Chapter 6

In Vitro Blood Compatibility of Novel Hydrophilic Chitosan Films for Vessel Regeneration and Repair

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1. Introduction

Tissue and organ failure treatments include drug therapy, surgical repair, medical devices but they do not always provide satisfactory restoration of organ function.

At present transplants represent an actual solution in treating organ failure once overcoming immunological rejection even though its application is largely affected by the paucity of available donors.

In the vascular field, currently, the best graft performance is given by saphenous vein autografts [1] whose main failures are related to thrombosis development, emboli production, and intimal hyperplasia. Synthetic non-bio-resorbable vascular prosthesis (such as Dacron® or extended-PTFE) exhibited very low incidences of thrombosis or hyperplasia and showed good clinical results in medium- and large-diameter graft sites.

Strategies based on polymeric materials (synthetic or natural) appear to be a valid alternative for the production of tissue graft materials. However, synthetic polymers are not able to induce any biological response leading to tissue regeneration, due to the lack of biomimetic activity. On the contrary, natural biodegradable polymeric supports, resembling extracellular matrix component, can provide a useful platform in tissue engineering and regenerative medicine applications [2-5].

Among them, chitosan (CS) a biodegradable [6], non-amphiphilic polymer of D-glucosamine obtained by partial de-acetylation of chitin [3], has shown interesting properties including biomimetism due to the similarity of is structure with that of glycosaminoglycanes [4].

Kind *et al*. [7] reported that it promotes plasma protein adsorption, platelet adhesion and activation, and thrombus development [8]. The positive charged CS surface induces a great degree of platelet adhesion. In fact, the Food and Drug Administration approved its use as haemostatic dressing for reducing haemorrhage [9-11]. Furthermore, it has been reported that the negatively charged-modified surface of CS prolongs clot formation after re-calcification of plasma [12].

Up to now, few data, often conflicting, on the haemocompatibility of negative charged-modified surfaces of CS films are available[11,12].

In a previous work [13] we developed novel CS hydrogel prepared in the presence of phosphate salts and relatively high amount of disaccharides such as D-(+)raffinose or D-(+)saccharose and investigated the physico-chemical characteristics as well as the cytocompatibility of films obtained with this hydrogel. These sugars were not retained in the final structure of the film but were able to act as viscosity modifiers during the solidification/gelation process. The interference of salts and disaccharides resulted in smooth, amorphous film with improved hydrophilicity and cytocompatibility compared to CS films produced with the same procedure but in low viscosity milieu. Differentiated human cells showed a great affinity for these sugar-modified chitosan (smCS) films, thus suggesting their candidature as promising biomaterial for tissue regeneration and repair.

The aim of the present study was to investigate qualities and aspects of the haemocompatibility (platelet activation, haemolysis and activation of coagulation cascade) of smCS films produced according to Bettini*et al.* [13]. Moreover, the cytotoxicity of fragmented smCS was investigated in view of its bio-resorbability.

These films were compared to materials able to activate platelets and induce thrombus formation such as plastic (standard polystyrene for cell culture) and glass (cover slips) as well as a material able to trigger cell death such as latex.

2. Methods

2.1. Production of sugar-modified chitosan films

Chitosan solution was prepared as described in [13]. Briefly, four grams of chitosan powder (Chitosan 95/50 HMC$^{+,}$ Germany) were dissolved in a 1% (w/v) acetic acid aqueous solution until complete dissolution. Dibasic sodium phosphate (7.5 mM), sodium dihydrogen phosphate (22 mM), potassium dihydrogen phosphate (1.5 mM), sodium chloride (125 mM) andpotassium chloride (2mM) were then sequentially added. The solution was filtered under vacuum using a 0.8 μM filter. Finally, D-(+) raffinose pentahydrate (290 mM) or D-(+) sucrose (290 mM) were added to the solution and allowed to dissolve for 2 hours under gentle

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stirring. About one mL of this solution was poured into a circular mould (1 cm diameter) and dried at 45 °C for 45 minutes in a ventilated oven. The obtained dry film was placed in a 5% (w/v) KOH aqueous solution for 12 hours then, washed in distilled water until neutrality of the wash water.

2.2. Wettability

Contact angle measurements were performed at room temperature with a goniometer (AB Lorentzen & Wettre, Germany) on the surface of smC film in comparison to glass cover slip and plastic (standard polystyrene culture plates) to evaluate the wettability of the surface. Briefly, a drop (4 μL) of human serum was placed on the surface of the specimen. Images of the serum drop were recorded within 10 seconds of deposition by means of a digital camera (FinPix S602 Zoom, Fuji film, Japan). Digital pictures were analysed by ImageJ 1.43v software (NIH, USA) for angle determination. At least five measurements, taken at different positions on each specimen, were carried out on both left and right side of the drop and averaged.

2.3. Atomic force microscopy

Atomic force microscopy (AFM) images of the films were analysed by AFM Nanoscope IIIA (Digital Instruments Inc., USA). Point probe silicon cantilever tip was used in contacting mode by the accompanying software to determine the surface roughness of investigated surfaces. The roughness parameters of each sample was evaluated on three scanned areas of 10μm x 10μm each.

2.4. Procurement and processing of blood perfusates

This procedure was conducted in accordance with the tenets of the Declaration of Helsinki. Following the indication of Italian DLgs no.196/03 (Codex on Privacy) in order to guarantee the respect of the privacy of the patients and the confidentiality of the donors' information. Blood (3.5-4 mL/test) was drawn by venipuncture from four healthy volunteers and added with tri-sodium citrate (0.109 M, 3.2% final concentration) in a 9:1 volumetric ratio to prevent coagulation. Whole blood was used for the haemolysis and thrombus formation tests.

Platelet-rich plasma (PRP) was obtained by centrifugation (400xg for 10 minutes, at room temperature) while platelet-poor plasma (PPP, platelets less than 10.000/μL) by centrifugation at 2000xg for 20 minutes at room temperature.

Coagulation- and factor XII-assays were performed with platelet-poor plasma isolated from whole blood. For platelet function studies, PRP was volume adjusted with PPP to obtain a final physiologic stock platelet count of 3 10^5 platelets μL^{-1}.

2.5. Cell proliferation

Human endothelial cells derived from foetal umbilical vessels (HUVEC) were provided by the American Type Culture Collection (Rockville, MD, USA). Cell monolayer were cultured

in complete medium (D-MEM containing antibiotics and 10% foetal calf serum) supplemented with 50 μg mL^{-1} of endothelial cell growth factor (Sigma-Aldrich, USA) and kept in a incubator at 37 °C in a water-saturated atmosphere with 5% CO_2. Endothelial cells were seeded onto smCS films as well as on tissue culture plates (TCPS, Corning, USA) or glasses (20x20 mm, ForLab, Carlo Erba, Italy,) at a density of 1–2.5 10^4 cells cm^{-2} in 24-well plates. After 1, 3 and 7 days, the monolayer was rinsed twice with phosphate buffer solution, PBS, and cells detached from the substrate by 0.02% trypsin in PBS. The number of adherent cells was then, counted with a Burkerhaemocytometer.

2.6. Cell morphology

For morphological characterization, endothelial cells cultured on smCS films were examined by contrast-phase microscopy. After 7 days, the cell monolayer adherent to the film was gently washed with PBS three times. Then, the film was fixed with 2.5% glutaraldehyde in PBS for 1 h at 4 °C. After thorough washing with PBS, the cells were dehydrated through graded alcohol series and positioned under the microscope (Zeiss AxioPhot, Germany) for observation and image recording (Zeiss AxioCam, Germany).

2.7. Cytotoxicity test

Endothelial cells were grown until confluence. The smCS films was cut in small pieces (0.5x0.5 mm) and placed in direct contact with the cell layer for 72 hours. Cells were detached and the resulting suspension was counted in a Burkerhaemocytometer after proper dilution.

Duplicate cell counts on each suspension from 3 culture wells were performed for each substrate investigated. Not less than 50 cells were scored for each counting. Counts from triplicate seeding differed by not more than 10% among replications throughout the experiments.

2.8. Haemolysis assay

Two positive controls, copper and deionised water, and a negative control, glass cover slip, were used in this study, SmCS films were dried and washed three times with PBS and then sterilized by soaking in 75% (v/v) ethanol for 15 minutes. Then, washed 5 times in sterile PBS and kept in the same buffer until use. Thereafter, the samples were put in vacutainers containing sodium citrate (0.109 M, 3.2% w/v final concentration) (Greiner Bio-One International AG, Austria) in which 3.5 mL of healthy volunteers blood was finally collected. The substrates were incubated with blood at 37 °C, with gentle shaking twice every 30 minutes. After 3 hours, 1.5 mL of each vacutainer was centrifuged at 740xg for 10 minutes at room temperature. The obtained pellet was re-centrifuged at 3000g for 15 minutes at room temperature. The haemolyses was quantified on a ADVIA 2120 system (Siemens-Bayer, Germany) using a colorimetric assay.

2.9. Coagulation assays

Human whole blood (3.5 mL) from a healthy volunteer was collected and mixed with an aqueous solution containing sodium citrate, then the human whole blood was centrifuged at 1500g for 15 min at room temperature to separate the blood corpuscles, and the resulting

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PPP was used to study the coagulating ability of the CS film. All tests were performed on *IL Coagulation and ELECTRA™* system (Instrumentation Laboratories, USA). The level of Prothrombin Time (PT), activated Partial Thromboplastin Time (aPTT) and Thrombin Time (TT) were determined by using three different kits (Instrumention Laboratory USA): HemosIL ™ RecombiPlasTin 2G is a high sensitivity thromboplastin reagent based on recombinant human tissue factor (RTF) for the quantitative determination in human citrate plasma of Prothrombin Time (PT); HemosIL ™ SynthASil is a high synthetic phospholipids reagent for the *in vitro* determination of APTT (Activated Partial Thromboplastin Time). After incubation at 37 °C for an optimized period of time, calcium is added to trigger the coagulation process and the time required for clot formation is determined; HemosIL ™ Thrombin Time was used for the determination of TT in human citrated plasma.

2.10. Erythrocytes adhesion assay

SmCS films (10x10 mm) were equilibrated in PBS for 1 hour at 37°C. A washed-erythrocytes stock suspension containing 3 10^5 mL^{-1} was poured on plastic and smCS film surfaces and incubated for 30 minutes. The incubation volume was kept low (100 μL) to (a) minimize the floating population of erythrocytes and (b) maintain the total erythrocytes count at a level such as to prevent saturation-levels of adhesion and (c) to prevent other still suspended erythrocytes from contacting the surface. After that, the specimens were rinsed with PBS, fixed with glutaraldheyde and detached from surface with 1% sodium dodecyl sulphate, SDS. Ten microliters of recovered erythrocytes suspension were counted with a Burkerhaemocytometer.

2.11. Platelet adhesion assay

SmCS films, plastic and cover slips glass were sterilized with 75% (v/v) ethanol solution. Then air dried under a laminar-flow hood and rehydrated with 1 mL of sterile PBS for 1 hour. The surfaces were overlaid with 300 μL PRP at 37 °C for 2 hours. Then, the films were washed three times in PBS with mild shaking to remove non- or poorly-adherent platelets. After that, the specimens were rinsed with PBS, fixed with glutaraldheyde and cells detached with 1% SDS. Ten microliters of recovered platelets suspension were counted with a Burkerhaemocytometer.

3. Platelet immunofluorescence

The platelet count in the PRP was adjusted to 300.000 μL-1 by dilution with homologous PPP. After 1 hour contact of PRP with the different specimens at 37 °C, samples were washed with PBS, followed by fixation with 3% (w/v) paraformaldehyde and incubated with 1% (w/v) BSA in PBS. Labelling of the platelets was performed with a mouse monoclonal antibody CD62P (anti-P-Selectin, Santa Cruz Biotechnology, USA) at dilution 1:100, followed by 1:200 diluted monoclonal goat anti-mouse IgG antibody, FITC conjugated (Sigma-Aldrich, USA).

4. Platelet morphology

The platelets-coated testing surfaces were fixed with freshly prepared 2.5% glutaraldehyde for 20 minutes. After washing with PBS, the samples were dehydrated in a graded-ethanol series (50, 70, 90, and 100% v/v) for 15 minutes each and allowed to dry at room temperature. The platelet-attached surfaces were carbon sputter coated under vacuum to a thickness of 100–200 Å and examined at 10 kV using a Cambridge StereoScan 200 microscope (Cambridge Scientific Instruments, UK).

4.1. Platelet aggregation

The blood samples were collected in tubes containing PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone) as anticoagulant. Platelet aggregation was measured by means of light transmission aggregometry using Born's turbidimetric procedure and the PPACK-4 Platelet Aggregation Chromogenic Kinetic System (Helena Laboratories, USA). Briefly, 250 μL of PRP were incubated with specimen surfaces for 10 (baseline) and 60 minutes. Thereafter,the PRP were placed in a cuvette containing a metal stir bar in the absence or in the presence (positive control) of the pro-aggregation agent, adenosine diphosphate (ADP) 20 μM. Upon the addition of ADP the platelets started to aggregate thus increasing light transmission through the sample. The degree of platelet aggregation was expressed as the maximum percentage change in light transmission from PPP used as baseline. The obtained values were expressed as mean of two measurements.

4.2. Complement activation assay

The test, based on Complement Reagents Kit (Siemens Healthcare Diagnostic, Germany) was performed on BCT Siemens coagulometer (Siemens, Germany). The test focused on the ability of the complement system to lyse a standard suspension of sheep erythrocytes, sensitized with a rabbit anti-serum against sheep erythrocytes. Briefly, 1 mL of fresh blood samples previously incubated for 1 hour with different substrates were incubated with sensitized erythrocytes to investigate the complement activation. Diminished levels of individual components (e.g. due to prior activation by a foreign surface) result in a prolongation of the time taken for lyses. The time necessary for the lyses of a defined amount of erythrocytes is used as basis for determining the complement activity [14,15]. The results were evaluated using a reference curve prepared by a serial dilution of standard plasma with isotonic saline to give 100% of complement activity, 75% (75% of plasma + 25% saline) down to 10% of complement activity (10% of plasma + 90% saline).

4.3. Statistical analysis

Data were expressed as means ± standard deviation (SD). Where not differently stated, measurements were conducted at least in triplicate. Chi-square test or Student's t-test on unpaired data was used to assess the statistical significance of the difference between the results obtained from the tested specimens (Kaleida-Graph, Synergy Software, USA). Statistical significance was assumed at a confidence level of 95% ($p < 0.05$).

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5. Results

5.1. Physico-chemical characterisation

As already stated, the sugar added to the chitosan solution during the preparation of the smCS film was not retained in the final structure of the film.This assumption was mainly based on FT-IR spectra analysis for the identification of the absorption bands relevant to vibration of functional groups of chitosan [13]. The addition of phosphate salts and D-(+) raffinose to the chitosan solution used for film preparation led to non dramatic modifications in the IR spectrum of chitosan. The observation of the 1700-1500 cm^{-1} region evidenced that the amide I band (C=O in amide group) wavenumber was lower than the value for chitosan powder (1664 cm^{-1}) for all the prepared films and particularly for those prepared from a solution that did not contain the sugar [13]. This was interpreted as the result of a lower mobility of the C=O group in the film due to its involvement in the week bound formation in the solid structure. The incorporation of phosphate salts and significant amount of sugar in the chitosan solution used for film preparation reduced this effect. On the other hand, the amino group band of films prepared from a solution that did not contain the sugar was at a lower wavenumber (1588 cm^{-1}) than from chitosan powder (1592 cm^{-1}), while it was practically unchanged in film prepared from the sugar containing solution (1590 cm^{-1}).

D-(+)raffinose FT-IR spectrum evidenced characteristics bands at 2936 and 1649 cm^{-1}. Interestingly, no trace of this bands was found in the FT-IR spectra of the chitosan film prepared from solutions containing D-(+)raffinose. Similarly, no trace of the characteristic series of peaks between 2994 and 2914 cm^{-1} of the sucrose powder was found in the spectrum of the film prepared from a solution containing a high amount of sucrose [13].

These observations allowed to conclude that the excipients added to chitosan in the film forming solutions though not retained in the solid film, interact or interfere with chitosan chains during the film formation likely acting as viscosity modifiers during the solidification/gelation process.

5.2. Wettability

Contact angle measurements were performed by using serum droplets on plastic surface and on smCS film. As expected, plastic showed the least wettable surfaces with significantly higher contact angle (50° ± 6.3) compared to smCS film (15° ± 0.1) (Chi Square $P< 0.001$), thus confirming the high hydrophilicity of smCS [13].

The hydrophilicity of the sm CS film was also investigated by measuring the swelling index in water at the equilibrium according to the following equation:

$$S_w = \frac{W_s - W_d}{W_s} x100 \quad (1)$$

where W_s and W_d represent the weight of the fully hydrated and the dry film respectively. The smCS film afforded a degree of swelling at the equilibrium more than 3 order of magnitude (1285%) higher than that of the dry film. These data confirmed the very high hydrophilicity of the films obtained by adding a sugar to the solution used for the film preparation.

5.3. Roughness (AFM measurements)

The AFM analysis (Figure 1) revealed that the plastic specimen exhibited rather low surface roughness (average= 28 nm) in contrast to smCS film that showed a roughness approximately 1.7-fold higher, around 50 nm. It is interesting to note the almost regular appearance of groove and pits in smCS compared with plastic surface.

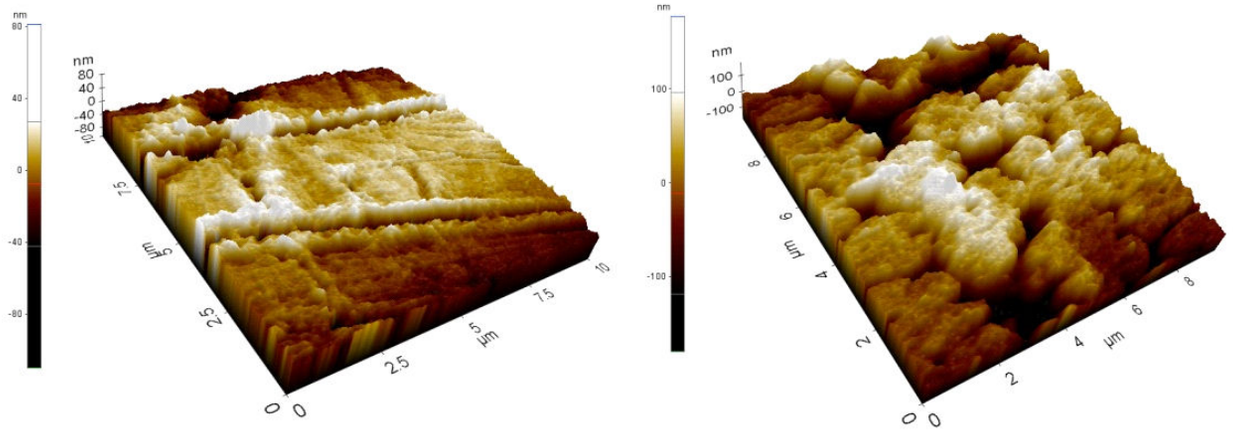

Figure 1. AFM 3D image of (A) standard colture plastic dish (plastic) and (B) smCS film surfaces.

5.4. Adhesion and proliferation assay of endothelial cells

As shown in Figures 2 endothelial cells attached (A), extended and proliferated (B) very well on all surfaces tested. Cell attachment (panel A, C) and proliferation (panel B) on smCS films were comparable to control cells grown on standard tissue culture surface (plastic). Contrast-phase microscopy showed that cells were well attached to the different surfaces and closely packed maintaining their original shapes. Moreover, endothelial cells did not evidence any morphological indication of cell death 72 hours after seeding (panel D). The counts of cells showed little variation for the three surfaces used. In the case of plastic surface (control) the growth of HUVEC reached the values of 21284 ± 650 cm^{-2}, while in the case of smCS reached a lower value of 19805 ± 305 cm^{-2} similarly to that obtained on glass surface (19543 ± 1050 cm^{-2}).

5.5. In vitro cytotoxicity

Data relevant to cell growth in the presence of small pieces of smCS film or latex (positive control) are reported in Table 1.

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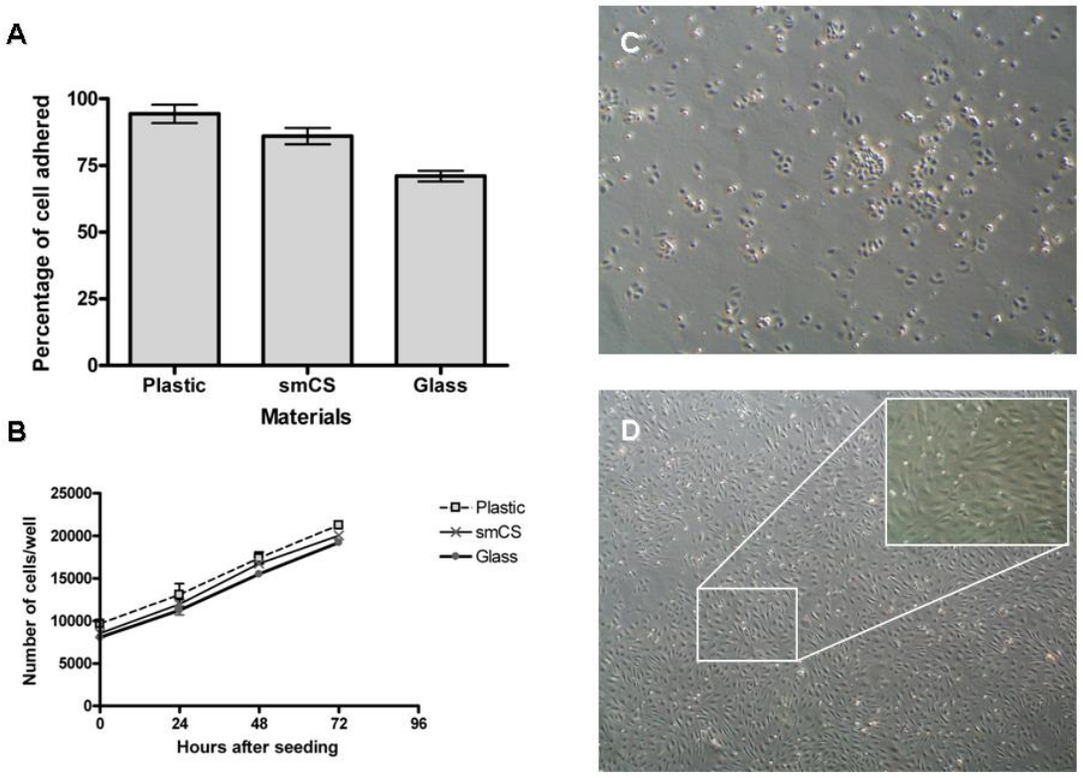

Figure 2. A) Percentage of cells adhered after 24 hoursand (B) proliferation assay of endothelial cells on the different surfaces tested. Pictures taken at the optical microscope, in phase contrast (40x), showing the morphology of endothelial cells 8 hours (C) and 72 hours (D) after seeding on smCS film.

	Hours after seeding			
	0	**24**	**48**	**72**
Latex (positive control)	10000 (± 239)	924 (± 25)	714 (±16)	50 (±30)
smCS	10000 (± 360)	11420 (±100)	16070 (±290)	18570 (± 200)

Table 1. Number of endothelial cells attached to the different substrates in the presence of latex or smCS film fragments.

The initial plating corresponds to the number of cells attached to the substrate 6 hours after their inoculation into the well. The measured plating efficiency was around 95%. When smCS fragments were present in the colture medium, a progressive increase of cell numbers was observed, while in the presence of latex a progressive detachment was noticed with almost all plated cells detached from the substratum after 72 hours.

These results indicate that smCS film were not cytotoxic while latex, as expected, was found to markedly affect endothelial cell survival.

5.6. Haemolysis assay

Haemolysis of red blood cells was used to evaluate the membrane damaging potential of the surface of the smCS film. Two positive controls, distilled water and Copper, and one negative control, glass, were used.

Substrate	Haemolysis (%)
Distilled Water	97 (± 5)
Copper	7 (± 2)
Plastic	5 (± 1)
Glass	2 (± 1)
smCS	1 (±3)

Table 2. Percentage of haemolysis measured on different substrates. The tested surfaces were incubated with whole blood for 1 hour. Distilled water was used as positive control.

As shown in Table 2, distilled water resulted in about 100% haemolysis, while Copper led to 7% haemolysis. Glass and smCS film caused negligible haemolysis (within the experimental error) indicating very low membrane damaging properties of smCS material.

5.7. Blood coagulation assay

The effects of the biomaterial on coagulation process were tested by means of the (aPTT), the (PT) and the (TT) selected as reliable measurements of the capacity of blood to coagulate through the intrinsic, extrinsic and common coagulation mechanisms, respectively. As shown in Figure 3 the values obtained for PT, TT and aPTT were similar to those observed for human plasma, thus indicating that all materials tested, including smCS, did not affect coagulation pathways.

Substrates	Erythrocyte lyses time (seconds)
Plasma (*control*)	35.4
Glass	38.2
Plastic	35.7
SmCS film	36.3

Table 3. Erythrocyte lyses time determined by plastic and glass surfaces in comparison with smCS film.

5.8. Complement activation assay

The erythrocyte lyses time observed and reported in Table 3 shows no significant difference among the material studied and the control. The data presented demonstrate that smCS is a nonreactive biomaterial that does not directly activate complement.

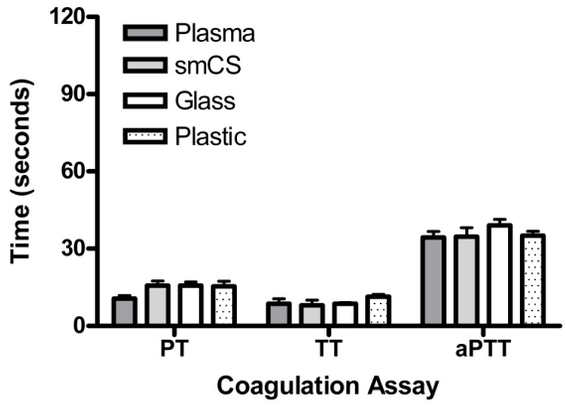

Figure 3. Effect of the different surfaces on coagulation time tested by means of the (aPTT), the (PT) and the (TT).

5.9. Erythrocytes and platelets adhesion assay

In Table 4 the number of cells detached with SDS from the different surfaces after adhesion test is reported. The smCS film presented a lower overall erythrocyte and platelet adhesion in comparison to plastic surface.

Materials	Surface Adhesion	
	Erythrocytes μL^{-1}	*Platelets* μL^{-1}
Plastic	40 (± 13)	11(± 8)
Glass	-	17 (± 6)
smCS	8 (±3)	5 (± 2)

Table 4. Numbers of erythrocytes and platelets adhered to the studied surfaces

The test showed a high significant difference in the number of adhered erythrocytes on materials studied ($p<0.0001$): the erythrocyte adhesion on smCS film was about 5 fold less than the adhesion on plastic surface. A similar behaviour was observed for platelets. In fact, the platelets recovered from plastic and glass surfaces ranged from 2 to 3 fold more than platelets recovered from smCS film surface.

5.10. Platelet aggregation

This test was performed in order to investigate the ability of plastic, smCS film and glass surfaces to induce platelet aggregation. The presence of ADP (adenosine-diphosphate, a pro-aggregation agent) determined a normal profile of platelets aggregation (range 90-95% after 5 minutes of incubation). Subsequently, the influence of materials on platelets aggregation in the absence of ADP was studied. There were no differences between materials ob-

served at baseline (10 min) and after 1 hour of incubation with the substrates. smCS induced slightly higher aggregation of platelets (5-6%) compared to plastic (2.5%) or glass (less than 2%). However, these differences have to be considered with caution, as the coefficient of variation estimated with human plasma in the absence of ADP was around 10%.

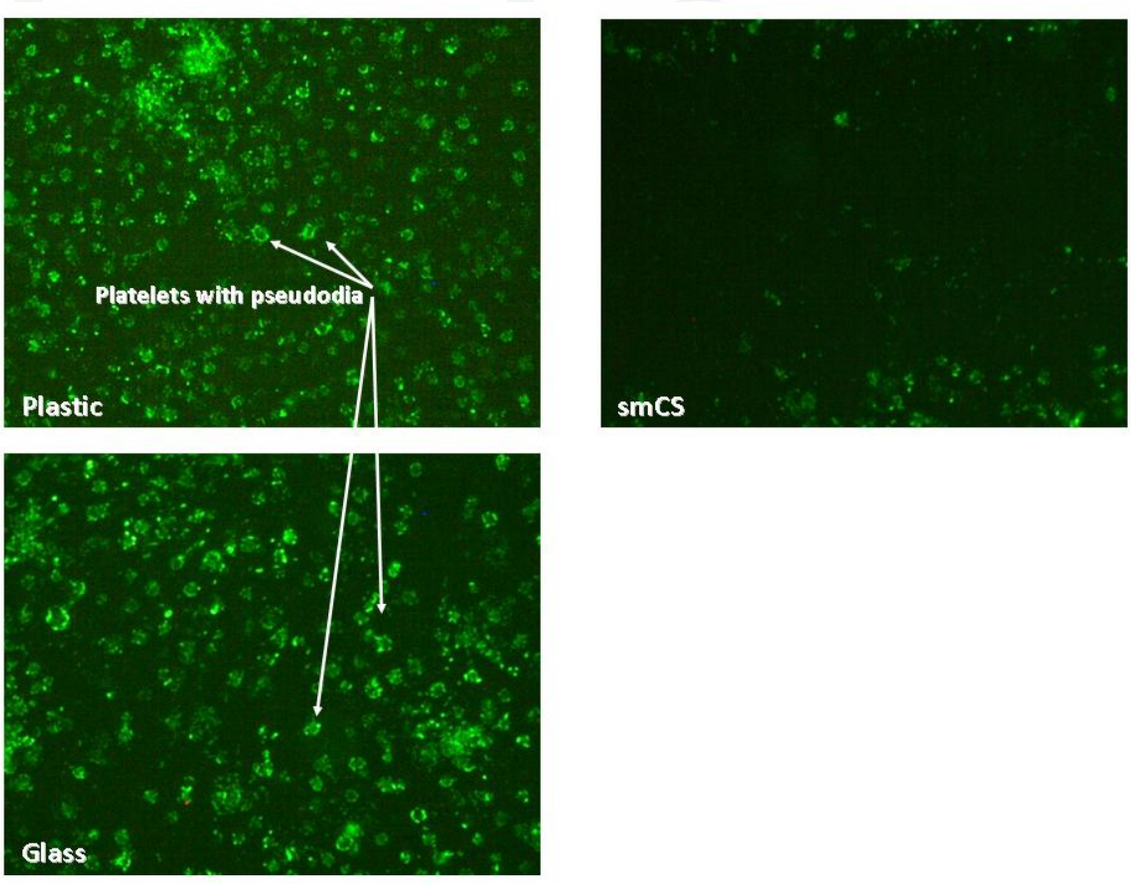

Figure 4. Fluorescence microscopy (100X)images of human platelets immunodecorated with CD62P (p-Selectin). Arrows indicate the presence of pseudopodia.

5.11. Platelet activation assay

Platelet activation was studied by the membrane expression of P-Selectin using the CD62P antibody. The expression of P-Selectin was evident on platelets adherent to plastic and glass surfaces and was negligible on platelets settled on smCS films (Figure 4).

On glass and plastic (see arrows) the analysis of morphology showed several fully spread platelets expressing pseudopodia with the occurrence of focal clumps. This was also evident when platelets were examined by Scanning Electron Microscopy, SEM (Figure 5B and 5C).

SmCS film (Figure 5A) induced very limited morphological changes over the 90 minutes of contact: platelets remained mostly discoid without the occurrence of pseudopodia.

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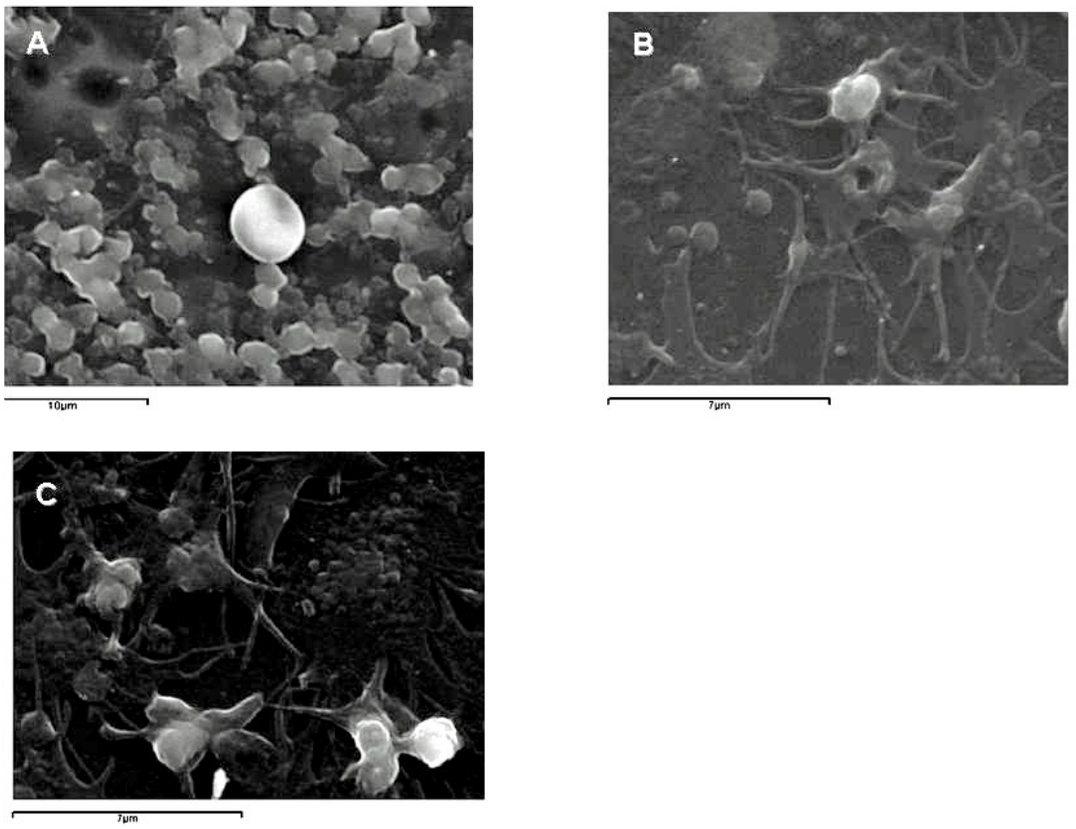

Figure 5. Scanning Electron Microscope images of platelets incubated onto different surfaces. (A) smCS film, (B) plastic, (C) glass.

6. Discussion

For blood-contact applications, haemocompatibility is largely determined by specific interactions with blood and its components [16]. Many, if not all, blood-contacting biomaterials are able to cause different undesired host responses like thrombosis, inflammatory reactions and infections.

The coagulation system and platelets are the main factors for thrombus formation on biomaterials and represent a major unmet problem in the design of vascular implants and blood-handling systems [17].

It is known that the endothelium is an active organ that maintains vessels integrity and prevent thrombosis and intimal hyperplasia [18,19]. Hence, biomaterials able to promote *in situ* endothelialisation of implants would be highly desirable.

Studies involving in vitro endothelialisation of grafts with cultured endothelial cells prior to implantation have shown that a confluent endothelium is able to prevent trombogenic

complications and improves long-term patency [20,21]. Thus, taking into account that the endothelialisation of the blood-contacting polymeric materials is an important pre-requisite for the success of the synthetic vascular grafts [22] we firstly investigated the ability of endothelial cells to adhere and proliferate on smCS film. The results obtained, in agreement with those shown in our previous paper [13], showed only little variation among the surfaces tested (glass, plastic and smCS). However, from the cell proliferation and morphology it was very difficult to discriminate difference in cytophilicity among the surfaces tested. Furthermore, the presence of fragmented smCS did not induce any decrement in the total number of endothelial cells compared to latex that, on the contrary, strongly affected cell survival.

The high hydrophilicity of smCS, indicated by the low contact angle, could ease the interaction with the bipolar extra-cellular matrix proteins such as fibronectin and vitronectin. Furthermore, the reduced cationic nature, due to a water shell does not allow anionic proteins such as collagen and fibronectin to dissociate from CS surface in a physiological environment. This aspect is in agreement with the conclusion of [23] who reported that a hydrophilic surface is good for anti-non-specific protein adsorption. It was recently reported that the affinity for water of the cell-material interface seems to be a chief parameter in controlling cell adhesion, migration and differentiation [24].

Stevens and George [25] recognized that cells are sensitive to microscale patterns of chemistry and topography, and Dalby [26] noted that cell behaviour is directly influenced by the surface structures such as grooves, pits, or ridges.

In this paper AFM images of smCS films evidenced a topographically patterned surface. In the light of the above reported literature, this observation can be used to speculate about the enhanced adhesion and proliferation of vascular cells compared to conventional, CS films previously observed in [13].

Surface properties such as wettability, surface topography and charge are known to affect endothelial cells attachment and growth [8]likely by altering the rate of the amount of adsorbed proteins and their conformational changes [27,28]. The effect of surface materials on erythrocyte aggregation and platelet adhesion/activation becomes a chief parameter in haemocompatibility studies.

Several years ago Malette and co-workers [29] ascribed the pro-coagulation properties of chitosan to the negative charged surface of erythrocytes, while [30]showed that chitosan may induce the adhesion of erythrocytes.

In the present study, the surface of smCS films induced only a limited erythrocytes agglomeration, thus indicating that smCS surface neither captures erythrocytes nor forms a three-dimensional network structure with these cells.

The lack of erythrocyte aggregation may be likely due to a polymer chains rearrangement that masks the cationic nature of chitosan surface. Such a rearrangement can be ascribed to the larger amount of water in smCS films as described in [13].

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One of the most important findings of this work is the observed difference in platelets morphology seeded on smCS in comparison with glass or plastic. On the latter surfaces platelets appeared flat with interconnecting pseudopodia coupled to strong P-Selectin membrane expression.On the contrary, the platelets on smCS films were discoidal, and neither pseudopodia formation nor a P-Selectin membrane translocation was observed.

This finding could be attributed to a new conformation of the adsorbed plasma proteins on glass or plastic that could have facilitated platelet aggregation. Indeed, it is well known that the surface topography can induce a spatial reorganization of adsorbed proteins as well as how this phenomenon occurs [31]. In contrast, when the adsorbed proteins maintain their native state, they do not support platelet adhesion and aggregation [32].

The absence of platelet activation on smCS surfaces suggests this outcome.

As far as the surface morphology is concerned, it has been reported that platelets adhere in similar manner on smooth and rough surfaces when tested under static conditions [33]. Similarly Ward et al. [34] concluded that it is not the roughness *per se* which affects the platelet adhesion.

One decade ago, Suzuki and Minami [35,36] showed that Chitosan depleted complement proteins from plasma, suggesting that chitosan activates complement. A greater depletion of complement activity was seen for a highly de-acetylated form of chitosan [36]. It is however, important to note that the results obtained about the complement activation were based on binding and depletion assays. This complement depletion can equally be explained by assuming a tight binding to the chitosan surface without activation [37].

The results presented here indicate that although large amounts of serum were deposited on smCS surface no activation of the complement system occurred, suggesting that the complement is not directly activated by the smCS surface in the process of blood coagulation.

Haemolysis testing of biomaterials has been advocated for, and used in, standard biological safety testing of materials for more than 30 years. The results of test for haemolysis should be considered with care even if they represent the only recommended test for some medical devices as stated in Part 4 of ISO 10993 guideline.

Different papers have reported that chitosan promotes surface-induced haemolysis likely through an electrostatic interactions [38]. In the present work, in the presence of whole blood smCS triggered less than 5% of haemolysis that, along with the low erythrocyte adhesion, indicates a wide safety margin in blood contacting applications and suitability for vascular implants.

In the process of haemostasis, the activation of platelet adhesion and aggregation could represent an initial and critical step. Here we showed that the surface of smCS films does not interfere with coagulation mechanism and supportswell endothelial cell adhesion and proliferation even if [39] reported that the haemostatic mechanism of chitosan may be independent of the classical coagulation cascade.

7. Conclusion

In this paper we demonstrated that the simple introduction of a viscosity modifier, such as a polysaccharide, during the process of production of chitosan films affords chitosan structures (smCS film) with improved capability to induce surface endothelialisation.

This structure is moreover, characterized by a high degree of haemocompatibility and does not induce clots formation.

These findings are of particular interest as they add new information with respect to the presently available literature and they put new light on the use of chitosan for producing surfaces that has to get in contact with blood.

As a matter of fact, the haemostatic properties of chitosan have to be considered more carefully as we have demonstrated that they could be dramatically reduced by an improvement of the hydrophilicity of the chitosan film surface.

Finally, from the results presented in this work, we can conclude that the sugar modified chitosan film could be envisaged as a new material for the design the luminal portion of vessel prosthesis based on a natural and bio-resorbable polymer.

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