Preliminary study on human protein adsorption and leukocyte adhesion to starch-based biomaterials

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In this study, the adsorption of human serum albumin (HSA), fibronectin (FN) and vitronectin (VN) onto the surface of novel biodegradable materials was evaluated by immunostaining. Specifically, polymeric blends of corn starch with cellulose acetate (SCA), ethylene vinyl alcohol copolymer (SEVA-C), and polycaprolactone (SPCL) were immersed in unitary and competitive systems; that is, binary and more complex protein solutions. For binary solutions, different HSA and FN protein distribution patterns were observed depending on the starch-based surface. Furthermore, the relative amount of proteins adsorbed onto starch-based surfaces was clearly affected by protein type: a preferential adsorption of VN and FN as compared to HSA was observed. On tests carried out with unitary, binary and more complex solutions, it was found that vitronectin adsorption ability was enhanced in competitive systems, which was associated with a lower amount of adsorbed albumin. In order to assess the effect of these human proteins on cell behavior, a mixed population of human lymphocytes and monocytes/macrophages was cultured over pre-coated SEVA-C surfaces. Through anti-CD3 and CD-14 monoclonal antibody labeling and cell counting, leukocyte adhesion onto pre-coated SEVA-C surfaces was analyzed. Based on the results, it was possible to detect albumin long-term effects and fibronectin short-term effects on cell adhesion proving that previously adsorbed proteins modulate leukocyte behavior.

1. Introduction

Protein adsorption from surrounding tissue fluids is the first very important phenomenon of the host-biomaterial interactions that may lead to implant integration or rejection [1–3]. Immediately upon contact with physiological solutions, many proteins adsorb to the implant surface, subsequently promoting nearby cells to interact with the material [4–11]. It can be said that when implanted, foreign materials interact indirectly with living tissues meaning that cell-surface interactions are mediated by proteins adsorbed from surrounding fluids [8, 9]. Together with other interfacial processes, protein adsorption will regulate cell function and the biocompatibility of the implant [1, 12], being responsible for homeostasis and tissue healing but also for adverse immunologic mechanisms [1, 13] such as, inflammation [10, 13–18] and thrombosis [10, 13–18].

To investigate further the phenomenon of protein adsorption and the effect of the surfaces on it, human proteins were used to coat different starch-based biodegradable polymeric blends. These novel biodegradable materials were recently shown to be biocompatible [19, 20] and cytocompatible surfaces [21], and have been proposed for a range of biomedical applications [22–25], including tissue-engineering scaffolding [26].

Proteins conventionally classified as adhesive and non-adhesive were used in this study. Fibronectin, a glycoprotein known to contain both RGD, PHSRN, and synergistic sequences [27, 28], influences several processes, such as cell attachment, migration, differentiation, and matrix assembly [27, 29, 30]. Vitronectin, also found in plasma and ECM, comprises an important domain, the RGD peptide that is known for mediating attachment and spreading of cells [29, 31]. On

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the other hand, by binding to plasminogen activation inhibitor-1, this protein can potentially regulate the proteolytic degradation of the extracellular matrix [29, 31, 32]. The third protein is human serum albumin that, although known to be depleted of adhesion ligands, was selected due to its presence in high concentrations in serum and its ability to bind other molecules, influencing competitive protein adsorption [33–35]. The typical concentration of these proteins in the human serum is 35, 0.4, and 0.3 mg/mL, respectively for HSA, FN and VN [33].

In this study, antibody labeling of specific biological molecules was selected to detect HSA, VN, and FN adsorption on the surface of starch-based materials by means of adapting a previously described technique [36]. The methodology is based on the initial binding of a primary antibody to the surface of the sample where the antigen is adsorbed. Following this, the surface is incubated with a second antibody solution resulting in the formation of a primary–secondary complex. If this secondary is conjugated with an enzyme, such as alkaline phosphatase, the addition of the corresponding substrate will produce a color change and, consequently, visualize the presence of the antigen on the surface [37].

Using the immunostaining technique, the relative presence of human albumin, fibronectin and vitronectin on the surface of different starch based polymers was determined. In addition, both distribution patterns and type of proteins that preferentially adsorb from human blood serum were analyzed. Furthermore, the effect of human proteins in modulating the interaction of a mixed population of leukocytes and the materials surfaces was investigated. For this purpose, monoclonal mouse anti-human CD3 and CD14 antibodies were used to label human lymphocytes and monocytes/macrophages, respectively.

2. Materials and methods

2.1. Starch-based materials

The materials used in this study were biodegradable polymeric blends of corn starch with: (i) cellulose acetate (SCA), (ii) ethylene vinyl alcohol copolymer (SEVA-C), and (iii) polycaprolactone (SPCL), supplied by Novamont (Italy). The amount of starch is 50% by weight (wt %) on SCA and SEVA-C and 30% wt on SPCL. Using conventional injection molding technology samples were processed into 10 mm circular discs. Efforts were made in order to obtain the most reproducible surfaces. Samples were sterilized by ethylene oxide as described in other works [38], washed, and all subsequent experimental procedures were performed under sterile conditions.

2.2. Protein adsorption assay

Human serum albumin, human fibronectin and human vitronectin were obtained from Sigma (UK). Human vitronectin was reconstituted to a final concentration of 2 μg/mL in tissue-culture-grade water and sterilized by filtration. Whole blood was collected from healthy un-medicated adult donors, coagulated and centrifuged at 2500 rpm during 5 min for serum separation. Protein solutions of different concentrations were prepared by diluting initial solutions in 0.1 M phosphate-buffered saline (PBS) solution without Ca^{2+} and Mg^{2+} and pH 7.4. HSA solutions used in the immersion experiments had concentrations of 1000, 70, and 35 μg/mL. The FN solutions had concentrations of 1, 0.8, and 0.4 μg/mL and VN solution were diluted to 0.6 and 0.3 μg/mL. Besides single protein solutions, competitive systems were studied: (i) binary solutions prepared to final concentrations of 1000 μg/mL of HSA and 1 μg/mL FN and (ii) also complex solutions with 1%, 10% and 20% (v/v) of human blood serum. All solutions were sterilized by filtration through 0.2 μm of pore size. Proteins were adsorbed to starch-based surfaces by transferring each sample to polypropylene tubes and immersing them in 2 mL of the prepared solution for 24 h at 37 °C. Batches of samples were also immersed in PBS solution without proteins to be used as control surfaces. After 24 h samples were fixed in 4% formaldehyde for 5 min, immersed in PBS and kept at 2–8 °C. Care was taken in order to prevent drying of the protein-coated surfaces before further analysis.

2.3. Detection of surface adsorbed proteins by immunostaining

Samples and control surfaces were removed from PBS solution and coated for 20 min with rabbit serum (1 : 10 dilution, Serotec, UK) in order to block later non-specific binding. Monoclonal mouse anti-human HSA, FN or VN antibodies (Dako, Germany) used as primaries in the immunostaining procedure were diluted to 1 : 1000. Discs were washed and, afterwards, coated with biotinylated rabbit anti-mouse immunoglobulins (IgG, Dako, Germany) diluted to 1 : 200. Both primary and secondary antibody solutions were diluted in PBS with 1% BSA and incubated for 30 min. Detection of proteins adsorbed onto starch-based surfaces was processed using the alkaline-phosphatase protocol. The incubation of alkaline phosphatase (Vector, UK) and alkaline phosphatase substrate (Vector, UK) solutions was performed for 30 min. Between each step of the immunostaining procedure, samples were repeatedly washed with PBS. For each assay, an additional control was prepared consisting of a protein-coated sample submitted to the same described procedure but instead of incubating with the primary, PBS was used. Three replicates for each experimental condition were prepared and the procedure repeated three to five times. Immunostaining results were observed using reflected light microscopy (Axioplan 2 Imaging, Zeiss, Germany) and image acquisition by digital camera equipment (AxioCam, Zeiss, Germany).

2.4. *In vitro* human monocytes/macrophages and lymphocytes isolation and culture

Whole blood was collected from healthy un-medicated, adult volunteers and anticoagulated with 0.002% of heparin. Human blood mononuclear cells were isolated by means of using a one-step gradient centrifugation method. Five milliliters of anticoagulated blood was carefully layered over 3 mL of LymphoSep Media (ICN,

USA) and centrifuged at 2500 rpm for 25 min at 4 °C. The interface was harvested, washed three times in PBS and cells were resuspended in 199-cell culture medium (GibcoBRL, USA) supplemented with 10% foetal bovine serum (FBS).

After isolation, cells were seeded onto the protein pre-coated SEVA-C surfaces at a density of 5×10^4 cells/mL (in 199 medium with 10% FBS). Samples were incubated for 30 min and 24 h at 37 °C in 5% $CO_2$2 and 100% humidity. After incubation, surfaces were fixed for 10 min in 4% formaldehyde, immersed in PBS and kept in the refrigerator at 4 °C.

2.5. Analysis of leukocytes attachment by immunostaining and haematoxylin staining

After leukocyte culture, fixed samples and controls obtained were removed from PBS solution, coated with rabbit serum (1 : 75 dilution, Serotec, UK) for 20 min and incubated overnight with purified monoclonal mouse anti-human CD3 or CD14 antibodies (1 : 1000 dilution, PharMingen, USA). Each disc was then coated with biotinylated rabbit anti-mouse IgG (1 : 200 dilution, Dako, Germany), followed by phosphatase alkaline (Vector, UK) and PA (Vector, UK) incubation, 30 min each. Between every step, samples were rinsed with PBS for 5 min. For each assay, an additional control was prepared, by replacing the incubation of the primary by PBS solution. Samples labeled with antibodies were then counterstained with haematoxylin (Sigma, UK). Surfaces were then rinsed in warm water for 1 min and analyzed using the reflected light microscope and digital camera for image acquisition.

2.6. Cell counting

In order to obtain lymphocyte, monocyte/macrophage, and total cell numbers, cell counting was performed in a two-step procedure: after labeling leukocytes with CD3 and CD14 primary antibodies and after haematoxylin staining. For each sample, counting was performed from 20 different and arbitrary fields of vision using KS400 3.0 image analysis software coupled to a transmitted light microscope (Zeiss, Germany) and digital camera (JVC, USA).

3. Results and discussion

3.1. Immunostaining analysis of protein adsorption

3.1.1. Unitary systems

In this study, immunostaining technique was used to analyze protein adsorption with respect to their proportional amounts in the human serum. 0.1 and 0.2% of those concentrations [33] were used. The immunostaining technique allowed qualitative and semi-quantitative analysis of different starch-based blends studied in terms of protein adsorption pattern and intensity (Figs. 1–3). In the first case, the aim was to detect different protein distribution patterns and to determine whether it was a material or protein dependent phenomenon. For all proteins studied it was observed that SCA and, to some extent, SPCL present a less homogeneous protein coating than SEVA-C. Starch and ethylene vinyl alcohol blends are inter-penetrating networks (INP). SPCL presents some complexion and interaction between the starch and PCL phases, while SCA is a non-miscible blend. Considering the order SCA, SPCL, and SEVA-C, we obtain an increase in miscibility and interaction between these natural and synthetic polymeric phases, explaining why proteins form a smooth and homogeneous protein coating at SEVA-C surfaces when compared with SPCL and SCA. By means of analyzing Figs. 1–3 it can be concluded that protein adsorption pattern is determined by the type of material. Whatever the protein used the same staining distribution for a certain starch-based surface was obtained. In terms of intensity of the staining, and

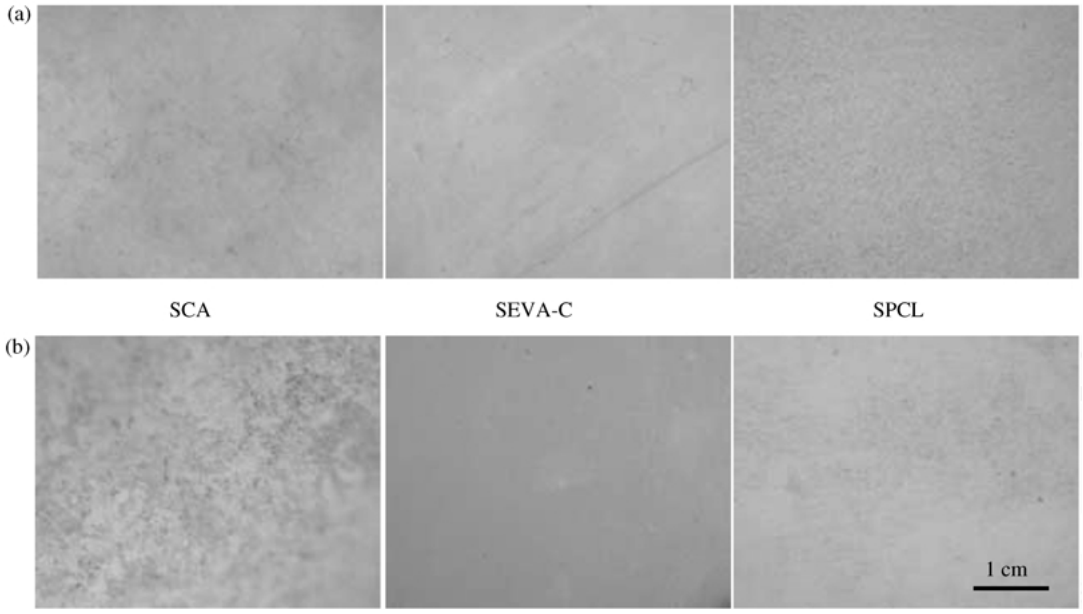

Figure 1 Immunostaining results (5 ×) of different starch-based materials (SCA, SEVA-C, and SPCL) immersed in two distinct human serum albumin solutions: 35.0 μg/mL – 0.1% of the concentration in human serum – (a) and 70.0 μg/mL – 0.2% of the concentration in human serum (b).

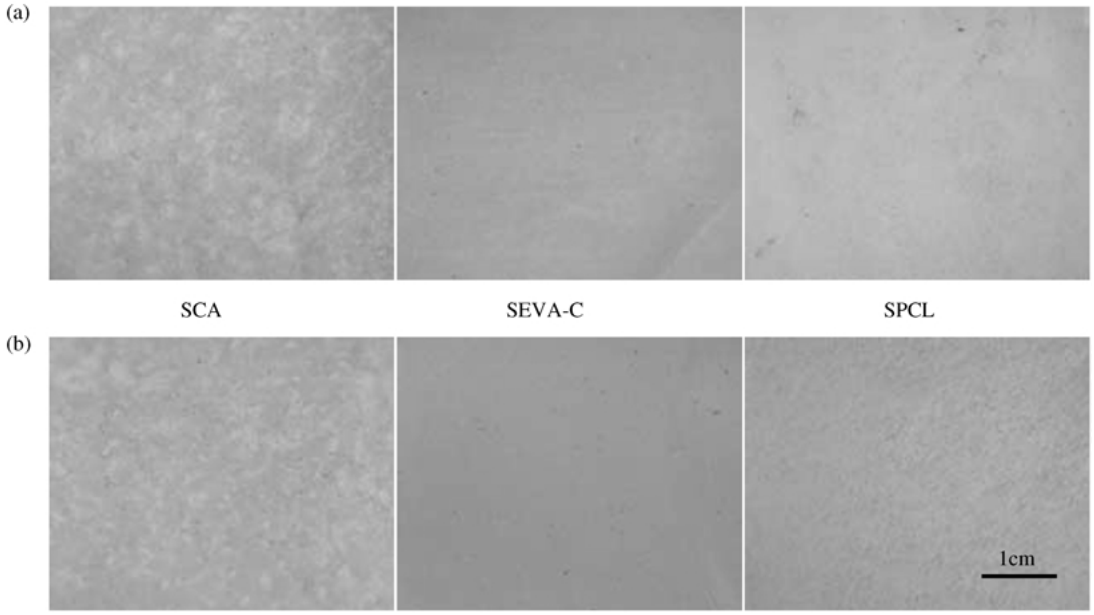

Figure 2 Immunostaining results (5 ×) of different starch-based materials (SCA, SEVA-C, and SPCL) immersed in two distinct human fibronectin solutions: 0.4 μg/mL – 0.1% of the concentration in human serum – (a) and 0.8 μg/mL – 0.2% of the concentration in human serum (b).

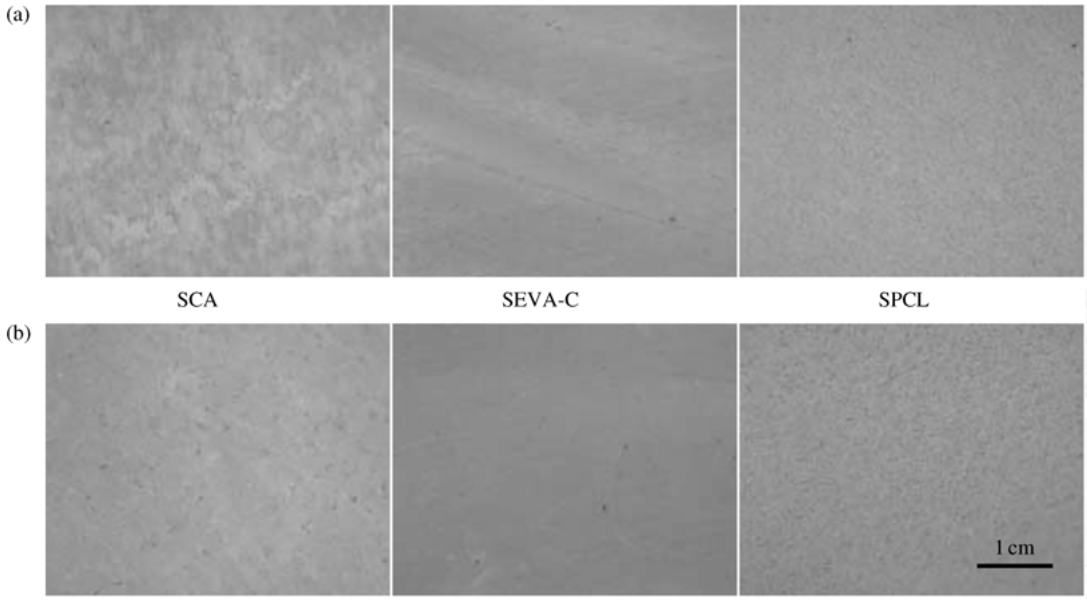

Figure 3 Immunostaining results (5 ×) of different starch-based materials (SCA, SEVA-C, and SPCL) immersed in two distinct human vitronectin solutions: 0.3 μg/mL – 0.1% of the concentration in human serum – (a) and 0.6 μg/mL – 0.2% of the concentration in human serum (b).

comparing Figs. 1(a), 2(a) and 3(a) with Figs. 1(b), 2(b) and 3(b) it is possible to observe that human albumin, fibronectin, and vitronectin at 35, 0.8 and 0.4 μg/mL are nonsaturating for almost all surfaces, once the protein concentration is doubled and a more intense staining is achieved. Furthermore, it can be observed that both fibronectin and vitronectin adsorb in higher amounts to the starch-based surfaces and at least 85 lower concentrations are used. These results indicate higher adsorption affinity of FN and VN to the materials studied.

3.1.2. Binary systems

In order to examine protein adsorption in a competitive environment, binary solutions of 1000 μg/mL of albumin and 1 μg/mL of fibronectin were prepared and samples were immersed in the solutions for 24 h at 37 °C. Fig. 4 presents the different starch-based surfaces previously immersed in the two-protein solutions and immunoassayed for HSA and FN. It was observed that, in opposition to single protein solutions (see Figs. 1 and 2), staining intensities were generally lower although higher albumin and fibronectin concentrations were used. In unitary systems, SEVA-C adsorbed more FN than HSA and in this assay showed a more intense staining for HSA. In addition, although SCA and SPCL present a general decrease in staining intensity, higher adsorptions are still obtained for fibronectin. In opposition to the previously discussed results, the binary solutions indicate low adsorption capabilities of fibronectin in competitive

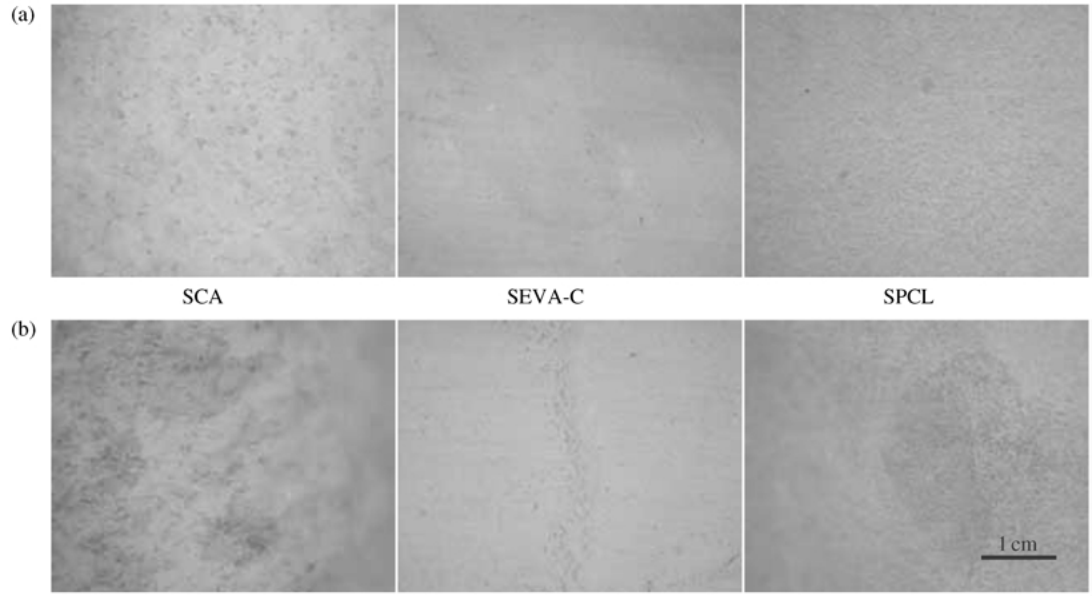

Figure 4 Immunostaining results (5 ×) of different starch based materials (SCA, SEVA-C, and SPCL) immersed for 24 h in a solution with both human serum albumin (1000 μg/mL) and human fibronectin (1 μg/mL) and stained for albumin (a) and fibronectin (b).

environment and, subsequently, a synergistic effect driven by the presence of albumin.

3.1.3. Complex systems

To complement the results obtained for binary systems, more complex protein solutions were also prepared and studied. Collected serum was diluted to 20%, 10% and 1% (v/v) in PBS without Ca^{2+} and Mg^{2+}. After immersion in serum solutions, surfaces were immunoassayed for HSA, FN, and VN (Fig. 5). Although it was not possible to observe any increase in staining intensity from less to more concentrated solutions, both 10% (results not shown) and 20% human serum solutions seemed, in quantitative terms, to stimulate higher protein adsorption than the 1% solution (results not

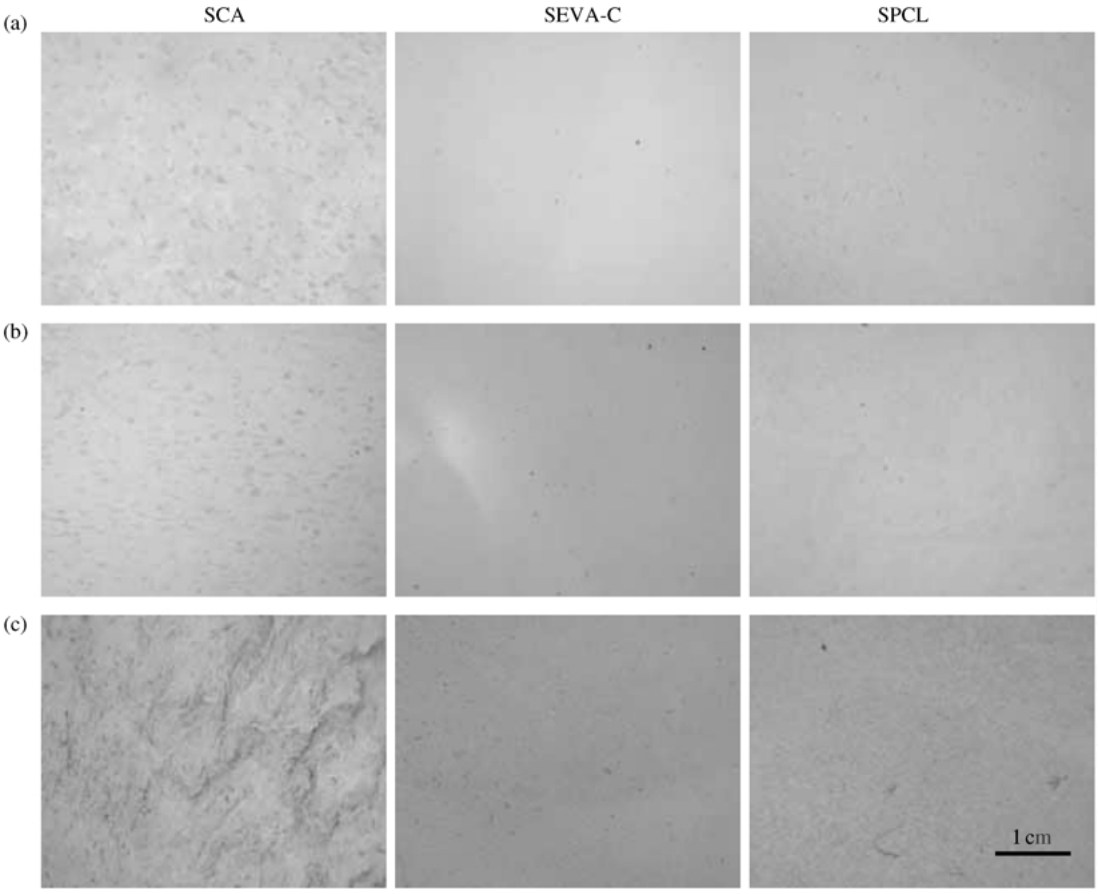

Figure 5 Immunostaining results (5 ×) of different starch-based materials (SCA, SEVA-C, and SPCL) immersed 20% (v/v) of human serum and stained for human serum albumin (a), human fibronectin (b) and human vitronectin (c).

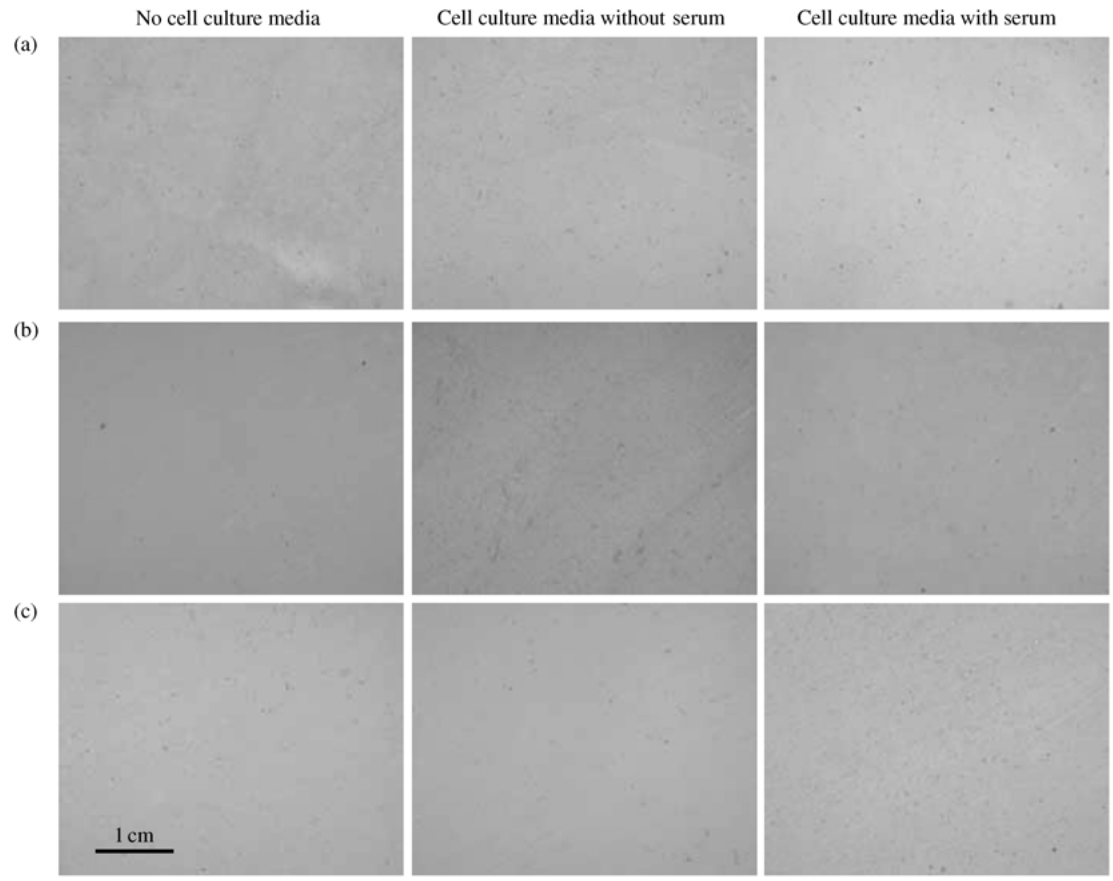

Figure 6 Immunostaining results (5 ×) of SEVA-C initially immersed in HSA (a), HF (b) and HV(c) at 0.2% of the concentration in the human serum, 70.0, 0.8 and 0.6 μg/mL, respectively, and then exposed 24 h to the cell culture media with and without foetal bovine serum.

shown). Analysis of the results leads to the conclusion that in opposition to vitronectin, albumin is the protein that is adsorbed to the smallest extent onto the starch-based materials. Alternatively, from the materials perspective, SPCL and SEVA-C were observed to present higher protein adsorption affinity when compared to starch cellulose acetate (SCA) polymeric blends (see Fig. 5).

3.2. Immunostaining analysis of leukocyte adhesion to protein coated surfaces

3.2.1. Effect of cell culture media, foetal bovine serum, and leukocyte culture onto pre-adsorbed proteins

In order to study cell-protein interactions on starch-based material surfaces the effect of cell culture media and foetal bovine serum were examined. Samples were immersed in protein solutions concentrated at 0.2% of the human serum and then placed in cell culture media with and without bovine serum. The main aim was to study the effect of cell culture media, but also the effect of foetal serum, as this is a highly concentrated complex protein solution where protein-protein interactions are certain to occur. Although no significant differences in staining intensities were observed for SEVA-C pre-coated with FN and VN (see Fig. 6), albumin antibody-labeled surfaces showed some loss of this protein after immersion in serum-supplemented media. This change may be a result of protein release from the SEVA-C surface to the surrounding solution or due to the coverage of this substrate with bovine foetal serum proteins.

After confirming that albumin, fibronectin and vitronectin are still present on the surface after cell culture immersion, the effect of previously adsorbed proteins on cultured cells was assessed. Fig. 7 shows SEVA-C surfaces exposed to 0.2% of protein concentrations, on which cells were seeded and then stained for the presence of HSA, FN, and VN. After 30 min and 24 h of leukocyte culture, fibronectin samples showed no interference determined by cell incubation. With respect to HSA and VN, results indicate protein desorption from the surface leading to the idea that fibronectin might be the more strongly adsorbed protein or at least less affected by cell culture interactions.

3.2.2. Immunostaining and cell density analysis of adherent leukocytes

To examine how adsorbed proteins on SEVA-C surface affect cell adhesion, samples were immersed in albumin, fibronectin and vitronectin solutions, concentrated at 0.2% of physiological levels. Than surfaces were used as substrates for cell culture and afterwards, antibody-labeling was performed. An example of the immunostaining results is presented in Fig. 8 where it is possible to observe lymphocytes and monocytes/macrophages (CD3 and CD14 positive stained cells, respectively) incubated with SEVA-C surfaces previously immersed in vitronectin solution. From the preliminary results obtained, it was not possible to observe inhibition of monocytes/macrophages or lymphocytes adhesion to SEVA-C surface driven by the presence of HSA, FN, nor VN.

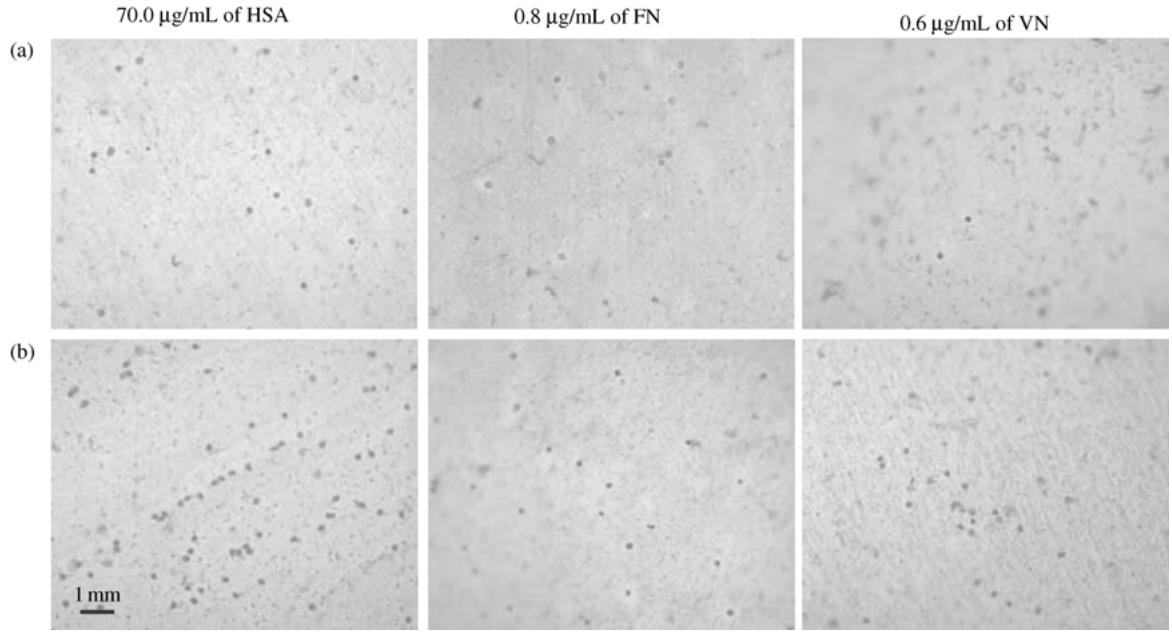

Figure 7 Protein immunostaining samples (20 ×) of SCA, SEVA-C and SPCL previously immersed in 0.2% of HSA, HF, and HV and then submitted to cell culture for 30 min (a) and 24 h (b).

To assess quantitatively the effects of albumin, fibronectin, and vitronectin on monocyte macrophage and lymphocyte adhesion to SEVA-C surfaces, CD3 and CD14 positive cells were first counted and then counter-stained with haematoxylin to determine total cell number. From Fig. 9(a) and (b), it is possible to see differences in cell number from 30 min and 24 h of cell incubation. In terms of specific populations, it is obvious the preference of monocytes/macrophages to adhere to SEVA-C surfaces when compared to CD3 positive cells. These observations conflict with established procedures regularly used to isolate lymphocytes from other leukocytes, which consider these CD3 positively stained cells not capable of adhesion to exposed surfaces [40]. After 24 h (Fig. 9(b)), it was possible to observe a cell number decrease for all the studied conditions. This behavior was not obtained for HSA previously immersed samples that exhibit a clear increase in total cell number, and in CD3 and CD14 positive cells. The decrease in cell number can be explained by these cells ability to detach from surfaces in order to adhere later or begin the apoptotic cycle [40, 41]. Once again, CD3 positive cells seem to adhere less than CD14 positive cells to SEVA-C surfaces (with or without proteins previously adsorbed on it). In summary, it might be said that vitronectin promotes a short-term effect in leukocyte adhesion and albumin was found to endorse long-term effects, subsequently leading to cell number increase after 24 h of cell culture.

4. Conclusions

In this study, the use of antibodies to label human proteins allowed to obtain reproducible results, useful for understanding protein adsorption on biodegradable surfaces. Single protein solutions lead to higher adsorptions of FN and VN followed by HSA. Preference of FN adsorption in comparison to HSA was also detected when studying binary systems, except for SEVA-C, which was the only material on which HSA

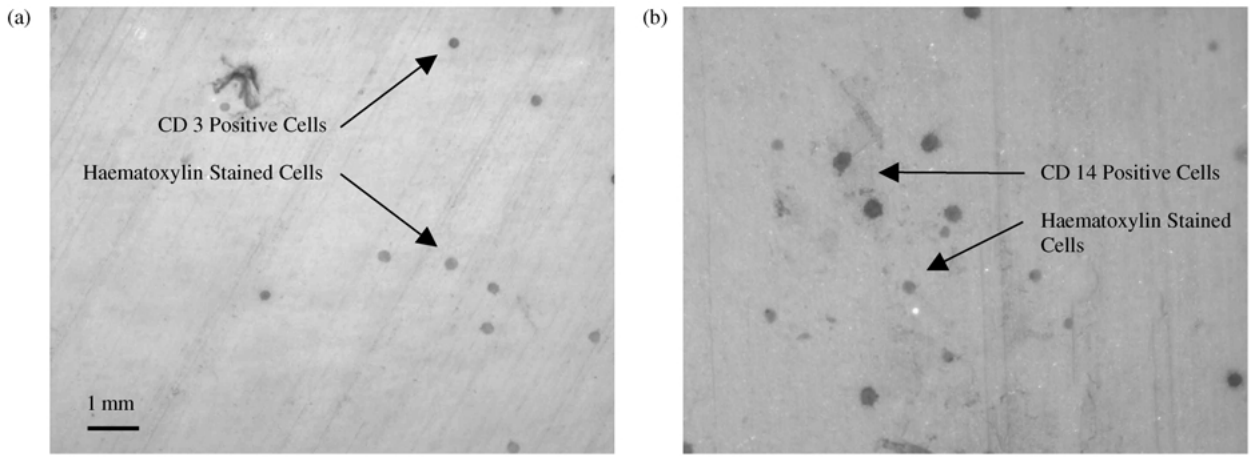

Figure 8 Example of immunohistochemical detection of human leukocytes cultured on SEVA-C surfaces previously immersed in 0.6 µg/mL of VN. Red cells consist of CD3 (a) and CD14 (b) positive cells; blue cells are a result of haematoxylin stain (20 ×).

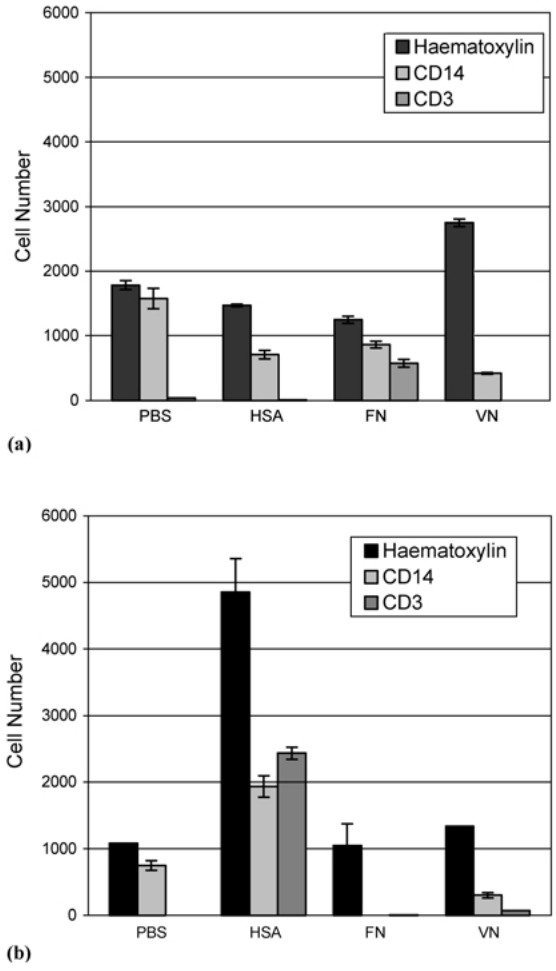

Figure 9 Total cell number, CD3 and CD14 positive cell numbers obtained for each different pre-treatment promoted to the surface of SEVA-C samples. Two different cell incubation periods are presented: 30 min (a) and 24 h (b).

preferentially adsorbed. When human blood serum was used as the protein source, differences in protein behavior were observed: in opposition to vitronectin and to fibronectin, the competitive potential of albumin to adsorb onto starch-based surfaces was decreased. Furthermore, SPCL presented the highest protein adsorption levels, even though for the studied serum concentrations surfaces showed, in general, good VN adsorption. These results support the idea that single solution studies are not good simulations of the real situation of the complex bio-enviromnent. Consequently, complex systems should be considered in future for protein adsorption studies to be carried out on starch-based biomaterials.

In the second part of this study, the effect of human albumin, fibronectin, and vitronectin on modulating leukocyte adhesion gave rise to interesting results. In fact, short and long-term effects in cell adhesion were found to be developed in the presence of vitronectin and albumin, respectively. In addition, it was shown that cell populations adhere to protein coated and non-coated surfaces, although monocytes and/or macrophages were found in higher numbers mainly for shorter incubation periods.

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