

The Inhibitory Effect of an RGD-Human Chitin-Binding Domain Fusion Protein on the Adhesion of Fibroblasts to Reacetylated Chitosan Films

Vera Carvalho · Lucília Domingues · Miguel Gama

Published online: 2 August 2008
© Humana Press 2008

Abstract Biomaterials used for tissue engineering applications must provide a structural support for the tissue development and also actively interact with cells, promoting adhesion, proliferation, and differentiation. To achieve this goal, adhesion molecules may be used, such as the tripeptide Arg-Gly-Asp (RGD). A method based on the use of a carbohydrate-binding module, with affinity for chitin, was tested as an alternative approach to the chemical grafting of bioactive peptides. This approach would simultaneously allow the production of recombinant peptides (alternatively to peptide synthesis) and provide a simple way for the specific and strong adsorption of the peptides to the biomaterial.

A fusion recombinant protein, containing the RGD sequence fused to a human chitin-binding module (ChBM), was expressed in *E. coli*. The adhesion of fibroblasts to reacetylated chitosan (RC) films was the model system selected to analyze the properties of the obtained proteins. Thus, the evaluation of cell attachment and proliferation on polystyrene surfaces and reacetylated chitosan films, coated with the recombinant proteins, was performed using mouse embryo fibroblasts 3T3. The results show that the recombinant proteins affect negatively fibroblasts anchorage to the materials surface, inhibiting its adhesion and proliferation. We also conclude that this negative effect is fundamentally due to the human chitin-binding domain.

Keywords Human chitin-binding module (ChBM) · RGD · Fibroblasts · Cell adhesion · Reacetylated chitosan (RC) films

Introduction

Chitosan is a polycationic biopolymer obtained by the N-deacetylation of chitin [1]. This polymer exhibits a number of characteristics that makes it attractive for different applications, namely on the pharmaceutical industry [2–11]. Due to its biocompatibility, biodegradability, and potential for formulation as flakes, membranes, sponges, fibers, gels, or particles, it is considered as a very interesting polymer also for biomedical applications.

In the biomedical and pharmaceutical fields, chitosan has been used in the preparation of artificial skin, surgical sutures, contact lenses, blood dialysis membranes, and artificial blood vessels, as antitumor, blood anticoagulant, antigastritis, hemostatic, hypocholesterolemic, and antithrombogenic agents, in drug- and gene-delivery systems and in dental therapy [1, 4, 7–10]. It also stimulates the immune system of the host against viral and bacterial infections [11].

The control of the physical, chemical, and biochemical properties of an implant surface is one of the most important issues in the design of biomedical devices, since the first interaction between a foreign body (implant) and the biological environment occurs at the interface. Surfaces that mimic the natural extracellular matrix (ECM) are of great interest for tissue engineering and regenerative medicine [12, 13].

Several strategies may be employed to improve the cell adhesion, proliferation, and differentiation on biomaterials, such as the reduction of unspecific protein adsorption, or the immobilization of adhesion molecules to ensure

V. Carvalho · L. Domingues · M. Gama (✉)
IBB, Institute for Biotechnology and Bioengineering, Centre
of Biological Engineering, University of Minho,
Campus de Gualtar, 4710-057 Braga, Portugal
e-mail: fmgama@deb.uminho.pt

controlled interaction between cells and synthetic substrates [14–18]. Adhesion motifs are present in ECM molecules such as laminin, vitronectin, and fibronectin. These proteins regulate the adhesion, migration, and growth of cells by binding to integrin receptors located on the outer cellular membranes [19, 20].

The peptide Arg-Gly-Asp (RGD) was found to be the major functional amino acid sequence responsible for cellular adhesion. This sequence can be used to elicit specific cellular responses [21]. It has been extensively demonstrated that RGD sequence improves cell adhesion, spreading, and proliferation in different materials [21–25].

The immobilization of fibronectin or RGD to the surface of a material can be achieved by different techniques [21], but most of these include complex chemical reactions and crosslinking processes, with low yields and often not successful [21, 26, 27]. An alternative method was developed, through the creation of fusion proteins containing carbohydrate-binding modules, to immobilize RGD on polysaccharide surfaces [22, 23]. In this work, the RGD peptide was fused with a human chitin-binding module by recombinant DNA technology.

A protein with homology to fungal, bacterial, or plant chitinases has been identified in humans. This enzyme, later identified as a chitotriosidase, is expressed by macrophages and its activity is dramatically elevated in patients with Gaucher disease [28, 29]. The human chitin-binding module (ChBM) is comprised by the 72 C-terminal amino acids of the human chitinase [30].

In this study we examine: (1) the production of two active fusion proteins containing RGD and/or a human chitin-binding module (RGDChBM and ChBM, respectively); (2) the effect of ChBM and RGDChBM on cellular adhesion to polystyrene plate (selected as a model surface, where the recombinant proteins were allowed to adsorb); (3) the effect of the recombinant fusion proteins on fibroblasts cultures; and (4) the effect of the proteins on fibroblasts adhesion to reacylated chitosan films.

Materials and Methods

Reagents

All reagents used were of laboratory grade and purchased from Sigma-Aldrich (USA), unless stated otherwise.

Construction of the Expression Plasmids

The DNA encoding for the chitin-binding module of the human chitinase (ChBM) was synthesized by GenScript Corporation (USA) with optimized codons for bacterial expression.

The genic fusion RGDChBM was obtained by PCR using the DNA encoding template for the ChBM. Briefly, the RGDChBM sequence was amplified using the forward primer 5'-CTTCCATGGCCAGAGGTGATCCGGAAGTGGCC-3' (*NcoI* restriction site is underlined and RGD sequence is in bold) and the reverse primer 5'-CCACTCGAGGTTCCAGGTGCAGCATTG-3' (the *XhoI* restriction site is underlined). The PCR was performed as follows: preheating at 98°C for 30 s; 40 amplification cycles at 98°C for 10 s, 70°C for 30 s, and 72°C for 10 s; and a final elongation period of 10 min at 72°C, using Phusion DNA polymerase from Finnzymes (Finland) in a My Cycler™ Thermal Cycler (Bio-Rad, USA).

The PCR amplified product RGDChBM was digested with *NcoI* (Roche Diagnostics GmbH, Germany) and *XhoI* (Roche Diagnostics GmbH, Germany) and then cloned, using T4 DNA ligase (Promega, USA), into the *NcoI* and *XhoI* restriction sites of the expression vector pET25b(+) (Novagen, USA), yielding the recombinant expression vector pET25RGDChBM. The synthesized sequence encoding for the ChBM was also cloned in pET25b(+) vector using *NcoI* and *XhoI* restriction sites, yielding the pET25ChBM recombinant expression vector. The nucleotide sequences of the recombinant constructs were analyzed by dideoxy nucleotide sequencing to ensure the lack of errors.

Expression and Purification of the Recombinant Proteins

Escherichia coli strain Tuner(DE3) (Novagen, USA), transformed with the recombinant expression vectors, was grown at 37°C in minimal medium M9 supplemented with 100 µg/ml ampicillin to an O.D._{260 nm} of 0.4. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM. The cells were harvested after 16 h incubation at 30°C by centrifugation (10 min at 10000g) and the cell pellet was resuspended in buffer A (20 mM Tris-HCl, 500 mM NaCl, pH 7.4 and 1 mM phenylmethylsulfonyl fluoride). The cells were disrupted by sonication (Branson Sonifier; duty cycle 50%; output 5) on ice and the insoluble debris removed by centrifugation (30 min, 10000g, 4°C). Purification was made by immobilized metal ion affinity chromatography, using a HiTrap™ Chelating HP 5 ml column (GE Healthcare, UK), following the manufacturer instructions. For that purpose, imidazole was added to the soluble fraction of the lysate, to a final concentration of 40 mM, and the pH was adjusted to 7.4. After purification, proteins were dialyzed against buffer A, filtered through a 0.22 µm millipore membrane (to sterilize the proteins), and stored and conserved at –20°C, until use. Concentration of the two proteins was estimated from the absorbance at 280 nm ($\epsilon_{\text{ChBM}} = 10345 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{\text{RGDChBM}} = 10345 \text{ M}^{-1} \text{ cm}^{-1}$).

Proteins were analyzed by SDS-PAGE, using 15% (w/v) acrylamide gels. Staining was carried out with 0.05% (w/v) Coomassie brilliant blue.

ChBM and RGDChBM Affinity Assays

To evaluate the carbohydrate-binding activity of ChBM and RGDChBM, solid-phase-binding assays were done using chitin or chitosan. These affinity assays were performed mixing by 0.5 ml purified protein (0.5 mg/ml) with 100 mg of chitin or chitosan, followed by 2 h incubation at 4°C. Then, the mixture was centrifuged (10000g, 10 min, 4°C), the proteins in the supernatant (not bound to the polysaccharide) were collected, and the proteins bound unspecifically were washed out with buffer A. The elution of the proteins bound specifically to chitin or chitosan was made using buffer A with 1% sodium dodecyl sulfate (SDS). Proteins that did not bind to chitin or chitosan were analyzed by 15% (w/v) SDS-PAGE acrylamide gel stained with 0.05% (w/v) Coomassie brilliant blue.

Cell Cultivation and Viability Evaluation

Mouse embryo fibroblasts 3T3 (ATCC CCL-164) were grown in Dulbecco's modified Eagle's media (Sigma-Aldrich, USA) supplemented with 10% newborn calf serum (Invitrogen, UK) and 1 µg/ml penicillin/streptavidin (DMEM complete medium) at 37°C in a 95% humidified air containing 5% CO₂. At confluency, 3T3 fibroblasts were harvested with 0.05% (w/v) trypsin-EDTA (Sigma-Aldrich, USA) and subcultivated in the same medium.

The viability of the 3T3 fibroblast cells was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich, USA). The MTT assay accurately measures the activity of living cells via mitochondrial dehydrogenase activity. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazon crystals that can be dissolved in DMSO, resulting in a purple solution that is spectrophotometrically measured. The increase in cell number results in an increase in absorbance. After 5 h of MTT incubation with cells, the light absorbance at 570 nm was measured by an ELISA 96-well plate reader.

Effect of the Recombinant Fusion Proteins on the Adhesion of Fibroblasts on Polystyrene Plates

The recombinant proteins were added to the wells (0.05 µg/well) of a 96-well polystyrene plate (Orange Scientific, Belgium) and left overnight for adsorption to the polystyrene, at 4°C. Then, the unbound proteins were collected to estimate, by absorbance at 280 nm, the amount of proteins bound to the polystyrene wells. Finally, the

wells were washed twice with PBS sterile solution. Fibroblasts were seeded in DMEM medium, with or without newborn calf serum, at a density of 2.5×10^3 cells/well or 5×10^3 cells/well, respectively, and incubated at 37°C, 5% CO₂. After 1 h incubation, the wells were washed twice with sterile PBS solution to remove free cells and DMEM complete medium was added.

Light microscope observations and MTT assay were carried out at 1, 5, 24, and 48 h after the cells seeding and the results compared to assays carried out with cells adherent to wells without adsorbed proteins.

To compare the effect of ChBM and RGDChBM on fibroblast adhesion to polystyrene wells to the effect of other RGD fused recombinant proteins, we tested four more recombinant proteins developed in our laboratory. Andrade et al. [31] produced a fusion protein with RGD and a cellulose-binding module (RGDCBM) and Moreira et al. [32] created a fusion protein with RGD and a starch-binding module (RGDSBM). The three carbohydrate-binding modules (ChBM, CBM, and SBM) and the three RGD fusion proteins (RGDChBM, RGDCBM, and RGDSBM), were tested as described above.

Cytocompatibility Tests

To evaluate the direct cytotoxicity of the recombinant proteins towards fibroblasts, 4×10^3 cells/well were seeded in a 96-well polystyrene plate (Orange Scientific, Belgium) and incubated at 37°C, 5% CO₂. After 5 h, the wells were washed twice with sterile PBS solution, to remove floating cells, and the medium was replaced. Then, the MTT test was carried out to quantify the cells adherent to the wells. At that time, 0.02 ml sterilized protein solution (0.5 mg/ml) or buffer A was added to the wells and the cells incubated 37°C, 5% CO₂. Cell proliferation and viability was followed by regular light microscope observations and by the MTT test. The results were compared to a control prepared with the same cell culture without the addition of proteins.

Live and Dead Assay

After the first hour of cell adhesion, a test was performed to verify whether the cells were alive or dead. The LIVE/DEAD[®] Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, UK) was used to determine cell viability. This kit provides two-color fluorescence cell viability assay, based on the simultaneous determination of live and dead cells with two probes that measure intracellular esterase activity and plasma membrane integrity. In a few words, the proteins (1 mg/well) were let to adsorb to the wells of a 6-well polystyrene plate (Orange Scientific, Belgium) overnight at 4°C. After the unbound proteins were

collected and the wells washed, the fibroblasts were seeded, 2×10^4 cells/well, in DMEM medium without serum and incubated at 37°C, 5% CO₂, for 1 h. Then, 100 µl of a solution of 2 µM calcein AM and 4 µM ethidium homodimer-1, in sterile PBS, were added to the wells, incubated for 30–45 min at 37°C, 5% CO₂, and visualized in a fluorescence microscope with or without the culture medium, to see the condition of the cells attached to the polystyrene wells.

Effect of the Recombinant Proteins on the Adhesion of Fibroblasts on Reacetylated Chitosan Films

Reacetylated chitosan (RC) films were obtained as follows: briefly, a 1% chitosan (from crab shells, purchased from Sigma-Aldrich (USA)) solution in 0.2 mM acetic acid was made. The resulting solution was autoclaved (20 min, 121°C, 1 atm) and 50 µl was added to each well of a 96-well polystyrene plate (Orange Scientific, Belgium). The plate was air-dried at room temperature. To obtain reacetylated films, 10 µl of acetic anhydride was added and further air-dried. The wells were then washed successively with PBS sterile solution.

The adhesion of fibroblasts on RC films was accessed as described previously for the polystyrene plates. Succinctly, proteins were let adsorbing to plates coated with RC films overnight, at 4°C. Then, the unbound proteins were collected and the wells washed twice with PBS sterile solution. Fibroblasts, 5×10^3 cells/well, were seeded in DMEM without serum. After 1 h incubation at 37°C, 5% CO₂, non-adherent cells were washed and DMEM complete medium was added.

Light microscope observations and MTT assay were carried out at 1, 5, 24, and 48 h after the cells were seeded and the results compared to cells adherent to control wells (wells without proteins and wells coated with RC films without proteins).

Results

Production of the Recombinant Proteins

The sequence encoding for the human chitin-binding module was used to obtain the genic fusion encoding for RGDChBM, by PCR. The two nucleotide sequences, ChBM and RGDChBM, were inserted in the *NcoI* and *XhoI* restriction sites of the expression vector pET25b(+). The pET25b(+) expression vector has a T7lac promoter and expresses a C-terminal hexa histidine tag fused with the recombinant proteins, allowing purification by immobilized metal ion affinity chromatography. It also contains a pelB leader sequence that targets the recombinant proteins

A
 Met Ala Pro Glu Leu Glu Val Pro Lys Pro Gly Gln Pro Ser Glu Pro Glu His Gly
Pro Ser Pro Gly Gln Asp Thr Phe Cys Gln Gly Lys Ala Asp Gly Leu Tyr Pro Asn
Pro Arg Glu Arg Glu Arg Ser Ser Phe Tyr Ser Cys Ala Ala Gly Arg Leu Phe Gln
Gln Ser Cys Pro Thr Gly Leu Val Phe Ser Asn Ser Cys Lys Cys Cys Thr Trp Asn
 Leu Glu Ile Lys Arg Ala Ser Gln Pro Glu Leu Ala Pro Glu Asp Pro Glu Asp Val
 Glu His His His His His His

B
 Met Ala Arg Gly Asp Pro Glu Leu Glu Val Pro Lys Pro Gly Gln Pro Ser Glu Pro
Glu His Gly Pro Ser Pro Gly Gln Asp Thr Phe Cys Gln Gly Lys Ala Asp Gly Leu
Tyr Pro Asn Pro Arg Glu Arg Glu Arg Ser Ser Phe Tyr Ser Cys Ala Ala Gly Arg
Leu Phe Gln Gln Ser Cys Pro Thr Gly Leu Val Phe Ser Asn Ser Cys Lys Cys Cys
Thr Trp Asn Leu Glu Ile Lys Arg Ala Ser Gln Pro Glu Leu Ala Pro Glu Asp Pro
 Glu Asp Val Glu His His His His His His

Fig. 1 Amino acid sequence and relevant characteristics of (a) ChBM and (b) RGDChBM. Underlined represent the 72 amino acids comprising the human chitin-binding module. Bold sequences represent the 6 histidine tag used for purification. Underlined and in bold amino acids represent the RGD sequence

produced to the periplasmic space. This pelB leader sequence is usually cleaved after the proteins are exported to the periplasmic space. Figure 1 shows the amino acid sequences and relevant features of the two recombinant proteins produced.

As shown in Fig. 2a, the two proteins were successfully expressed in *E. coli* Tuner(DE3), in the soluble fraction (Fig. 2a—lane 1), and almost completely purified from the *E. coli* contaminant proteins (Fig. 2a—lane 3). The bands seen in lane 3 (Fig. 2a) correspond to the processed fusion protein RGDChBM (where the pelB leader sequence was cleaved) and to the unprocessed protein (where the pelB leader sequence was not cleaved), with a higher molecular weight. About 0.5 mg of the recombinant proteins was obtained from 1 l of *E. coli* cultured in M9 minimal medium.

Figure 2b shows the results of the affinity assays analyzed by SDS-PAGE. As it can be seen, there is almost no binding of the proteins to chitosan (Fig. 2b—lane 3), while the recombinant fusion proteins binds strongly to chitin (Fig. 2b—lane 2), indicating that the human chitin-binding module is functional, maintaining its affinity and specificity towards chitin.

Effect of the Recombinant Proteins on the Adhesion of Fibroblasts on Polystyrene Plates

Proteins were left adsorbing to polystyrene wells overnight, at 4°C. The binding module used has specific

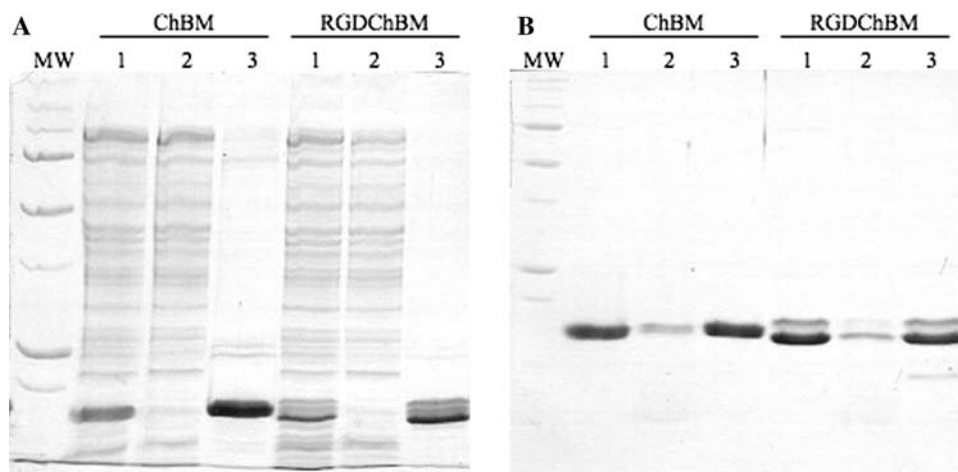


Fig. 2 (a) Protein expression and purification analysis by SDS-PAGE. MW—Molecular weight marker (250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, 20 kDa, 15 kDa) from Bio-Rad (USA); 1—Protein soluble lysate; 2—Flowthrough fraction from HisTrap™ HP 5 ml column; 3—Protein eluted with 300 mM Imidazole. (b) Binding activity and carbohydrate binding specificity of the recombinant proteins analysis by SDS-PAGE. MW—

Molecular weight marker (250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, 20 kDa, 15 kDa) from Bio-Rad (USA); 1—Soluble protein before carbohydrate adsorption; 2—Protein fraction after adsorption to chitin; 3—Protein fraction after adsorption to chitosan. The samples analyzed on lanes 2 and 3 are the supernatants (with non-adsorbed protein) obtained in the adsorption assays carried out, respectively, with chitin and chitosan

affinity for chitin, but about 15% of the recombinant proteins adsorbed (non-specifically) to the polystyrene wells. Although the final goal of the work is the binding of RGD peptides to partially reacylated chitosan-made materials, polystyrene was used as a model material to analyze the interaction of the proteins with fibroblasts (selected as model animal cells). Although realizing there are major differences in the two assays (ChBMs adsorb strongly, specifically, and with a specific orientation to chitin, as opposed to what happens in polystyrene), cell culture plates offer a convenient and simple way to observe the adsorption and morphology of cells. In Fig. 3a, we can see light microscopy photographs of fibroblasts 1, 5, 24, and 48 h after the cells were seeded, in DMEM complete medium. In Fig. 3b and c, the MTT absorbance results are shown for the assays with (Fig. 3b) or without (Fig. 3c) serum in the culture medium.

As it can be seen, the fibroblasts culture in control wells (without bound proteins or with buffer A), with or without serum, behaves normally: the cells adhere 1 h after seeding and proliferate, reaching confluency after 48 h. In the wells coated with the recombinant proteins, the initial value of attached cells is very low as compared to the control and cells do not proliferate (as evaluated by the MTT assay), especially in the medium without serum (Fig. 3c) where the absorbance values are lower. We can also see that the few adhered cells are still round, indicating a delay in adhesion and spreading.

In Fig. 4 we can see light microscopy photographs of fibroblasts seeded, in complete medium, on wells coated

with the six different recombinant proteins ChBM, CBM, SBM, RGDCBM, RGDCBM, and RGDSBM. The images clearly show that RGDSBM and RGDCBM improve cell adhesion and, on the contrary, the protein RGDCBM inhibits cell adhesion. Concerning the carbohydrate-binding modules (with no RGD bound), we can see that SBM and CBM neither improve nor hinder cell adhesion, taking as reference the effect of polystyrene plates on the fibroblasts adhesion. On the contrary, the ChBM does not allow the attachment of the cells.

Cytocompatibility Tests

Considering the poor adhesion of fibroblasts on the polystyrene surface, we decided to analyze the cytotoxicity of the recombinant proteins under study. Figure 5a shows light microscopy photographs of fibroblasts 24 h, 48 h, 96 h and 6 days after the proteins were added to the cell cultures. In Fig. 5b, the corresponding MTT assay results are presented.

As it can be observed, in the control wells, fibroblasts proliferated normally and neither cell death nor growth disorders were noticed; the cells have the typical fibroblast morphology, they are flattened and spread. After 48 h, the cultures reached confluency, as expected.

In the wells where the proteins were added, the cells appear morphologically normal but there is a progressive decrease in the cell number, as compared to the controls. In the first 72 h there is an increase of cell density (as

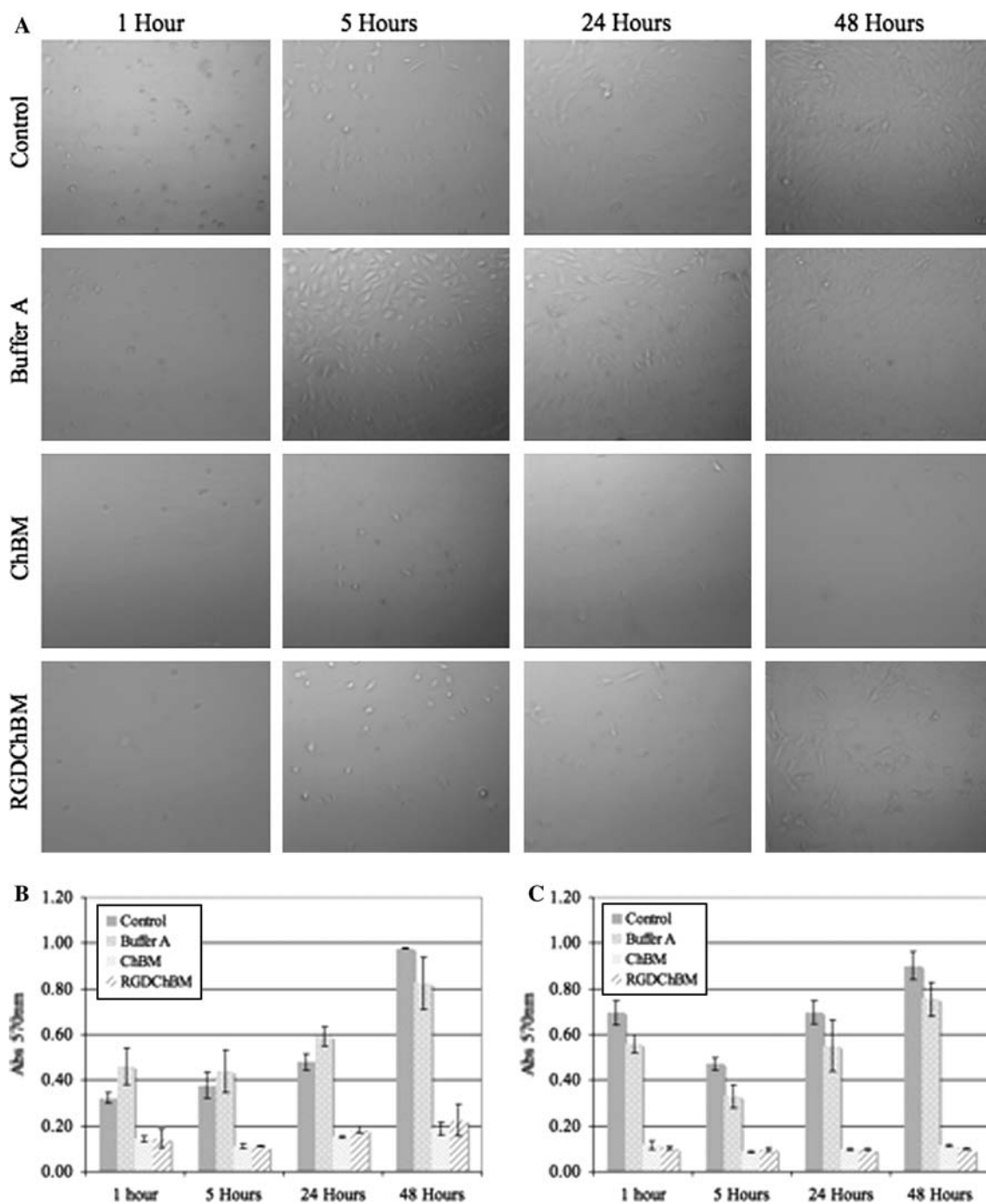


Fig. 3 Effect of the recombinant proteins coated on polystyrene wells. (a) Light microscope photographs 1 h, 5 h, 24 h, and 48 h after cells were seeded, in DMEM complete medium, on polystyrene wells coated or not with the two recombinant proteins, ChBM, RGDChBM

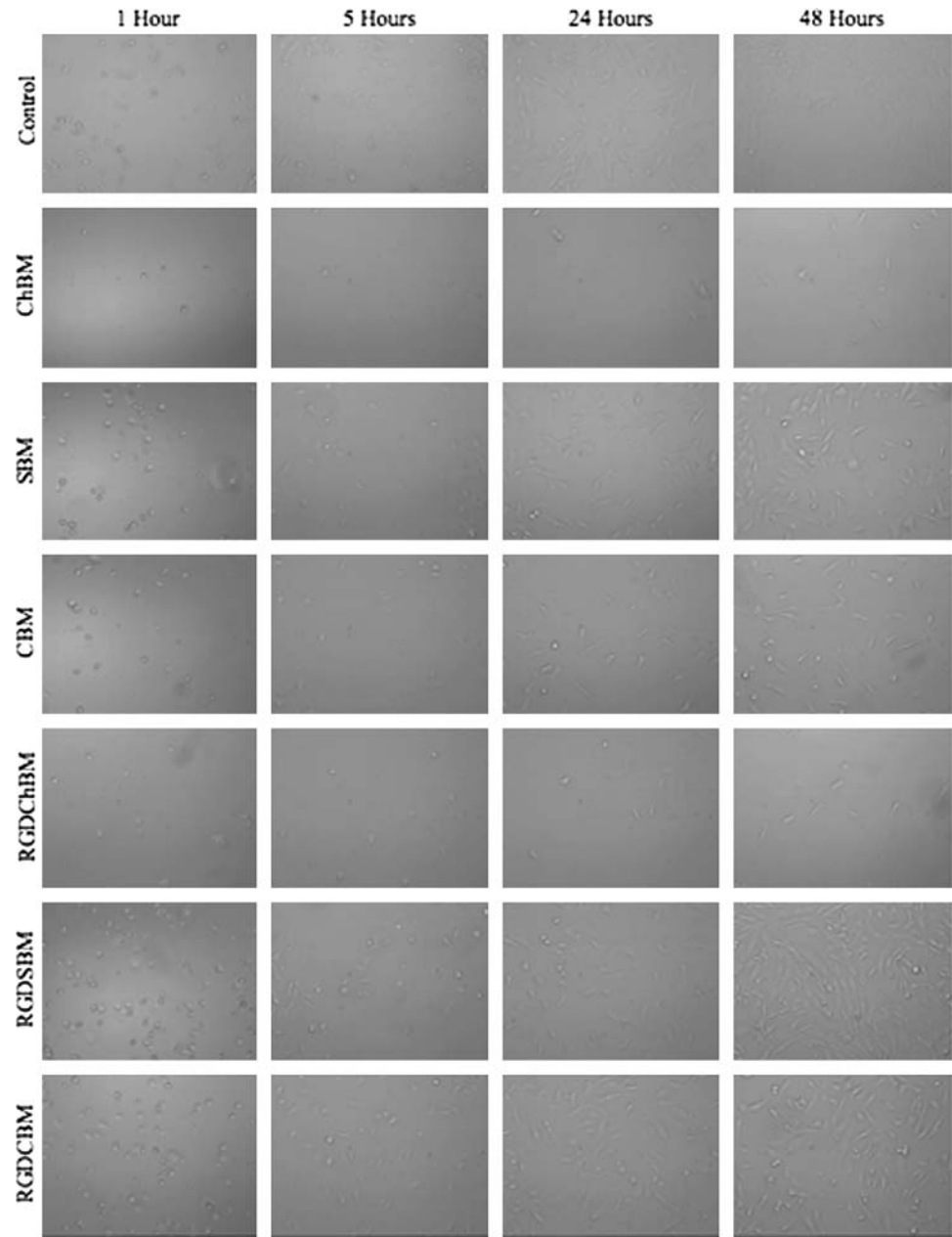
or with buffer A. MTT assay results after cell seeding, in (b) DMEM complete medium or in (c) DMEM without serum, on wells coated or not with ChBM, RGDChBM or buffer A

measured by the MTT assay) almost comparable to the control, but, after 6 days, the number of cells is considerably lower than on the control. Although this effect is observed with both recombinant proteins, it seems that RGDChBM has a less negative effect on the cells.

Live and Dead Assay

To estimate the viability of the cells during the initial stage of the interaction with the proteins (first hour of adhesion), we performed the live and dead assay.

Fig. 4 Light microscope photographs 1 h, 5 h, 24 h, and 48 h after cells were seeded, in DMEM complete medium, on polystyrene wells coated with the six recombinant proteins, ChBM, SBM, CBM, RGDChBM, RGDSBM, and RGDCBM



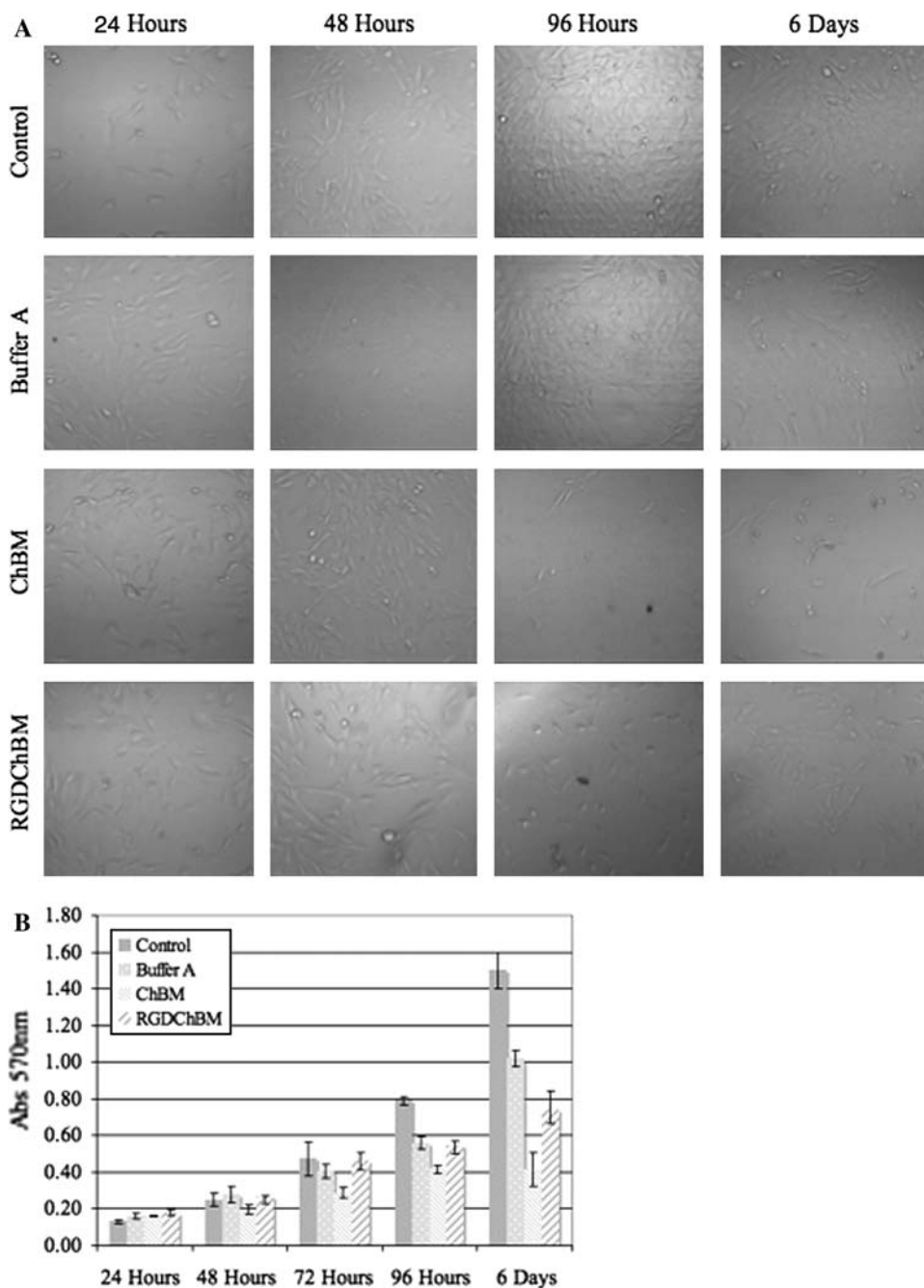
In Fig. 6, bright field and fluorescence images of the cells, in the presence and absence of culture medium, are shown. The presence of the culture medium is relevant to distinguish between cells in suspension and cells actually adhered to the wells. It can be seen that, in control wells (not coated with proteins) there is almost no alteration in the number of cells before and after the culture medium was discarded. Thus, the cells started adhering and spreading, and the number of living cells (stained in green) is far superior to the number of dead cells (stained in red). In the wells coated with ChBM and RGDChBM, the situation is rather different: a large number of round, not attached, cells can be observed; when the culture medium

is discarded, most of these cells are also removed. It can also be noticed that some of the cells that remain adherent to the coated wells turned red, which meant that they were dying.

Effect of the Recombinant Fusion Proteins on the Adhesion of Fibroblasts on Reacetylated Chitosan Films

To evaluate the effects of the recombinant proteins on the adhesion of fibroblasts to reacetylated chitosan (RC) films, proteins were left adsorbing overnight at 4°C. Polystyrene wells and RC films without adhered proteins were used as

Fig. 5 Effect of the recombinant proteins on cultured fibroblasts. **(a)** Light microscope photographs 24 h, 48 h, 96 h, and 6 days after the recombinant proteins were added. **(b)** MTT absorbance results



controls in these assays. Figure 7 presents the MTT absorbance values.

In the first 24 h, similar absorbance values were obtained in the different wells. After longer incubation times, only the cells cultivated on polystyrene wells, without recombinant proteins, proliferated significantly. In the wells coated with RC films, either with or without proteins, there is no significant increase in cell number. The few attached cells to these wells were always round, meaning there were not strongly attached. Thus, it seems that the recombinant fusion protein, RGDCBM does not improve fibroblast adhesion to the RC films.

Discussion

The sequence encoding for the human chitin-binding module was used to obtain a fusion protein, RGDCBM. The proteins ChBM and RGDCBM were successfully expressed in *E. coli* Tuner(DE3) (Fig. 2a), fused at the C-terminal to a hexa-histidine tag that allowed the purification using a nickel column (Fig. 2b).

The affinity assays performed (Fig. 3), showed that the human chitin-binding modules present in the recombinant proteins were active, binding specifically to chitin. We also found that the recombinant proteins were only eluted from

Fig. 6 Bright field and fluorescence photographs of fibroblasts with and without culture medium stained with LIVE/DEAD[®] Viability/Cytotoxicity Kit for mammalian cells. Live cells are stained in green and dead cells are stained in red

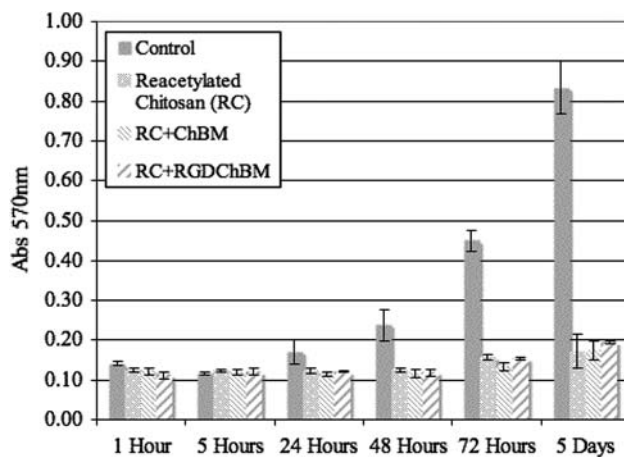
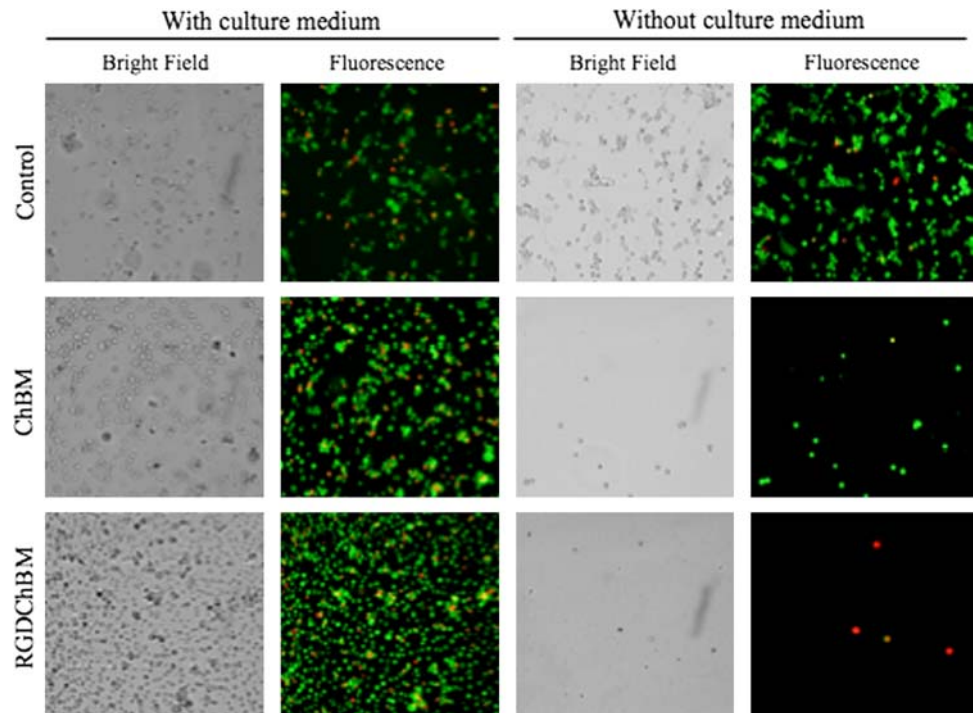


Fig. 7 MTT assay results after fibroblast seeding, in DMEM without serum, on wells coated with chitosan films, reacetylated chitosan (RC) films, RC films with linked ChBM, and RC with linked RGDChBM

chitin using harsh conditions, such as 1% SDS (data not shown) [33], indicating a strong interaction between the fusion proteins and chitin, as already stated by Tjoelker et al. [30]. This ability to bind strongly and specifically to chitin can also be used to successfully purify the recombinant proteins produced. Some authors have already shown that the chitin-binding module can be used as an affinity tag to purify fusion proteins expressed in different microorganisms [34–36]. Even though 1% SDS was used to elute the proteins from chitin, the specificity and ability of the recovered protein to bind to chitin was maintained (data not shown) [33].

Cell-surface interaction is very important for cell adhesion and growth, both microtopography and the surface chemistry influencing the cell-surface interactions. In a serum-containing medium, it is likely that the surface become covered with adsorbed proteins, with adhesion molecules, enhancing cell attachment.

By coating wells with the recombinant fusion protein we were expecting to provide a supply of adhesion molecules, in this case RGD, to the growing fibroblast culture in DMEM without serum, allowing its normal adhesion or even improving it. Other authors were able to improve the adhesion of cells to different biomedical materials, by coating them with fusion proteins containing carbohydrate-binding modules and RGD sequences. In 1995, Wierzbza et al. [37] described a fusion protein between a RGD and a cellulose-binding domain (CBD) from the *Cellulomonas fimi* endoglucanase A (CenA) that promoted the attachment of green monkey Vero cell to polystyrene and cellulose acetate. Wang et al. [23], used a self-designed bifunctional RGD-containing fusion protein (BFP) made of two GRGDS sequences separately fused to the C-terminus and N-terminus of the *Trichoderma koningii* cellobiohydrolase I gene cellulose-binding domain. This BFP, grafted on the Petri dish, improved human keratinocytes and dermal fibroblasts adhesion.

In our laboratory, two different fusion proteins containing carbohydrate-binding modules and RGD sequences were tested. Andrade et al. [31] tested a recombinant fusion protein (RGDCBM) between RGD and cellulose-binding module from the cellulosome of the bacteria

Clostridium termocellum and Moreira et al [32] developed a fusion protein, containing a C-terminal starch-binding module (SBM), from an α -amylase from *Bacillus* sp. TS-23 and an N-terminal RGD sequence (RGDSBM). Comparing to the control wells, the images obtained show (Fig. 4) that CBM and SBM have no significant effect on fibroblast adhesion; ChBM, on the other hand, seems to inhibit cell adhesion. We can also notice that RGDCBM and RGDSBM really improve cell adhesion and proliferation, while RGDChBM has the contrary effect. In fact, as can be seen in Figs. 3, 4, and 6, it seems that the recombinant proteins, ChBM and RGDChBM inhibit cellular adhesion to the wells, regardless of the presence of RGD sequence.

Chitosan is a biocompatible polymer. It was already shown that chitosan-based materials do not elicit allergic reactions after implantation, injection, or topical application in the human body. Chitosan is commonly obtained by the N-deacetylation of chitin. The degree of acetylation (DA) of chitosan represents the proportion of N-acetyl-D-glucosamine units relatively to the total number of units. This is a structural parameter that influences several physicochemical and biological properties. DA exerts influence in cell adhesion and proliferation, but does not change the cytocompatibility of chitosan [38]. Some authors have already studied the influence of chitosan DA on human fibroblast [39], keratinocytes [38], and chick dorsal root ganglion (DRG) neurons [40]. These authors conclude that, for some cell lines, cell viability and adhesion decrease as DA increases. Chatelet et al. [38] also shows that, whatever their DA, fibroblasts do not proliferate in chitosan, because they adhere very strongly to the material, interacting in a way that would inhibit their growth.

It is predictable that fibroblasts would adhere poorly to RC films, as our results also shows. In Fig. 6 it is visible that the number of cells adhered to RC films is very low and that this number does not increase over time. By using proteins that specifically and strongly bind to chitin we were aiming to improve the fibroblast adhesion and proliferation in RC films. ChBM could be used to modify chitosan with different DA, addressing fused bioactive peptides, such as RGD (tested in this work), to the surface of the biomaterial. Stable binding of RGD peptides to a surface is essential to promote strong cell adhesion that is fundamental for fibroblast growth. It is already known that simple adsorption can lead to poor cell attachment. However, RGDChBM do not seem to improve significantly cell adhesion to the RC films. In RC films with and without proteins, the cells (photographs not shown) show comparable morphology to the cells adhered to polystyrene wells coated with the recombinant proteins (Figs. 3 and 4): they are very few, round, and do not proliferate. It is not clear why ChBM, a chitin-binding module present in the human chitinase whose cDNA is the sixth transcript found in

macrophages [30], exhibits this surprising ability to inhibit fibroblast adhesion. Given its pI of about 6, its small size (72 amino acids), and amino acid composition, it would not be expected that ChBM had this kind of effect on the animal cells used. The reason for this effect is not clear at this stage.

Conclusions

Two recombinant proteins, containing a human chitin-binding module, were successfully expressed soluble and active in *E. coli*. These proteins were purified and used to test its effect on fibroblast adhesion. The recombinant ChBM affect negatively the fibroblasts adhesion. In the presence of ChBM and RGDChBM cells do not attach to surface of the biomaterials or seem to stop growing. It was already demonstrated that RGD sequences fused with carbohydrate-binding modules improve fibroblast adhesion to biomaterials, so it looks like, the chitin-binding module, fused or not with RGD peptide, interferes with the anchorage of the cells, fundamental to cell survival, cell division, and proliferation.

Acknowledgments Vera Carvalho was supported by the grant SFRH/BD/27359/2006 from Fundação para a Ciência e Tecnologia, Portugal. This study was supported by the Portuguese grant POCTI/BIO/45356/2002: “FUNCARB-FUNCionalização de materiais biomédicos usando um domínio de ligação a CARBohidratos”.

References

1. Tsigos, I., Martinou, A., Kafetzopoulos, D., & Bouriotis, V. (2000). Chitin deacetylases: New, versatile tools in biotechnology. *Trends in Biotechnology*, 18, 305–312. doi:10.1016/S0167-7799(00)01462-1.
2. Ruiz-Herrera, J. (1978) In: R. A. Muzzarelli, & E. R. Pariser (eds.), Proceedings of the International Conference on Chitin Chitosan (pp. 11–21). MIT Sea Grant Report 78–87, Sea Grant Information Center, MIT Sea Grant Program, Boston.
3. Hirano, S. (1996). Chitin biotechnology applications. *Annual Review Biotechnology*, 2, 237–258. doi:10.1016/S1387-2656(08)70012-7.
4. Dodane, V., & Vilivalam, V. (1998). Pharmaceutical applications of chitosan. *Pharmaceutical Science & Technology Today*, 1, 246–253. doi:10.1016/S1461-5347(98)00059-5.
5. Shahidi, F., Arachchi, J. K. V., & Jeon, Y. (1999). Food applications of chitin and chitosan. *Trends in Food Science & Technology*, 10, 37–51. doi:10.1016/S0924-2244(99)00017-5.
6. Ilum, L., Farraj, N. F., & Davis, S. S. (1994). Chitosan as a novel nasal delivery system for peptide drugs. *Pharmaceutical Research*, 11, 1186–1189. doi:10.1023/A:1018901302450.
7. Felt, O., Buri, P., & Gurny, R. (1998). Chitosan: A unique polysaccharide for drug delivery. *Drug Development and Industrial Pharmacy*, 24, 979–993. doi:10.3109/03639049809089942.
8. Madihally, S. V., & Matthew, H. W. T. (1999). Porous chitosan scaffolds for tissue engineering. *Biomaterials*, 20, 1133–1142. doi:10.1016/S0142-9612(99)00011-3.

9. Shigemasa, Y., & Minami, S. (1995). Applications of chitin and chitosan for biomaterials. *Biotechnology & Genetic Engineering Reviews*, *13*, 383–420.
10. Koide, S. S. (1998). Chitin-chitosan: Properties, benefits and risks. *Nutrition Research*, *18*, 1091–1101. doi:10.1016/S0271-5317(98)00091-8.
11. Peluso, G., Petillo, O., Ranieri, M., Santin, M., Ambrosio, L., Calabró, D., et al. (1994). Chitosan-mediated stimulation of macrophage function. *Biomaterials*, *15*, 1215–1220. doi:10.1016/0142-9612(94)90272-0.
12. Stevens, M. M., & George, J. H. (2005). Exploring and engineering the cell surface interface. *Science*, *310*, 1135–1138. doi:10.1126/science.1106587.
13. Lutolf, M. P., & Hubbell, J. A. (2005). Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nature Biotechnology*, *23*, 47–55. doi:10.1038/nbt1055.
14. Sakiyama-Elbert, S. E., & Hubbell, J. A. (2001). Functional biomaterials: Design of novel biomaterials. *Annual Review of Materials Research*, *31*, 183–201. doi:10.1146/annurev.matsci.31.1.183.
15. Hubbell, J. A. (1999). Bioactive biomaterials. *Current Opinion in Biotechnology*, *10*, 123–129. doi:10.1016/S0958-1669(99)80021-4.
16. Elbert, D. L., & Hubbell, J. A. (1996). Surface treatments of polymers for biocompatibility. *Annual Review of Materials Science*, *26*, 365–394.
17. Hubbell, J. A. (1995). Biomaterials in tissue engineering. *Bio/Technology*, *13*, 565–576. doi:10.1038/nbt0695-565.
18. Ikada, Y. (1994). Surface modification of polymers for medical applications. *Biomaterials*, *15*, 725–736. doi:10.1016/0142-9612(94)90025-6.
19. Plow, E. F., Haas, T. A., Zhang, L., Loftus, J., & Smith, J. W. (2000). Ligand binding to integrins. *The Journal of Biological Chemistry*, *275*, 21785–21788. doi:10.1074/jbc.R000003200.
20. van der Flier, A., & Sonnenberg, A. (2001). Function and interactions of integrins. *Cell and Tissue Research*, *305*, 285–298. doi:10.1007/s004410100417.
21. Hersel, U., Dahmen, C., & Kessler, H. (2003). RGD modified polymers: Biomaterials for stimulated cell adhesion and beyond. *Biomaterials*, *24*, 4385–4415. doi:10.1016/S0142-9612(03)00343-0.
22. Hsu, S. H., Chu, W. P., Lin, Y. S., Chiang, Y. L., Chen, D. C., & Tsai, C. L. (2004). The effect of an RGD-containing fusion protein CBD-RGD in promoting cellular adhesion. *Journal of Biotechnology*, *111*, 143–154. doi:10.1016/j.jbiotec.2004.03.014.
23. Wang, T. W., Wu, H. C., Huang, Y. C., Sun, J. S., & Lin, F. H. (2006). The effect of self-designed bifunctional RGD-containing fusion protein on the behavior of human keratinocytes and dermal fibroblasts. *Journal of Biomedical Materials Research. Part B: Applied Biomaterials*, *79B*, 379–387. doi:10.1002/jbm.b.30552.
24. Yang, F., Williams, C. G., Wang, D., Lee, H., Manson, P. N., & Elisseff, J. (2005). The effect of incorporating RGD adhesive peptide in polyethylene glycol diacrylate hydrogel on osteogenesis of bone marrow stromal cells. *Biomaterials*, *26*, 5991–5998. doi:10.1016/j.biomaterials.2005.03.018.
25. Lévesque, S. G., & Shoichet, M. S. (2006). Synthesis of cell-adhesive dextran hydrogels and macroporous scaffolds. *Biomaterials*, *27*, 5277–5285. doi:10.1016/j.biomaterials.2006.06.004.
26. Ugarova, T. P., Zamarron, C., Veklich, Y., Bowditch, R. D., Ginsberg, M. H., Weisel, J. W., et al. (1995). Conformational transitions in the cell binding domain of fibronectin. *Biochemistry*, *34*, 4457–4466. doi:10.1021/bi00013a039.
27. Chiba, M., Malik, S. W., & Specks, U. (1996). Microtiter plate immunoassay for the evaluation of platelet adhesion to fibronectin. *Journal of Immunological Methods*, *191*, 55–63. doi:10.1016/0022-1759(96)00021-X.
28. Renkema, G. H., Boot, R. G., Muijsers, A. O., Donker-Koopman, W. E., & Aerts, J. M. F. G. (1995). Purification and characterization of human chitotriosidase, a novel member of the chitinase family of proteins. *The Journal of Biological Chemistry*, *270*(5), 2198–2202. doi:10.1074/jbc.270.5.2198.
29. Boot, R. G., Renkema, G. H., Strijland, A., van Zonneveld, A. J., & Aerts, J. M. F. G. (1995). The human chitotriosidase gene nature of inherited enzyme deficiency. *The Journal of Biological Chemistry*, *270*, 26252–26256. doi:10.1074/jbc.270.5.2198.
30. Tjoelker, L. W., Gosting, L., Frey, S., Hunter, C. L., Trong, H. L., Steiner, B., et al. (2000). Structural and functional definition of the human chitinase chitin-binding domain. *The Journal of Biological Chemistry*, *275*, 514–520. doi:10.1074/jbc.275.1.514.
31. Andrade, F. K., Moreira, S. M. G., Domingues, L., & Gama, F. M. P. (2008) Improving the affinity of fibroblasts for bacterial cellulose using carbohydrate-binding modules fused to RGD. *Journal of Biomedical Materials Research: Part A*.
32. Moreira, S., Andrade, F., Domingues, L., & Gama, M. (2008) Development of a strategy to functionalize dextrin-based hydrogel for animal cell cultures. Submitted.
33. Carvalho, V., Gama, M., & Domingues, L. (2007). Cloning, expression and characterization of a chitin-binding domain. *Australian Journal of Biotechnology*, *131S*, S254–S262.
34. Chong, S., Mersha, F. B., Comb, D. G., Scott, M. E., Landry, D., Vence, L. M., et al. (1997). Single-column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene*, *192*, 271–281. doi:10.1016/S0378-1119(97)00105-4.
35. Babu, K. S., Antony, A., Muthukumar, T., & Meenakshisundaram, S. (2008). Construction of intein-mediated hGMCSF expression vector and its purification in *Pichia pastoris*. *Protein Expression and Purification*, *57*, 201–205. doi:10.1016/j.pep.2007.10.004.
36. Khatuntseva, S. A., Eldarov, M. A., Redo, V. A., & Skryabin, K. G. (2008). Purification and immobilization of recombinant variants of *Brevundimonas diminuta* glutaryl-7-aminocephalosporanic acid acylase expressed in *Escherichia coli* cells. *Journal of Biotechnology*, *133*, 123–126. doi:10.1016/j.jbiotec.2007.08.044.
37. Wierzbza, A., Reichl, U., Turner, R. F. B., Warren, R. A. J., & Kilburn, D. G. (1995). Adhesion of mammalian cells to a recombinant attachment factor, CBM/RGD, analyzed by image analysis. *Biotechnology and Bioengineering*, *46*(3), 185–193. doi:10.1002/bit.260460302.
38. Chatelet, C., Damour, O., & Domard, A. (2001). Influence of the degree of acetylation on some biological properties of chitosan films. *Biomaterials*, *22*, 261–268. doi:10.1016/S0142-9612(00)00183-6.
39. Hamilton, V., Yuan, Y., Rigney, D. A., Puckett, A. D., Ong, J. L., Yang, Y., et al. (2006). Characterization of chitosan films and effects on fibroblast cell attachment and proliferation. *Journal of Materials Science. Materials in Medicine*, *17*, 1373–1381. doi:10.1007/s10856-006-0613-9.
40. Freier, T., Koh, H. S., Kazazian, K., & Shoichet, M. S. (2005). Controlling cell adhesion and degradation of chitosan films by N-acetylation. *Biomaterials*, *26*, 5872–5878. doi:10.1016/j.biomaterials.2005.02.033.