody to the chitosan film. After rinsing with PBS (B), the BS3 solution was injected (C). No change in the QCM signal was visible due to the low mass of the BS3 molecule. Injection of the antibody (D) caused a significant decrease in the frequency signal, indicating an increase of mass at the sensor. In parallel, the dissipation increased indicating that the surface is becoming more viscoelastic. Only a small amount of the immobilized antibody could be removed after rinsing with PBS (E), indicating that the antibody is firmly attached to the chitosan layer. These results demonstrate that the chitosan membranes avoid non-specific adsorption of antibodies and that the BS3 linker successfully mediates the coupling of antibodies to the membranes.

3.2 Bioactivity and Capture Specificity of the Antibody-Chitosan Conjugate

To test the bioactivity of the immobilized antibodies, modified membranes were seeded with SaOs-2 cells, a very well characterized cell type. CD29 was used to recruit and attach SaOs-2 and CD3 as a negative control. CD29 antigen is a subunit that makes up an integrin heterodimer and that is present in the surface of many cells including Saos-2. It is expected that anti-human CD29 antibody mediates SaOs-2 attachment to the chitosan films. CD3 was used as a negative control since it is not expressed by SaOs-2 cells and in principle it does not have any influence in cell attachment.

Very poor attachment of SaOs-2 cells was observed onto non-modified chitosan membranes (Fig. 4a) or anti-human CD3 modified membrane (Fig. 4b) even after 24 h incubation (Fig. 4c). Cells readily attached to chitosan membranes modified with the anti-human CD29 (Fig 4d). In fact, after 24 h in culture (Fig. 4e) cells proliferated and spread over the surface, even in the absence of serum, These results demonstrate the validity of our approach to couple antibodies on chitosan surfaces retaining their bioactivity. The fact that no cell attachment was observed on the negative controls suggests that the attachment of SaOs-2 to CD29 membranes was achieved due to the presence of the antibody.
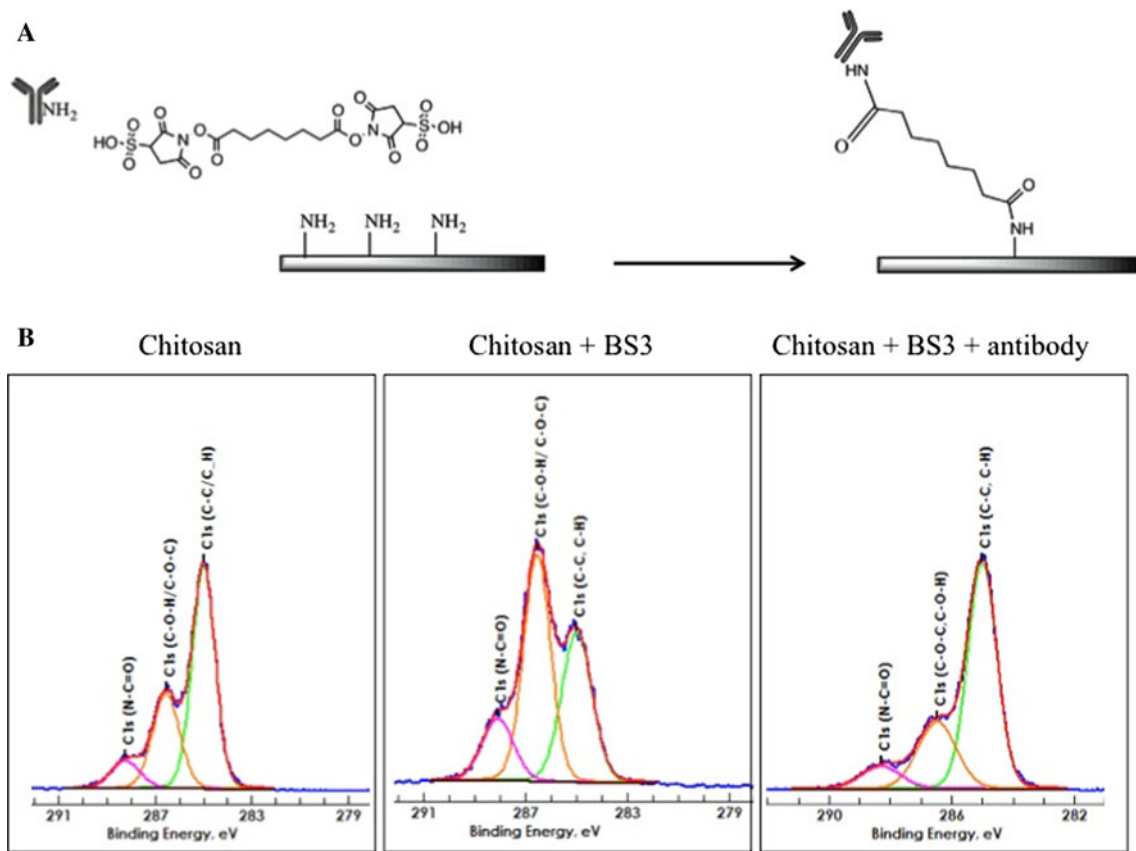
Fig. 2 **a** Schematic representation of immobilization procedure. IgG antibody is mixed in BS3 solution, and react with the amino groups in the chitosan membrane, **b** C1s high-resolution spectrum of chitosan membranes, chitosan membranes incubated with BS3 and chitosan membranes with immobilized antibody

Table 1 Relative peak composition of C1s high resolution spectra

	Chitosan (%)	Chitosan + BS3 (%)	Chitosan + BS3 + antibody (%)
N–C=O	9.7	15.6	9.3
C–O–C/C–O–H	30.4	48.3	26.4

3.3 Microcontact Printing of Antibodies and Cell Patterning

Micropatterning of cells is a critical step for studies in the fundamental biology of cell–cell and cell-surface interactions and provides new tools for spatially controlled tissue engineering. The flexibility of sizes and shapes of the patterns have allowed generation of patterned cell co-cultures and more control in cell proliferation and differentiation. The culturing of patterned cells requires in most cases non-adhesive surfaces for further modification for cell attachment in specific regions [30, 31].

In the current work chitosan represents an attractive cell repellent surface. The results inhere presented shown that cells only attach when they recognize an antibody on the membrane. Antibody immobilization on chitosan membranes could then be used as support to design cell patterns and spatially control cell attachment.

μCP was used for spatial control over antibody attachment and thus spatially control in cell attachment on the membrane. A PDMS stamp with 10 μm diameter cylindrical pillars with 10 μm spacing was used to create "spots" of anti-human CD29.

SaOs-2 cells were seeded onto the substrate patterned with antibodies and incubated at 37 °C.

After 1 h the medium was changed and non-attached cells were removed. A cell pattern was obtained where the SaOs-2 cells were selectively attached to the areas were the antibody had been printed (Fig. 5).

3.4 Cell Capture Selectivity

Following characterization and bioactivity tests, antibody modified membranes were used for cell capture selectivity experiments. In particular, the selection of ASCs from a co-culture of SaOs-2 and ASCs was attempted.
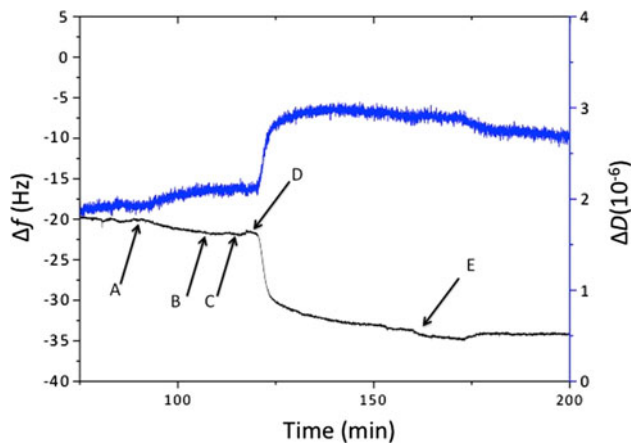
Fig. 3 QCM curves of antibody adsorption and coupling (fifth overtone). The frequency change of the sensor is shown in the black curve and the dissipation in the blue curve. (A) Antibody (10 µg/ml) in PBS, (B) PBS, (C) BS3 in PBS (0.35 mM), (D) Antibody (4 µg/ml) in BS3 (0.35 mM), (E) PBS were separately injected into the reaction cell and the resulting frequency changes were measured

ASCs have been described as mesenchymal stem cells (MSCs). According to the International Society for Cellular Therapy guidelines, the criteria that define MSCs are adherence to plastic, multipotent differentiation potential and specific surface antigen expression (CD73, CD90, CD105) [28]. From the referred it is known that CD105 is not expressed by SaOs-2 and highly expressed by ASCs.

Anti-human CD105 antibody was immobilized onto the chitosan films for capturing ASCs. After 4 h and 24 h incubation, samples were washed, stained and imaged to evaluate attached cells. Cells were labeled with DAPI to stain the nucleus and CD105-FITC to label the cells expressing CD105 antigen.

As expected SaOs-2 and ASCs showed distinguishable results for CD105-FITC immunostaining. SaOs-2 did not label for CD105 (Fig. 6a) whereas ASCs revealed positive for this marker (Fig. 6b). When the mixed cell population is cultured in TCPS, both cells attach (Fig. 6c) due to unselective surface. Modified chitosan films successfully performed positive selection for CD105 cells (Fig. 6d) and a purified cell population from the initial mixture was obtained. When CD105 was previously immobilized in the surface nearly all the attached cells were positive for the marker indicating that cells that do not express this antigen were removed from the mixture. Such result shows that antibody modified chitosan films are suitable to capture a
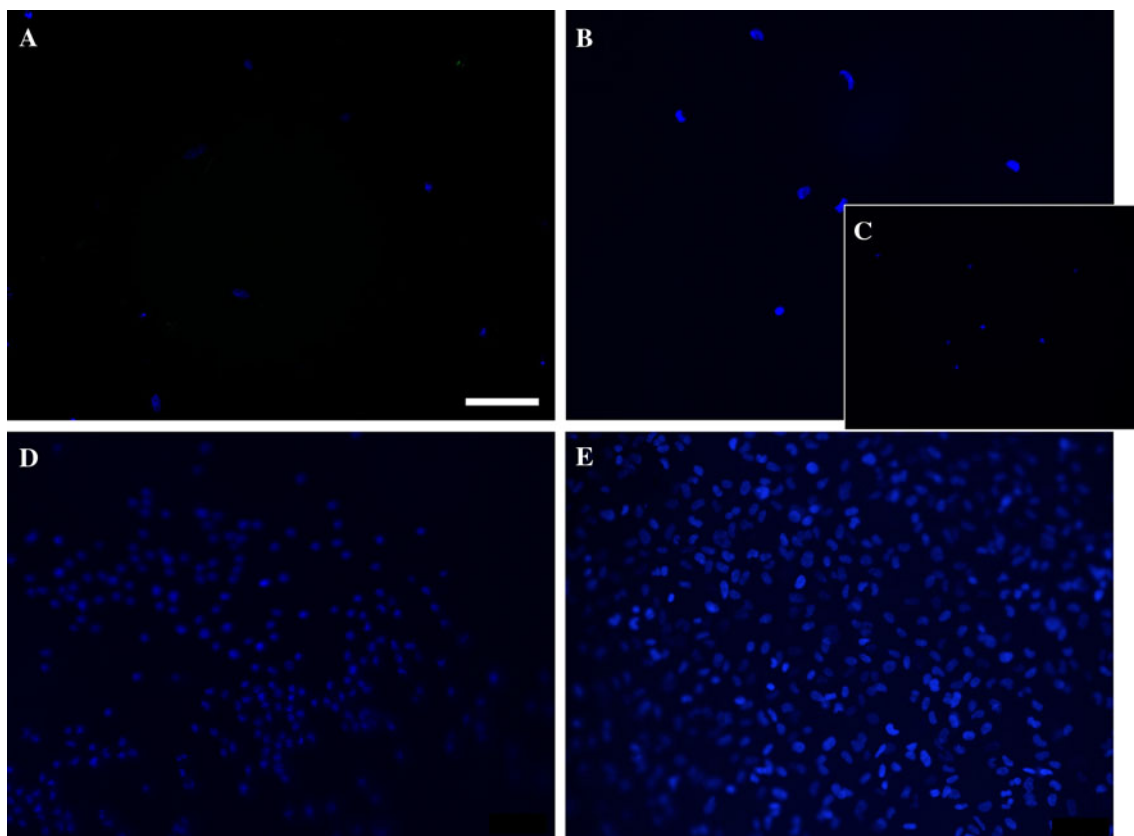
Fig. 4 SaOs-2 nuclei identified by DAPI staining. **a** Non-modified chitosan (4 h incubation), **b** chitosan modified with anti-CD3 (4 h incubation), **c** chitosan modified with anti-CD3 (24 h incubation), **d** chitosan modified with anti-CD29 (4 h incubation), **e** chitosan modified with anti-CD29 (24 h incubation)
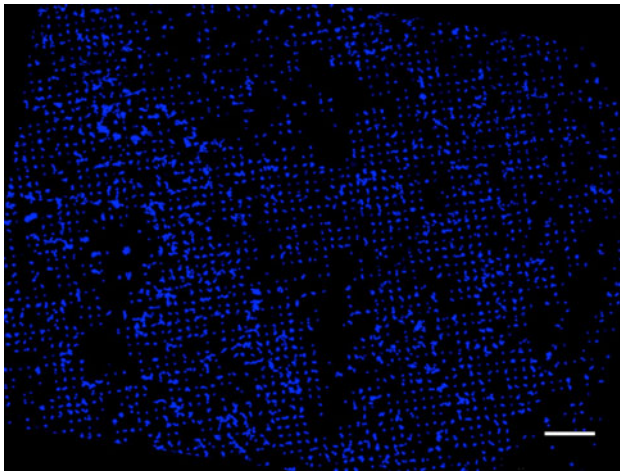
**Fig. 5** SaOs-2 nuclei identified by DAPI staining. *Scale bar* 100 μm

specific cell subpopulation from a heterogeneous cell population. In addiction was proved that cells remain viable and spread over the surface after 24 h incubation (Fig. 6e). Non-modified membranes revealed negligible cell attachment after 4 and 24 h incubation (results not shown) in agreement with the statement that the cell attachment is mediated by the presence of an antibody. Considering the results herein presented it is acceptable to consider that the chitosan films modified with antibodies succeeded as an effective method for cell selection avoiding attachment of non desired cells.

Flow cytometry was additionally performed to reinforce these results. SaOs-2, ASCs and the mixture of cells after cultured on modified membranes and TCPS were analysed by flow cytometry.

Cytometry analysis to the mixture of cells (1:1) revealed that 49.41 % were CD105 positive. Such result was expectable since only ASCs express this marker (Fig. 7a). TCPS is known to support good attachment of adherent cells thus it is not cell selective. As shown in Fig. 7b, cells that were recovered from TCPS after 72 % still express CD105 in approximately half (39.44 %) of the cell population, there is still a mixed cell population.

Corroborating results from immunostaining positive selection of CD105+ cells was successfully performed using the developed membranes coupled with CD105 antibody (Fig. 7c). From the recovered cells about 100 % of the cell population is positive for the marker. This result confirms the suitability of the presented system for
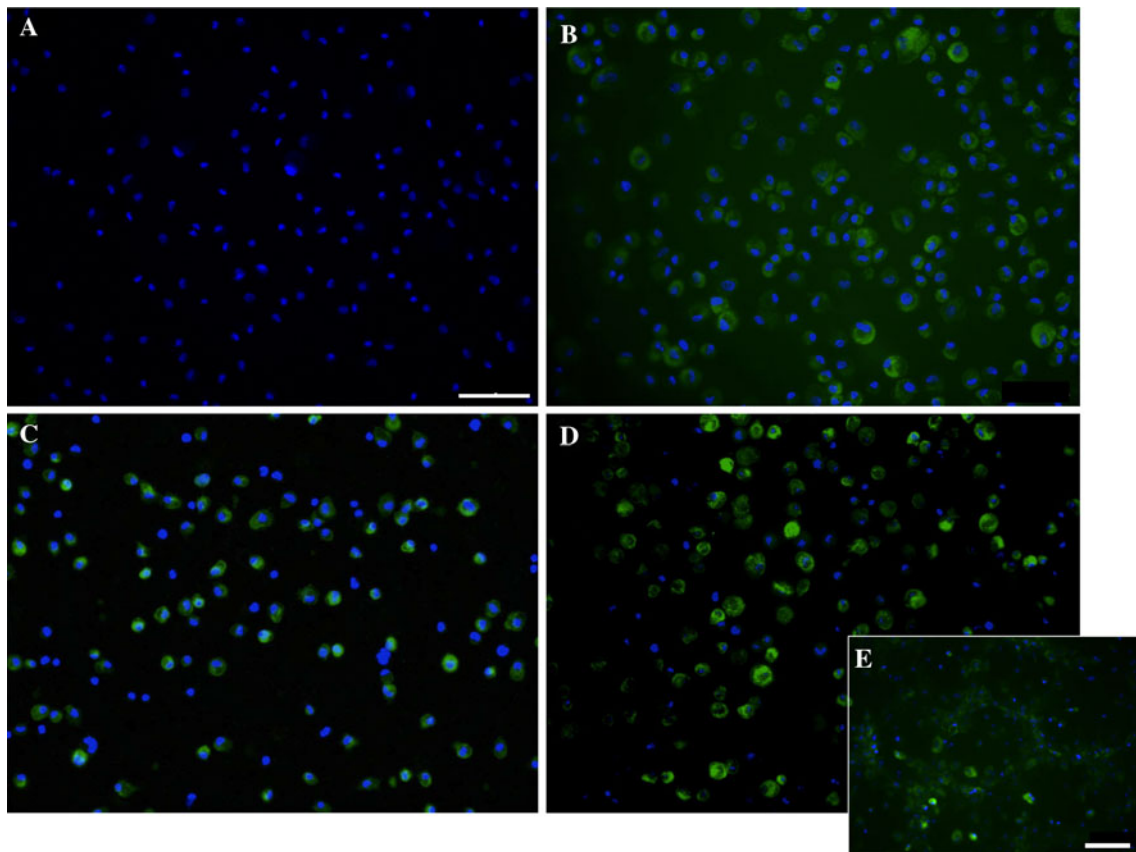
Fig. 6 Cells identified by DAPI in the nuclei and labelled by CD105-FITC. **a** SaOs-2 on TCPS (4 h seeding), **b** ASC on TCPS (4 h seeding), **c** ASC + SaOs-2 seeded on TCPS (4 h seeding), **d** ASC + SaOs-2 seeded on CD105 modified membrane (4 h seeding), **e** ASC + SaOs-2 seeded on CD105 modified membrane (24 h seeding). *Scale bar* 50 μm

Fig. 7 Representative histograms for flow-cytometry analysis of CD105+. **a** Initial cell population (ASCs:SaOs-2, 1:1), **b** cells collected from TCPS after 72 h incubation, **c** cells collected from CD105 modified chitosan membranes, **d** scheme of experiment: Following trypsin digestion, SaOs-2 and ASCs were mixed (1:1) and analyzed by flow cytometry. The mixture was plated in TCPS and chitosan films modified with CD105. After 72 h incubation (37 °C, 5 % CO_2) cells were trypsinized and analyzed by flow cytometry
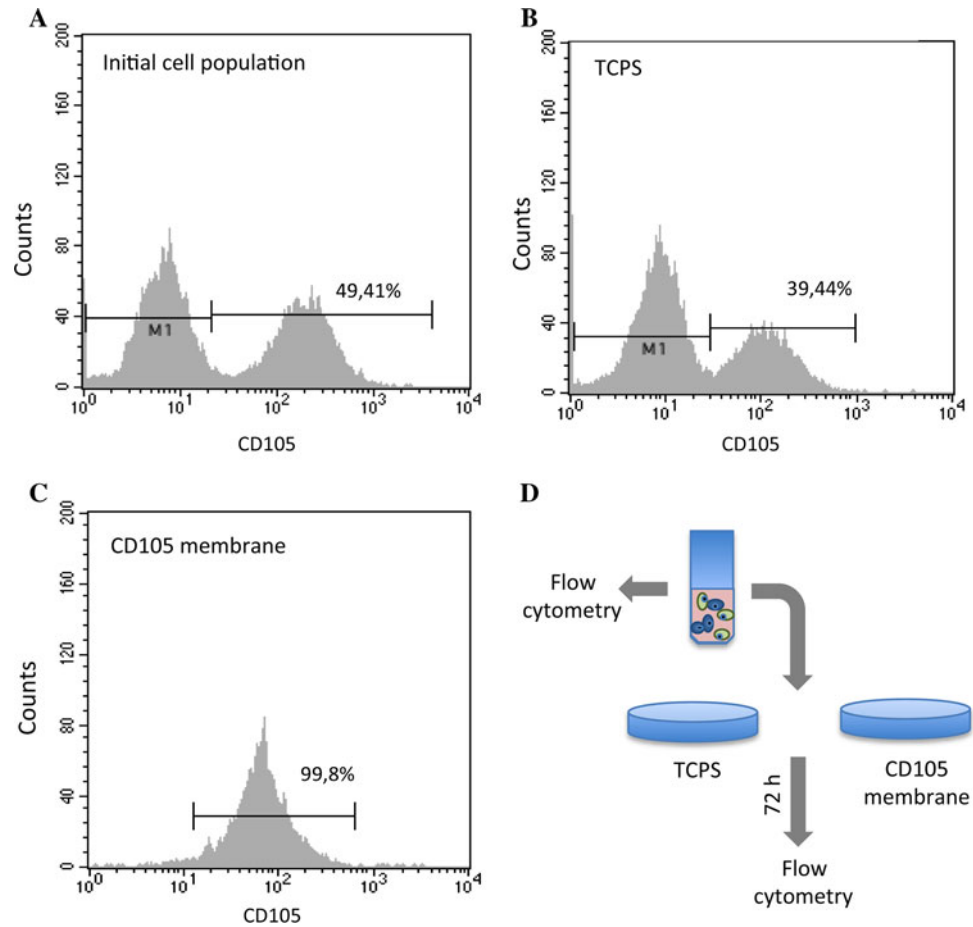
selection of CD105+ cells. In addition, the isolation procedure did not affect cell proliferation (Fig. 6e). Thus, the system is capable of effectively isolating viable cells for subsequent cultivation and further applications.

The most common and effective method currently reported for isolation of a specific cell type involves the use of magnetic beads coated with antibodies for target cells. This technique requires the attachment of magnetic beads to the cells that may be undesirable for cell-based therapeutic applications [15, 16].

Microfluidic devices incorporating antibodies or micropillars in the microchannel are able to overcome the limitations of classical immunomagnetic separation technology [13, 14]. Still they have limitations in the sense that the cells selected in the system need to be later released and collected from the device to be used in many therapeutic strategies. Biomaterials functionalized with specific molecules that promote adhesion and cell separation in situ as the system reported in this work could be promising candidates for biomedical applications that require specific cell populations. Recently Khademhosseini and collaborators reported a similar study, where hyaluronic acid and heparin-based hydrogels functionalized with CD34 antibodies were used to promote adhesion and spreading of EPCs

[18]. The present report adds further evidence on the use of this type of system for cell separation from a mixed cell population.

Compared with currently available devices found in literature the system herein proposed besides effective it is relatively low cost using a biomaterial with easy chemistry that can be later implantable [10–16]. The method proposed in this work allows the isolation of a target cell type from a mixed cell population with relatively high purity, decreasing cell handling where recruited cells can be used straightaway.

4 Conclusions

Chitosan films were successfully grafted with antibodies using the BS3 linker. The immobilized antibodies remained functional and mediated selective cell attachment to the chitosan. Using µCP to create antibody patterns, cell attachment was spatially controlled. An attempt has been made with the present work to overcome the complex process of cell isolation and selection by using implantable biomaterials that are able to recruit and select cells to attach. A major challenge in the techniques currently

available for cell separation is the lack of methods to achieve nondestructive release of cells. The use of antibody coated chitosan surfaces seems a promising approach to rapidly capture and seed a specific cell type directly to biomaterials, from a heterogeneous cell population avoiding additional cell manipulation.

Additional studies will be performed aiming to select specific cell types in designed patterns for cell co-cultures. The developed system could be in principle applied in 3D structures to be used in tissue engineering strategies. Also, the concept could be easily adapted to other substrates, either than chitosan. Further studies could be envisaged to assess the use of different immobilized bioactive cues to recruit circulating stem cells in vivo.

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