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RESEARCH ARTICLE

Immobilization of fibronectin in chitosan substrates improves cell adhesion and proliferation

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Abstract

Covalent grafting of biomolecules is a strategy to improve the biocompatibility and bioactivity of materials. However, it is critical to maintain the biological activity of the biomolecule upon its attachment to the surface. In the present study we compared the biological properties of chitosan, in which the surface was enriched with fibronectin (Fn), using two methodologies: chemical immobilization, using a water-soluble carbodiimide; and simple adsorption. X-ray photoelectron spectroscopy studies confirmed the successful immobilization of Fn onto modified membranes. SaOs-2 cells were seeded onto these surfaces to assess the biological consequences of such modifications. The presence of Fn stimulated cell adhesion on chitosan. It was found that after 7 days of culture in the presence of covalently attached Fn, the cells are confluent; significantly fewer cells were detected in unmodified film and in film with adsorbed Fn. This result is consistent with the fact that considerable desorption of Fn from chitosan takes place within 24 h in culture medium. This study showed that Fn may be easily covalently attached onto chitosan substrates, improving the biological performance of the material. The technique could find applications in tissue-engineering strategies, as the surface modification of chitosan-based substrates could be carried out in more complex geometries, such as in scaffolds or particles. Copyright © 2010 John Wiley & Sons, Ltd.

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1. Introduction

One of the major goals in biomaterials science is to accurately control protein adsorption, the cellular interactions with implantable surfaces and, ultimately, the host response.

Chitosan is a natural polysaccharide composed of glucosamine and *N*-acetyl glucosamine, linked in a $\beta(1-4)$ manner. It has been widely investigated for bone implant and tissue-engineering applications because of its ability to enhance wound-healing rates, support osteoconduction and provide antimicrobial properties (Malafaya *et al*., 2008; Nettles *et al*., 2002). Despite these favourable characteristics, limitations in cell adhesion and contradictory biological responses have been reported (Lopez-Perez *et al*., 2007; Mori *et al*., 1997; Silva *et al*., 2008). Different approaches have focused on improving cellular behaviour and tailoring surface functionality, using techniques such as plasma modification (Lopez-Perez *et al*., 2007; Silva *et al*., 2008), blending (Coutinho *et al*., 2008) and chemical reactions (Kast and Bernkop-Schnurch, 2001)· (Alves and Mano, 2008). The decoration of the surface with signalling molecules, proteins or small peptides is also used as a strategy to control and direct cell responses to biomaterials' surfaces (De Mel *et al*., 2008; Patel *et al*., 2007; Ho *et al*., 2005; Hirano and Mooney, 2004). There are three major methods of surface immobilization of biomolecules: (a) physical adsorption; (b) physical 'entrapment', i.e. barrier systems or hydrogels; and (c) covalent attachment, i.e. using solid surface chemistry, hydrogels or soluble polymer conjugates. Covalently

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immobilized proteins are more complex to obtain but can bypass some of the limitations related with the adsorption method, such as desorption or undesired and time-dependent conformational changes (Garcia *et al*., 1998; Vallieres *et al*., 2007).

A critical issue of the covalent immobilization strategy is the conservation of biomolecule bioactivity. Covalent coupling of proteins and small peptides is dependent on the presence of appropriate functional groups on both the target molecule and the polymeric material. Different coupling reagents and crosslinkers have been used to bind specific biomolecules on functional surfaces (Ho *et al*., 2005; Pieper *et al*., 2000; Barbucci *et al*., 2005; Sharon and Puleo, 2008). Fibronectin (Fn) is frequently studied because of its implication in numerous physiological processes, such as wound healing, cell adhesion, migration, differentiation and survival (Risau and Lemmon, 1988; Miyamoto *et al*., 1998). Fn is a large protein (450 kDa) component of the extracellular matrix (ECM), which is composed of homologous repeating structural motif designed type I, II and III modules. These, in turn, are grouped together into functional domains with specific biological activities. Of the more than 15 different FnIII modules, only FnIII10 contains the RGD (Arg–Gly–Asp) cell adhesion sequence located on an exposed loop that extends away from the body of the protein. Several studies have focused on the use of short bioadhesive oligopeptides to regulate cellular activities. The availability of the RGD-binding region is in many cases sufficient for cell adhesion (Aota *et al*., 1994; Olbrich *et al*., 1996; Garcia *et al*., 2002) and may replace RGD-containing proteins. Previous studies have demonstrated that the immobilization of RGD onto chitosan could improve cell attachment.(Ho *et al*., 2005; Tigli and Gumusderelioglu, 2008) Notwithstanding, the use of the protein instead of short peptides may provide important secondary actions, which regulate cell binding, signalling and, subsequently, cell proliferation and differentiation. One example is the modulator effect of the RGD and PHSRN (Pro–His–Ser–Arg–Asn) amino acid sequences of Fn. The synergy between these two domains is not achieved when using peptide immobilization approaches. Therefore, as an alternative strategy towards the enhancement of the biological properties of chitosan substrates, we have focused on the use of the entire molecule.

In this study, Fn was tethered via two different methods: physical adsorption; and covalent binding, using a water soluble carbodiimide. EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide] reacts preferentially with the carboxyl groups forming *O*-acylisourea, which can readily react with the amino groups available on the chitosan surface (Olbrich *et al*., 1996). Moreover, desorption of Fn was assessed and its bioactivity on chitosan membranes was investigated by culturing adhesion-dependent cells over different time periods.

2. Materials and methods

2.1. Preparation of chitosan membranes

Chitosan (CHIT; Sigma-Aldrich, Germany) with a degree of deacetylation (DD) of approximately 80%, was purified as described elsewhere (Mano, 2008). The purified chitosan was dissolved at a concentration of 2% w/v in 2% v/v acetic acid and stirred overnight. The solution was cast in glass coverslips with 13 mm diameter (Agar Scientific, UK), dried overnight and neutralized with 0.1 M NaOH. The samples were sterilized using ethylene oxide.

2.2. Protein attachment

2.2.1. Covalent immobilization of fibronectin

The molecules selected for this study were bovine serum albumin (BSA) and human serum fibronectin (Fn; Sigma-Aldrich, Germany). 2-(*N*-Morpholino)ethanesulphonic acid (MES; Fluka, Germany) buffer was used prepare the protein solutions.

The procedure used for protein immobilization was based on studies previously performed to immobilize peptides on amino-phase substrates (Pieper *et al*., 2000; Nakajima and Ikada, 1995). Briefly, EDC (Sigma, Germany) and *N*-hydroxysuccinimide (NHS; Aldrich, Germany) were used to pre-activate the proteins by reaction with their carboxyl groups (intermediate reaction). This step was performed during 20 min at room temperature (RT) with shaking. Afterwards, the protein solutions were added to the materials to react with the amino groups available on the chitosan surfaces (Scheme 1).

The immobilization conditions were determined using BSA, a well-studied protein often used as a model molecule. Parameters such as incubation time and temperature were optimized and 4 h and room temperature (RT), respectively, were subsequently selected. Different reaction times were tested: 4, 12 and 24 h. The optimal concentrations of EDC/NHS were also determined. Concentrations of 2 mM EDC and 5 mM NHS were chosen to immobilize human Fn (20 μg/ml) on chitosan surfaces.

2.2.2. Adsorption of fibronectin

Adsorption studies were performed by incubating 20 μg/ml Fn solutions on chitosan membranes at room temperature for 4 h. Tissue culture polystyrene (TCPS; Sarstedt, Germany) discs were used as control surfaces.

2.3. Protein quantification and surface characterization

Protein immobilized on the surface was assessed by coupling a depletion method with a protein assay, as follows. After incubation, the remaining Fn in

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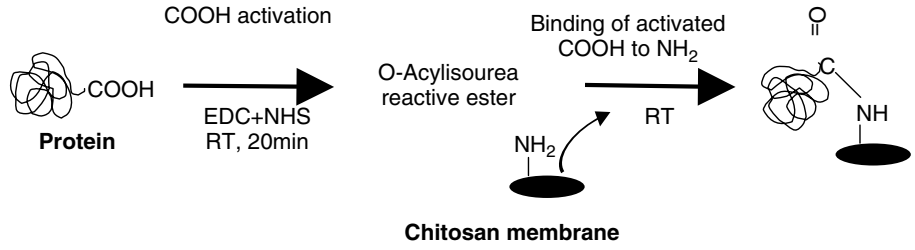

Scheme 1. One-step EDC reaction with carboxyl from the protein and amine group from chitosan

solution was assessed by a colorimetric method for total protein quantification, using the Bradford assay (Sigma, Germany) and reading emission (595 nm) in a microplate reader (BioTek, USA). The obtained value was subtracted from the initial value of protein in solution that corresponds to the protein bound on the membrane. Optical density values were converted to mass per area, by preparing a standard curve and considering the area of the chitosan membranes.

X-ray photoelectron spectroscopy (XPS) was used to further characterize unmodified and modified membranes. XPS was performed using a VG Escalab 250 iXL ESCA instrument (VG Scientific) with monochromatic Al Ka radiation ($h\nu = 1486.92$ eV) and a take-off angle of 90° relative to the sample surface. The measurement was carried out in constant analyser energy (CAE) mode, with 100 eV pass energy for survey spectra and 20 eV pass energy for high-resolution spectra. The C1s peak was resolved into three peaks at 285.0 eV.

2.4. SaOs-2 cell studies

2.4.1. Cell culture on chitosan-modified membranes

To evaluate the biological activity of bound and adsorbed Fn, cell culture studies were performed with SaOs-2 human osteoblast-like cells, which have been well characterized in the literature [European Collection of Cell Cultures (ECCC), UK]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, USA), supplemented by 10% heat-inactivated fetal bovine serum (FBS; Biochrom AG, Germany) and 1% antibiotic-antimicotic (Gibco, USA).

Following treatment, the membranes were washed in phosphate-buffered saline (PBS) solution to remove any unbound proteins. Cell adhesion studies were performed using 1×10^5 cells/ml in: (a) complete growth medium; and (b) serum-free medium. Non-treated chitosan membranes and TCPS discs were used as control surfaces. Cells were incubated at 37 °C, 5% CO_2 for 4 and 24 h. The effect of adsorbed and immobilized Fn on the cell response to chitosan membranes was further assessed in terms of cell proliferation. These experiments were performed for 7 days in FBS-supplemented media.

2.4.2. Viability and morphology of cells

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium (MTS) assay (Promega, Madison, USA) was performed to evaluate cell viability. Briefly, after the each time period, fresh culture media without FBS and phenol red was added to the cells. MTS was used in a 5 : 1 ratio to the culture medium and incubated with the membranes for 3 h (37 °C, 5% CO_2). After the incubation period, the optical density (OD) was read at 490 nm in a microplate reader (Bio-Tek, USA).

Phalloidin and DAPI staining to visualize actin cytoskeleton and to label the DNA, respectively, were conducted as outlined by the supplier's protocol (Sigma, Germany). Briefly, membrane-adherent cells were washed with PBS, fixed in freshly prepared 4% formaldehyde/PBS v/v for 30 min at room temperature (RT), and washed extensively in PBS to remove all traces of the fixative. The cells were then stained with 50 μg/ml fluorescent phalloidin-conjugate solution in PBS for 45 min at RT. DAPI labelling solution (0.5 μg/ml) was incubated for 3 min at room temperature. The membranes were washed in PBS to remove remaining staining solutions and imaged using a fluorescent microscope (Zeiss, Germany).

2.5. Protein desorption

Desorption was investigated to understand whether the methodologies rendered different binding forces between Fn molecules and chitosan surfaces. For that, membranes with protein adsorbed, immobilized and without Fn, were incubated in complete DMEM (supplemented with FBS) for 24 h at 37 °C. Afterwards, the membranes were washed with PBS and incubated with mouse anti-human Fn (1 : 100, Sigma, USA) for 30 min at RT. After washing with PBS, the membranes were incubated with goat anti-mouse Alexa-Fluor 488 secondary antibody (Invitrogen, USA), for 30 min at RT. Labelled samples were analysed using a fluorescence microscope (Zeiss, Germany).

2.6. Statistical analysis

The effect of the immobilization approach on the binding of Fn and the response of cells in terms of adhesion and proliferation to the different surfaces was evaluated and results represented in terms of mean ± SD ($n = 3$). Groups

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were compared using independent paired *t*-test, with $p < 0.05$ and $p < 0.01$ indicating statistical significance.

3. Results and discussion

Taking into consideration that only multimodular proteins can allow cells to dynamically self-regulate stimuli received from their environment, the aim of this work was to immobilize whole molecules and, thus, attempt to maintain their bioactivity. Adsorption and covalent immobilization of Fn were performed to modify the surface of chitosan membranes. In order to examine the influence that both Fn–chitosan systems have on cell adhesion, proliferation and morphology, a model osteoblastic cell line was used.

3.1. Immobilization and adsorption of fibronectin on chitosan surfaces

In the present study, Fn was immobilized on CHIT by formation of amide bonds with the amino groups of CHIT and carboxyl groups of Fn. EDC and NHS were used in this reaction to form intermediate reactants (pre-activated proteins). The amounts of immobilized and adsorbed proteins were evaluated using an indirect method (Figure 1). The results of Fn quantification indicated that the proteins were successfully grafted to the membranes. The amounts of Fn bound by covalent immobilization and by adsorption were, respectively, 11.8 μg/cm^2 and 10.6 μg/cm^2. Nevertheless, these values do not translate into significant differences (*t*-test; $p < 0.05$ and $n = 3$).

The presence of protein on the different surfaces was accessed with XPS. Analysis of Table 1 clearly indicates the increase in nitrogen atomic percentages (N%) when comparing non-modified CHIT to that of Fn pre-treated surfaces. The N% of CHIT increased 2.9% when Fn was immobilized (CHIT–Fn-immob) and 5.1% when Fn was adsorbed (CHIT–Fn-adsorbed), with respect to non-modified CHIT membranes. The high-resolution S2p spectrum (data not shown) provided further insights into the nature of the established bonds. The S2p spectra for both modified surfaces revealed an additional peak at 163.8 eV, which is likely to represent the disulphide bonds on the Fn molecule.

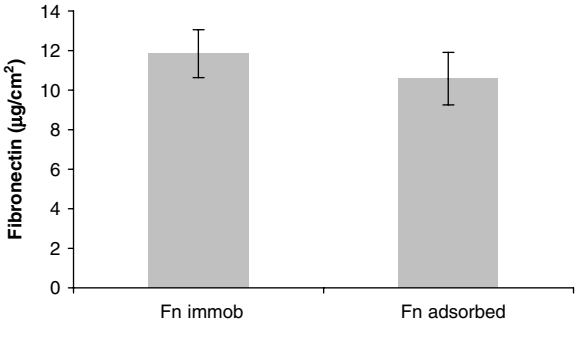

Figure 1. Quantity of Fn chemically immobilized and adsorbed on CHIT membranes after 4 hours incubation (n=3)

Table 1. Average atomic composition and atomic rations of fibronectin immobilized (CHI Fn-immob), fibronectin adsorbed (CHIT Fn-ads) and unmodified CHIT (CHIT) membranes

Conditions	C (%)	O (%)	N (%)	Ratio C : N	Ratio C : O
CHIT	61.8	31.1	6.4	9.7	2.0
CHIT–Fn–ads	64.5	21.7	11.5	5.6	2.9
CHIT–Fn–immob	66.1	23.0	9.3	7.1	2.9

Figure 2A presents the high-resolution XPS spectra of the C1s region for the unmodified CHIT, CHIT–Fn-adsorbed and CHIT–Fn-immob. Three components in the high-resolution XPS spectra in the C1s region of the CHIT membrane were isolated. The first, with an energy of 285.01 eV, was assigned to the C–C and C–H bonds, which overlap C–NH_2 chemical binds; the second, at an energy of 286.49 eV, was assigned to C–O, C–OH and C–N–C═O; and the final component, at an energy of 288.10 eV, was attributed to C═O and N–C═O. It is clear that, after modification of the membrane with Fn, the relative intensity of the peak at 286.49 eV decreases, due to the decrease of the relative amount of – C–OH groups found essentially on CHIT.

Similar analysis was performed for the N1s peak (Figure 2B). In the spectra of non-modified CHIT, the N1s region contains one dominant signal at 399.33 eV, which is characteristic of NH_2 bonds. Two other components at higher binding energies (400.27 and 402.05 eV) showed that nitrogen is also involved in more oxidized environments, such as C–N–O bonds, being consistent with the presence of Fn on the surface. Moreover, CHIT membranes with immobilized Fn showed an additional peak contributing to N1s, with binding energy at 397.52 eV. The higher energy of these bonds was attributed to amine and amide bonds, suggesting that Fn was covalently bound to the CHIT surface. Both adsorption and covalent immobilization of Fn could, in principle, be performed on non-flat surfaces. This could be particularly useful on CHIT structures for tissue-engineering applications with more complex geometries, such as scaffolds (Malafaya *et al*., 2008; Tuzlakoglu *et al*., 2004) or particles (Cruz *et al*., 2008).

3.2. Bioactivity of immobilized and adsorbed fibronectin

3.2.1. Cell adhesion to modified surfaces

Cell adhesion was determined 4 h after seeding. Figure 3 shows representative results of SaOs-2 cell attachment on surfaces with adsorbed (Figure 3A) and immobilized Fn (Figure 3B). Actin microfilaments of the cytoskeleton were labelled with phalloidin and the DNA stained with DAPI. The morphology of osteoblast-like cells was visualized by fluorescence microscopy.

After incubation for 4 h, osteoblast-like cells were well spread, presenting the typical elongated morphology

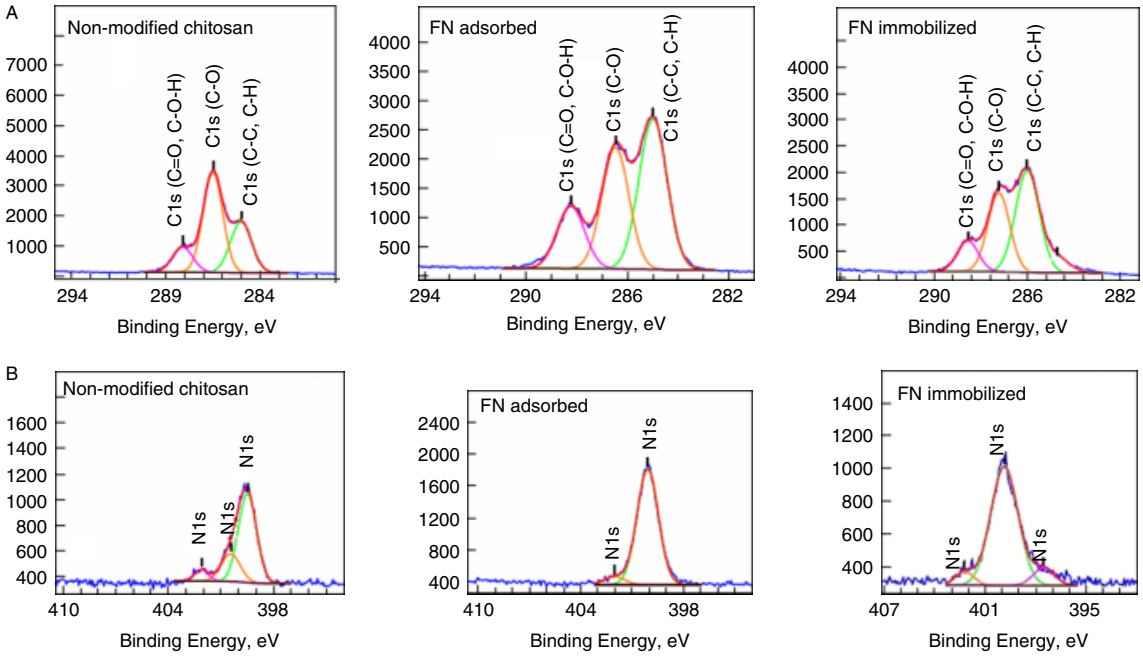

Figure 2. High-resolution XPS spectra. A) C1s region of non-modified CHIT, CHIT with adsorbed Fn and CHIT with immobilized Fn; and B) N1s region of non-modified CHIT, CHIT with adsorbed Fn and CHIT with immobilized Fn

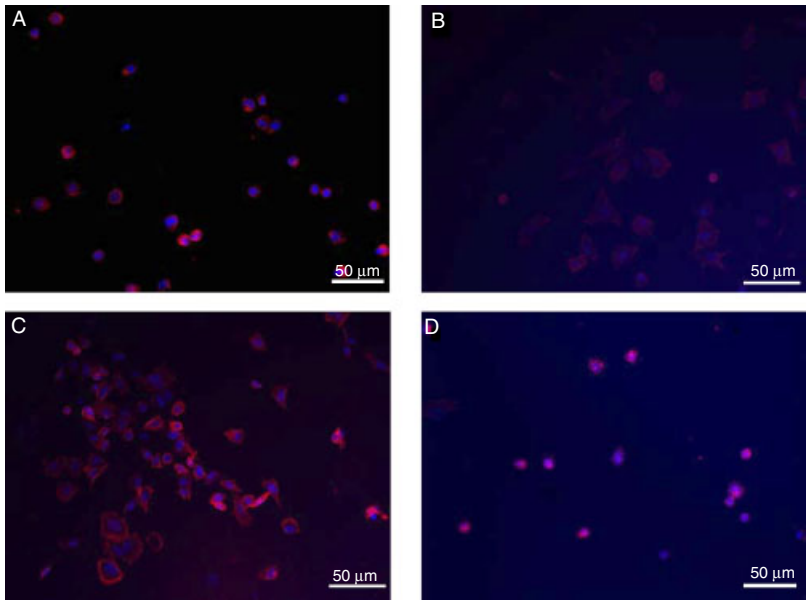

Figure 3. SaOs-2 cultured on CHIT membranes for 4 hours (×20). A) Non-modified CHIT; B) CHIT with adsorbed Fn; C) CHIT with immobilized Fn; and D) TCPS. Cells cultured in serum-free media. Scale bars correspond to 50 µm

when cultured in the CHIT/Fn surfaces, even in the absence of FBS. In opposition to CHIT with adsorbed or immobilized Fn, few cells adhered to the non-modified CHIT membranes and most of those presented round morphology.

Cell adhesion and morphology for Fn-adsorbed and Fn-immobilized surfaces were almost identical, indicating that the two methods were similar in enhancing the cells' adhesive activity and shape. These data indicate that Fn-tethered surfaces do improve cell adhesion, mainly in early culture periods.

Previous studies justify the use of small peptides, due to the complex conformation of proteins and the difficult immobilization of these molecules without lost of bioactivity (Garcia *et al.*, 1998; Vallieres *et al.*, 2007; Kowalczynska *et al.*, 2005), although synthetic peptides lack the complete specificity and function of native ECM proteins (Akiyama *et al.*, 1995; Tosatti *et al.*, 2004). A major limitation of using short RGD peptides is the lack of integrin specificity. The detection of other integrins that can bind to Fn demonstrates the potential of the use of this protein to mediate cell adhesion migration

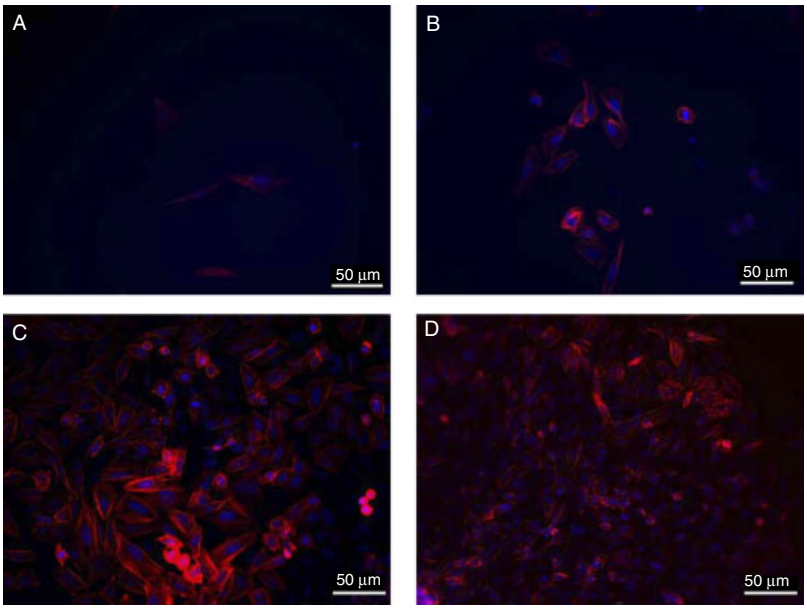

Figure 4. SaOs-2 proliferation on CHIT membranes after 7 days of culture (×20). A) Non-modified CHIT; B) CHIT with adsorbed Fn; C) CHIT with immobilized Fn; and D) TCPS. Scale bars correspond to 50 µm

and proliferation (Moursi *et al.*, 1997; Wu *et al.*, 1992). Herein, Fn was immobilized to enable the presentation of multiple cell binding sites. One of the important results of this study is the successful application of a simple method to overcome those limitations with great enhancement in cell–CHIT interactions.

3.2.2. Cell proliferation

To further analyse the bioactivity of these surfaces, cell proliferation was examined. Cells cultured on Fn-immobilized surfaces displayed a clear enhancement in cell proliferation after 7 days of culture when compared to that of Fn-adsorbed surfaces (Figure 4).

A surface with Fn covalently attached showed high proliferation during days 1–7, as shown by the MTS assay (Figure 5). Although both surfaces exhibit similar OD at the early culture stage, the number of cells decreased on CHIT surfaces with adsorbed Fn for the later time period. Although both modified surfaces had high initial adhesion efficiency, the cells did not proliferate over the 7 days of culture if Fn was just previously adsorbed. This cell response is in agreement with work already published (Biran *et al.*, 2001). This reduced proliferation over the culture experiments may be explained by Fn desorption or a loss in bioactivity when Fn is adsorbed. Various studies have been focused toward understanding the biochemical activity of proteins in the adsorbed state (Castner and Ratner, 2002) and the *in vitro* or *in vivo* biological response when in contact with biological fluids. Proteins selectively accumulate in the biomaterial surfaces from complex mixtures such as serum. We hypothesize that, during this period, the competitive adsorption of serum proteins induces desorption of pre-adsorbed Fn, followed by preferential adsorption of non-adhesive proteins to the surface (Curtis, 1984). Fn desorption tests were performed to validate this hypothesis.

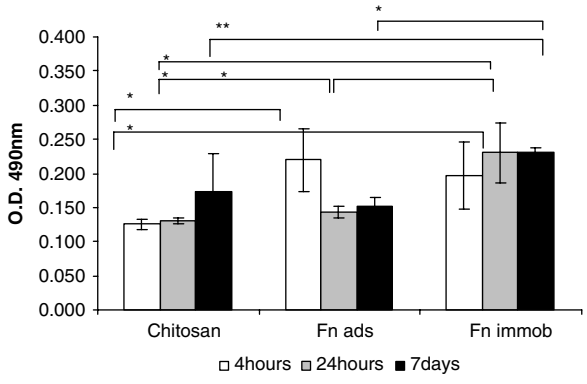

Figure 5. Cell viability and proliferation of human osteoblast like cells determined by MTS. Error bars represent means ± SD for n=3. Significant differences: *$p<0.01$; **$p<0.05$

3.2.3. Protein desorption studies

The resistance of immobilized and adsorbed Fn to desorption/exchange was assessed, aiming to further understand the results obtained in the cell studies. The presence of Fn on CHIT films after immersion in DMEM with 10% FBS for 24 h was assessed by fluorescence microscopy. Comparison of fluorescence signals induced by the labelled antibody revealed differences between the amounts of Fn on the surface (Figure 6).

After 24 h in culture medium, the staining intensity was higher on surfaces with immobilized Fn (Figure 6C) when compared with adsorbed Fn (Figure 6B). The images from fluorescence microscopy support the hypothesis of protein desorption/exchange. The results suggest that CHIT membranes with immobilized Fn resisted desorption better than those with adsorbed protein, enabling a

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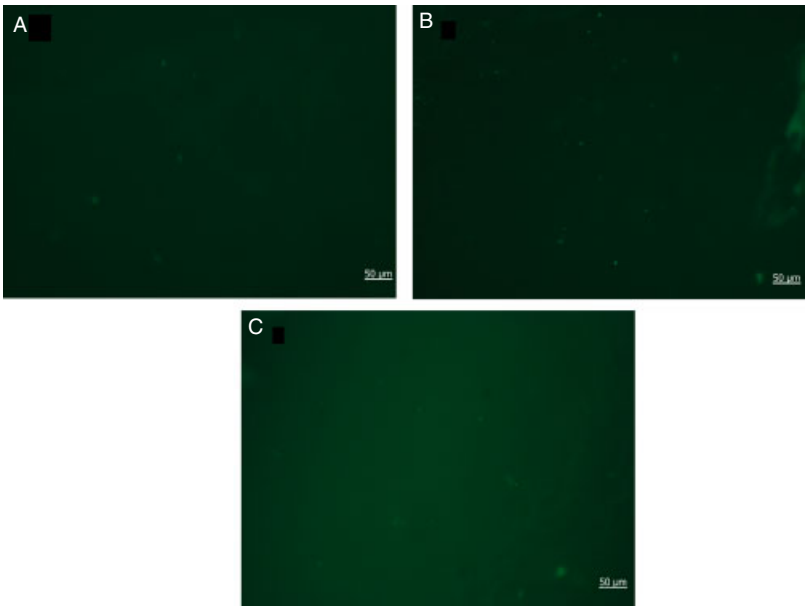

Figure 6. CHIT membranes fluorescently labelled for fibronectin (×20).: A) Non-modified CHIT; B) CHIT with adsorbed Fn; and C) CHIT with immobilized Fn, after 24h immersion in culture medium. Scale bars correspond to 50 μm

longer-term presence of the protein on the CHIT surfaces. These results could explain the superior results of cell proliferation on CHIT with chemically immobilized Fn.

4. Conclusions

In this study, we pin-pointed the importance of the covalent immobilization method of bioactive moieties, namely Fn, to enhance the rate of cell proliferation on CHIT surface. With a simple and reproducible approach, we were able to show that surfaces containing amino groups were modified by covalently grafting proteins that enhanced cell proliferation.

The results from these *in vitro* studies suggested that the presence of Fn strongly influences cell adhesion to CHIT surfaces. Even in the presence of serum in the medium but in absence of Fn, cells exhibited small adhesion ability. Although cell adhesion after 4 and 24 h do not reveal significant differences using covalent immobilization or the adsorption method, we have found that covalently immobilized Fn significantly improved proliferation when compared to surfaces with adsorbed protein or to non-modified membranes. These studies have demonstrated the ability to modulate the surface to promote cell adhesion and accelerate cell proliferation. Results from the protein desorption assay suggest that CHIT membranes with immobilized Fn resisted desorption better than those with adsorbed protein, enabling a longer-term presence of the protein on the CHIT surfaces. Using a low-cost and simple methodology, we were able to greatly increase the biological performance of amino containing surface materials. This approach could, in principle, be applied to the grafting of other biomolecules that possess a free carboxyl group.

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