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Recombinant carbohydrate-binding modules for biomedical applications
Biocompatibility of polysaccharide-based materials

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Doutoramento em Engenharia Química e Biológica
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Trabalho efectuado sob a orientação do
Doutor Miguel Gama
Doutora Margarida Casal

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The most exciting phrase to hear in science, the one that heralds the most discoveries, is not "Eureka!" but "That's funny..."

(Isaac Asimov)

AGRADECIMENTOS

Ao longo destes anos muitos foram os que me ajudaram e contribuíram para que eu chegasse ao fim desta etapa.

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Aprendi muito ao longo destes anos, mas o grande ensinamento que tiro desta aventura é que, o importante é ter bons contactos, porque quando não sabemos ou não temos alguma coisa, o importante é conhecer alguém que saiba ou tenha...

Para vencer - material ou imaterialmente - três coisas definíveis são precisas: saber trabalhar, aproveitar oportunidades e criar relações. O resto pertence ao elemento indefinível, mas real, a que, à falta de melhor nome, se chama sorte.

(Fernando Pessoa)

ABSTRACT

Recombinant carbohydrate-binding modules for biomedical applications. Biocompatibility of polysaccharide-based materials.

The development of biomaterials for medical applications envisages the design of three-dimensional structures – the scaffolds. These structures, mimicking the biological structures and interacting with the surrounding tissues through biomolecular recognition, elicit cellular responses mediated by specific interactions. Among the different scaffolds used in biomedicine, the materials based on polysaccharides present promising characteristics, due to their biocompatibility, hydrophilicity, degradability and appropriate mechanical properties, allowing for a favorable controlled interaction with living systems.

Recombinant proteins are widely used in the biomedical field, namely in the fuctionalization of biomaterials. It is well established that Carbohydrate-Binding Modules (CBMs) present in several glycanases are structural and functionally independent of the catalytic module; therefore, their application as fusion partners may be exploited, contributing to protein expression, solubilization, purification, and finally for the functionalization of polysaccharide-based materials. This is the main subject of this thesis: the evaluation of the potential of CBMs as tools for the improvement of the biocompatibility of polysaccharides.

One of the molecules often used to improve cells adhesion is the peptide Arg-Gly-Asp (RGD). The RGD sequence, present in several proteins of the extra-cellular matrix (ECM), is a ligand for integrin-mediated cell adhesion; this sequence was recognized as a major functional group responsible for cellular adhesion. Several polysaccharide-based materials have been produced recently at the DEB-UM laboratories, namely dextrin based hydrogels and bacterial cellulose scaffolds. In this study, recombinant proteins containing a CBM with starch affinity were fused to the bioactive molecule RGD, using recombinant DNA technology, in order to functionalize dextrin-based hydrogels.

The general introduction of this thesis is presented in chapter 1 and includes a bibliographic revision of: 1) the applications of polysaccharides as biomedical biomaterials (this revision is restricted to the dextrin and bacterial cellulose (BC) based materials, the ones that were used in this work); 2) the strategies available for the production of recombinant proteins, using bacterial systems; and 3) a state of the art on the CBMs and their applications.

The chapter 2 describes the development of a methodology for the expression and purification of the recombinant protein CBM-RGD, which has a CBM from the human protein laforin fused to a RGD sequence. Different commercial heterologous *Escherichia coli* expression systems (pET 29a, pET 25b and pGEXT41) were used in order to obtain high levels of soluble protein. Despite the use of the periplasmatic secretion approach (pET25) or the fusion of CBM with enhancing solubility tag (GST), the recombinant proteins were always obtained in the insoluble fraction. The utilization of CHAPS and arginine allowed the protein solubilization and purification, but not the production of functional protein with starch binding ability. Using the pET29a vector, the recombinant proteins were obtained in inclusion bodies (IB). After

solubilization and refolding, the CBM was recovered and showed starch affinity. This is the first report on the expression of the functional CBM from the human protein laforin.

The chapter 3 describes the production of recombinant proteins containing a bacterial CBM, which belongs to an α-amylase from *Bacillus sp.* TS-23. This protein, like the laforin CBM, also has starch affinity, being designated a Starch-Binding Module (SBM). The recombinant SBM and RGD-SBM proteins were cloned, expressed, purified and tested *in vitro*. The evaluation of cell attachment, spreading and proliferation on the dextrin-based hydrogel surface activated with recombinant proteins were performed using mouse embryo fibroblasts 3T3. The results showed that the RGD-SBM recombinant protein improved, by more than 30%, the adhesion of fibroblasts to dextrin-based hydrogel. In fact, cell spreading on the hydrogel surface was observed only in the presence of the RGD-SBM. The fusion protein RGD-SBM provides an efficient way to functionalize the dextrin-based hydrogel, improving the interaction with cells.

The characterization of dextrin-vinyl acrylate (dextrin-VA) and dextrin-hydroxyethylmethacrylate (dextrin-HEMA) hydrogels was presented in a previous study carried out at the DEB-UM laboratories. In this work (chapter 4) the *in vivo* biocompatibility and degradability of these hydrogels are reported. The histological analysis of subcutaneous implants of these hydrogels, featuring inflammatory and resorption events in mice, was carried out over a period of 16 weeks. While dextrin-HEMA hydrogel was quickly and completely degraded and reabsorbed, dextrin-VA degradation occurred slowly, apparently through an erosion controlled process. A thin fibrous capsule was observed 16 weeks post-implantation, surrounding the non-degradable hydrogel. In the case of the degradable material, only a mild inflammatory reaction was observed, with few foamy macrophages being detected around the implant. This reaction was followed by complete resorption, with no signs of capsule formation or fibrosis associated with the implants. Altogether, these results strongly suggest that the dextrin hydrogels are fully biocompatible, since no toxicity on the tissues surrounding the implants was found. Moreover, it may be speculated that a controlled degradation rate of the hydrogels may be obtained, using dextrin with grafted HEMA and VA in different proportions.

Chapter 5 presents the evaluation of Bacterial Cellulose – NanoFibers (BC-NFs) nanotoxicology. BC is a promising material for biomedical applications, namely due its biocompatibility. Although BC has been shown to be neither cytotoxic nor genotoxic, the properties of isolated BC-NFs on cells and tissues has never been analysed. Considering the toxicity associated to other fibre-shaped nanoparticles, it seems crucial to evaluate the toxicity associated to the BC-NFs. The results from single cell gel electrophoresis (also known as comet assay) and the *Salmonella* reversion assay showed that NFs, produced from BC by a combination of acid and ultrasonic treatment, are not genotoxic under the conditions tested. A proliferation assay using fibroblasts and CHO cells reveals a slight reduction in the proliferation rate, although no modification in the cell morphology is observed.

Overall, this work reports the successful expression and isolation of the atypical human CBM, from the protein laforin. It provides a contribution to the development of a strategy based on the use of CBMs as tools for the modification of the surface properties of biomaterials, improving the interaction with cells. Finally, this work characterizes biocompatibility aspects of biomaterials currently under development at DEB-UM laboratories.

RESUMO

Utilização de módulos recombinantes de ligação a carbohidratos para aplicações biomédicas. Biocompatibilidade de materiais baseados em polissacarídeos.

O desenvolvimento de biomateriais para aplicações biomédicas centra-se no desenho de estruturas tri-dimensionais – *scaffolds* – capazes de mimetizar as funções biológicas e interagir com os tecidos envolventes, através do reconhecimento biomolecular. Entre os diferentes materiais usados para produzir *scaffolds*, os constituídos por polissacarídeos (como é o caso dos hidrogeis de dextrino e os materiais de celulose bacteriana - BC) apresentam características promissoras devido à sua biocompatibilidade, hidrofilicidade, degradabilidade e propriedades mecânicas, permitindo a sua utilização biomédica.

As proteínas recombinantes são amplamente usadas em biomedicina, nomeadamente na funcionalização de diversos biomateriais. Sabe-se que os módulos de ligação a carbohidratos (CBMs), presentes em várias glicanases, são estrutural e funcionalmente independentes do domínio catalítico. Assim, a sua utilização em proteínas de fusão tem sido explorada, com o propósito de facilitar ou aumentar a expressão, solubilidade e purificação das proteínas. Uma das moléculas frequentemente usada para melhorar a adesão celular é o péptido *Arg-Gly-Asp* (RGD). Esta sequência, presente em diversas proteínas da matriz extra-celular, é um ligando para adesão celular mediada por integrinas, sendo reconhecido como o principal grupo funcional na adesão celular. Nos últimos anos, foram produzidos nos laboratórios do DEB-UM diversos materiais à base de polissacarídeos, nomeadamente hidrogeis de dextrino. Neste trabalho, usando tecnologia de DNA recombinante, foram produzidas proteínas bi-funcionais constituídas por um CBM (com afinidade para o amido) fundido com a molécula bio-activa RGD, com o propósito de os funcionalizar. Pretende-se assim melhorar a interacção do material com as células, favorecendo a adesão celular pela interacção com a molécula RGD que por sua vez está ligado ao material através do CBM.

Na Introdução geral desta tese (capítulo 1) apresenta-se: 1) uma revisão sobre biomateriais baseados em polissacarídeos (em particular dos hidrogels de dextrino e das nanofibras (NFs) de celulose bacteriana); 2) as estratégias usadas para produzir as proteínas recombinantes em sistemas de expressão bacterianos; 3) e uma revisão sobre os CBMs e as suas aplicações.

O segundo capítulo descreve a metodologia desenvolvida para a expressão e purificação da proteína de fusão CBM-RGD, pertencendo este CBM à proteína humana laforina. Foram utilizados diferentes sistemas comerciais para expressão heteróloga em *Escherichia coli* (pET 29a, pET 25b e pGEXT41), com o intuito de obter elevados níveis de proteína solúvel. Os sistemas de expressão que permitem a secreção das proteínas para o espaço periplasmático (pET25) ou a fusão com a GST (pGEXT4 1), um *tag* que potencia a solubilidade das proteínas, conduziram à obtenção de proteínas insolúveis. A adição de CHAPS e arginina ao tampão de lise, embora resultando num aumento da solubilidade, não permitiu a obtenção de proteína funcional, isto é, com afinidade para o amido. Usando o vector pET29a, a proteína foi obtida em corpos de inclusão que, depois de solubilizados e submetidos ao processo de

refolding, permitiram obter proteína funcional com afinidade para o amido. Este é o primeiro relato da expressão funcional deste CBM humano.

No capítulo 3 descreve-se a produção de proteínas de fusão contendo um CBM bacteriano, da α-amilase do *Bacillus sp.* TS-23. Este CBM também apresenta afinidade para o amido, sendo por isso designado por SBM (*Starch-binding module*). As proteínas recombinantes SBM e RGD-SBM foram produzidas usando um sistema de expressão de *E. coli*. O seu efeito na adesão, *spreading* e proliferação celular foi avaliado *in vitro,* usando fibroblastos de embrião de rato 3T3. Os resultados mostraram que o tratamento do hidrogel de dextrino com RGD-SBM melhorou a adesão celular em mais de 30%. Para além disso, só na presença da proteína foi possível observar as células alongadas na sua superfície. Assim, a proteína de fusão revelou-se eficiente para funcionalizar o hidrogéis de dextrino.

A caracterização dos hidrogéis de dextrino-vinil acrilato (dextrino-VA) e dextrino-hidroxietilmetacrilato (dextrino-HEMA) foi objecto de estudo em trabalhos anteriores, também desenvolvidos no DEB-UM. Neste trabalho (capítulo 4) apresentam-se os resultados da caracterização de biocompatibilidade e degradação destes hidrogéis *in vivo*. A análise histológica de implantes subcutâneos em ratinhos permitiu estudar os eventos de reabsorção e a resposta inflamatória. De acordo com os resultados, a degradação e reabsorção dos géis de dextrino-HEMA ocorre rapidamente; a degradação dos géis de dextrino-VA é mais lenta, devendo-se principalmente a processos de erosão. Após 16 semanas, foi observada uma fina cápsula fibrosa a rodear o implante não degradável. No caso do gel degradável, observou-se uma resposta inflamatória de baixa intensidade, sendo detectados alguns macrófagos com material fagocitado a envolver o implante. Esta reacção foi seguida pela completa reabsorção do material, não havendo sinais de formação de qualquer cápsula fibrosa. Estes resultados sugerem que os hidrogéis de dextrino são biocompatíveis, uma vez que não foram detectados sinais de toxicidade nos tecidos que envolviam o material. Os resultados sugerem também que é possível obter hidrogéis com velocidades de degradação controlada, usando dextrino substituído com HEMA e VA em diferentes proporções.

O capítulo 5 apresenta o estudo da nanotoxicidade de NFs de celulose bacteriana. A BC apresenta grandes potencialidades para aplicações biomédicas, sendo descrita como um material não citotóxico ou genotóxico. No entanto, o efeito das NFs, isoladas por tratamento ácido e ultrasons, nas células e nos tecidos não foi descrito. Considerando a toxicidade associada a outros nanomateriais com forma de agulha, o estudo da nanotoxicidade destas fibras torna-se crucial. Os resultados obtidos no ensaio cometa e de reversão da *Salmonella* mostraram que as NFs produzidas a partir da BC, não são genotóxicas na condições utilizadas. Para além disso, os resultados obtidos nos ensaios de proliferação celular usando fibroblastos e células CHO mostraram que, apesar de uma ligeira redução na proliferação, não são detectadas diferenças morfológicas.

Em resumo, este trabalho descreve, pela primeira vez, a expressão funcional do CBM atípico da proteína humana laforina. Este trabalho também contribui para o desenvolvimento de ferramentas que utilizam os CBMs recombinantes para a modificação das propriedades da superfície de materiais. Por último, são caracterizados aspectos da biocompatibilidade de materiais que estão a ser desenvolvidos nos laboratórios do DEB-UM.

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List of Abbreviations

AMPs	Antimicrobial peptides
APS	Ammonium persulfate
BC	Bacterial cellulose
BSA	Bovine serum albumin
CBHI	Cellobiohydrolase I
CBM	Carbohydrate-binding module
CbpA	Cellulose-binding protein A
CBS	Calf bovine serum
cDNA	Complementary deoxyribonucleic acid
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHO	Chinese hamster ovary
CPII	Cell proliferation inhibitor index
cryoSEM	Cryo-scanning electron microscopy
DS	Degree of substitution
DSP	Dual specific phosphatase
3D	Three dimensional
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleosides
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
ELP	Elastin-like polymers
ER	Endoplasmic reticulum
GHs	Glycosyl hydrolases
GST	Glutathione S-transferase
HAp	Hydroxyapatite
H&E	Haematoxylin- eosin
HEMA	Hydroxyethyl mehacrylate
His-tag	Polyhistidine-tag
IB	Inclusion bodies
IKVAV	Ile-Lys-Val-Ala-Val (signaling domain)
IPTG	Isopropyl-D-thiogalactopyranoside
LB	Luria broth
MALDI-TOF	Matrix-assisted laser desorption/ ionization-time of flight
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
MW	Molecular weight
NFs	Nanofibres
NPs	Nanoparticles
4 NQO	4-Nitroquinoline 1-oxide
OPH	Organophosphate hydrolase
OS	Osmotic solution
PAS	Periodic acid-Schiff
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG	Poly(ethylene glycol)
PMSF	Phenylmethylsulfonyl fluoride
PLA2	Phospholipase A2
PLGA	Poly (lactic/glycolic acid)
RGD	Arg-Gly-Asp (signaling domain)
rhEGF	Recombinant human epidermal growth factor
SBM	Starch-binding module
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SWNTs	Single-walled carbon nanotubes
TCPP	Cell culture polystyrene plate
TEM	Transmission electronic microscopy
TEMED	N,N,N',N'-Tetramethylenethylenediamine
VA	Vinyl acrylate
YIGSR	Tyr-Ile-Gly-Ser-Arg (signaling domain)

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SCOPE and COLLABORATORS

The main subject of this thesis is the evaluation of the Carbohydrate Binding Modules (CBMs) potential as tools for the improvement of the biocompatibility of polysaccharides-based materials. In addition, the biocompatibility of dextrin hydrogels and bacterial cellulose nanofibres, materials developed at DEB-UM laboratories, was also analysed.
Chapter 1 presents a revision of these subjects, namely 1) polysaccharide-based biomaterials; 2) strategies to express recombinant proteins using *Escherichia coli* systems; and finally, 3) a revision on CBMs. This subchapter is an adaptation of a book chapter accepted for publication.

The second chapter describes the strategies used to produce the bi-functional recombinant protein containing a human CBM from laforin fused to a RGD sequence. This work was performed in collaboration with the Biology Department of Universidade do Minho and the Biomolecular Biotechnology Unit of Biocant.

Chapter 3 describes the strategy to functionalize dextrin-based hydrogel using a recombinant protein containing a starch-binding module (SBM). The SBM of α-amylase from *Bacillus* strain TS-23 was fused to the RGD sequence by recombinant DNA technology and tested, *in vitro,* using mouse embryo fibroblast 3T3 cells. This work has been published on BMC Biotechnology Journal.

In this work the *in vivo* biocompatibility of the dextrin hydrogels was also investigated. The *in vitro* characterization of dextrin-based hydrogel was the aim of a previous work. However, the *in vivo* biocompatibility and degradability of those hydrogels were not evaluated; therefore, in the chapter 4 of this thesis is presented the study of the *in vivo* biocompatibility and degradability of dextrin-hydrogels implanted subcutaneously, in mice. These results were accepted for publication on Journal of Bioactive and Compatible Polymers and this work was performed in collaboration with Immuno-Phisiology and Pharmacology Department of Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

It is well known that nanomaterials with needle-like shape, such asbestos fibres, are citotoxic and present genotoxicity for cells. Because BC-nanofibres present similar structure it seems important to study its potential genotoxicity. In the chapter 5 the results from *in vitro* assays to evaluate BC-NFs effect on cells are presented. This study, performed in collaboration with the Biochemistry Department of Universidade Federal do Rio Grande do Norte (Brazil), was published on Toxicology Letters.

In the last chapter of this thesis (chapter 6) are presented the final remarks of this work and the future perspectives.

Chapter 1.

GENERAL INTRODUCTION

1.1

Polysaccharide-based biomaterials

The development of tissue engineering strategies is based on the design of three-dimensional structures made from natural or synthetic materials, termed scaffolds. Hydrogels are a class of hydrophilic polymeric scaffolds, with remarkable features from the perspective of biological mimicking. Among the materials used in the development of hydrogels, polysaccharide-based materials have been referred as promising materials, presenting appealing properties for biomedical applications.

In the following chapter an overview of biomaterials and tissue engineering developments, in particular the advantages and applications of polysaccharide-based hydrogels and bacterial cellulose biomaterials will be presented.

Biomaterials and Tissue Engineering

Biomaterials must be especially suitable for the intimate contact with living tissues, ideally mimicking the biological properties. They are used as drug delivery carriers, tissue engineering scaffolds, and biomedical devices. Thus, biomaterials are revolutionizing many aspects of preventive and therapeutic healthcare. With huge potential quality-of-life benefits owing to the many applications in the biomedical area, biomaterials are the focus of major research efforts with progresses in this field requiring a multidisciplinary approach. Indeed, the research on biomaterials gathers contributes from the materials science, chemical engineering, medical engineering, and pharmacology. The development of biomaterials for medical applications has focused on the design of biomimetic materials that interact with the surrounding tissues through biomolecular recognition, eliciting cellular responses mediated by specific interactions (Shin *et al.*, 2003). Currently, engineering of hard (*i.e.*, bone, teeth, and cartilage) and soft tissues (*i.e.*, skin and internal organs) encompasses the use of scaffolds, growth factors, and stem cells. Among the various materials assayed as scaffolds - assisting the cell proliferation and organ development - hydrogels have gained the preference of many researchers.

Scaffolds are ideally three-dimensional, highly porous structures with interconnected porosity. They are conceived as templates to guide the growth of tissue in the body, as delivery vehicles for transplanted cells and as drug carriers, activating specific cellular functions in a localized region, and ultimately regenerating the tissues (Murphy and Mooney, 1999; Thanos and Emerich, 2008; Vacanti *et al.*, 1998). Therefore, scaffolds may be implanted into a tissue defect without cells or bioactive compounds in its formulation, with the tissue regeneration depending only on the ingrowth of the surrounding tissue. Alternatively, the scaffolds may be loaded with cells or compounds, before implantation, improving the rate of tissue ingrowth, vascularization, and cell differentiation (Widmer *et al.*, 1998) (Figure 1).

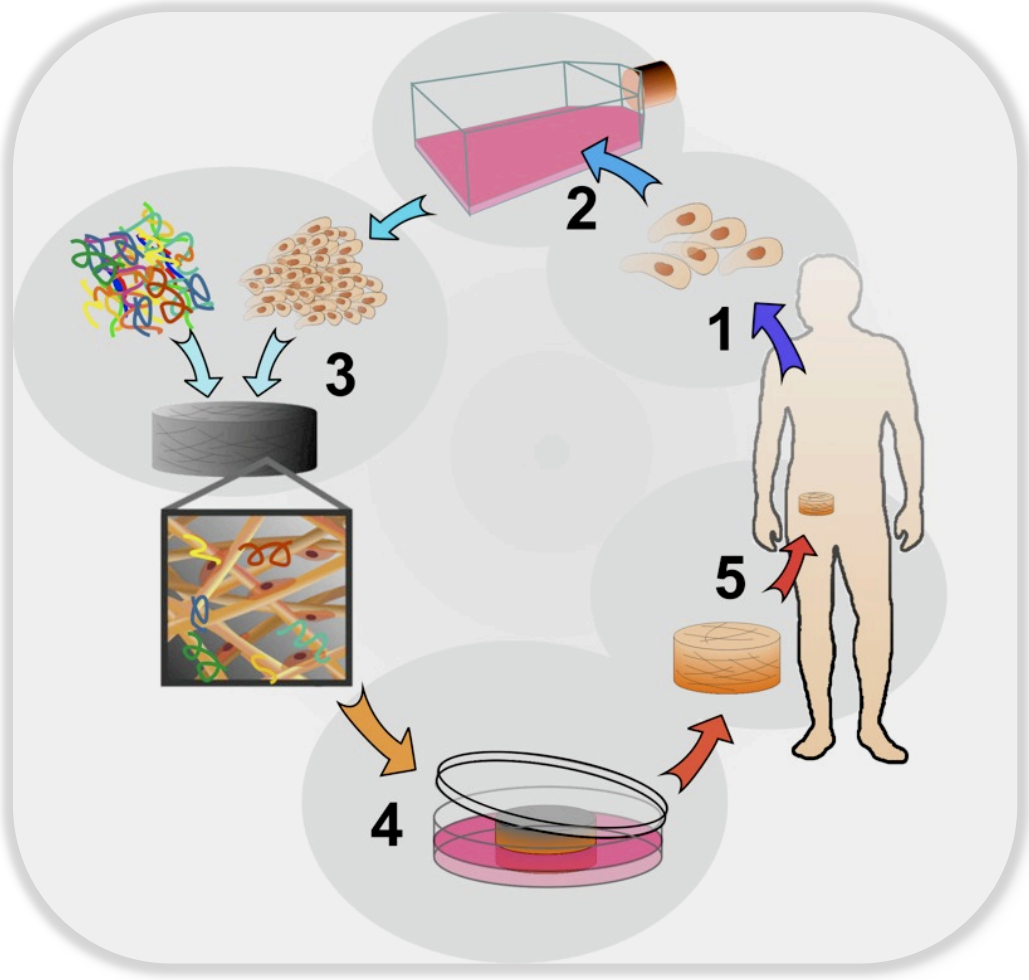


Figure 1 - Schematic representation of the application of a hydrogel scaffold in tissue engineering. 1) Patient cells (e.g., stem cells) collected and 2) grown *in vitro*. 3) Then, the cells are mixed with the hydrogel network, 4) Grown and/or differentiated in a specific culture medium; finally, the scaffold is implanted in the patient. (From www.centropede.com/UKSB2006/ePoster/images/background/TE_model_large.jpg).

The selection of a scaffold material is both critical and difficult. The sophisticated smart materials used in the biomedical applications must meet strict criteria, namely convenient mechanical properties and degradation rate, biocompatibility, porosity and interconnectivity, functional properties related to the interaction with cells and the release of pharmaceuticals, among others (Peppas *et al.*, 2000). Candidate materials include: 1) synthetic polymers, such as polylactic acid (PLA), polyglycolic acid (PGA), poly(lactide-co-glycolide) acid (PLGA), ethylene oxide block copolymers); 2) inorganic materials, such as tricalcium phosphate, calcium carbonate, non-sintered hydroxyapatite; and 3) natural polymers, such as fibrin, collagen, gelatin, hyaluronan. Indeed, natural polymers have

played an important role in these efforts, and recombinant polymers that combine the beneficial aspects of natural polymers with many of the desirable features of synthetic polymers have been designed and produced.

Besides biocompatibility, the biomaterial biodegradability is generally desirable for tissue engineering applications. Ideally, the degradation rate also matches the neo tissue formation rate, performing as a template (Ma, 2008).
Several materials have been exploited as scaffolds for tissue regeneration, each one presenting advantages and/or disadvantages, depending on the specific application. For instance, certain metals are an excellent choice for medical implants, due to their superior mechanical properties (Catledge *et al.*, 2004); of course, they are not a good choice for scaffold applications because of the lack of degradability in biological environment (Liu and Ma, 2004). In addition, certain inorganic/ceramic materials, such as hydroxyapatite (HAP) or calcium phosphates, having good osteoconductivity, have been considered for mineralized tissue engineering; however, they are of limited application because of its brittleness and poor processability into highly porous structures. In contrast, polymers present great design flexibility. Their structure can be tailored to the specific needs, and therefore have been extensively studied in various tissue engineering applications, including bone tissue engineering (Huang *et al.*, 2007; Liu and Ma, 2004; Meinel *et al.*, 2005; Rice *et al.*, 2005)

The overall goal of tissue engineering is to create functional tissue grafts that can regenerate or replace defective or worn out tissues and organs. Examples of grafts, now in pre-clinical studies or clinical use, include engineered skin, cartilage, bone, blood vessels, skeletal muscle, bladder, trachea, and myocardium (Grayson *et al.*, 2008).

Hydrogels as scaffolds for biomedical applications

Hydrogels are a class of materials that swell under conditions of excess of water (or biological fluids), holding a large amount of water in the wetstate. Chemical crosslinks (covalent bonds) or physical junctions (e.g. secondary forces, crystallite formation, chain entanglements) provide the hydrogels unique swelling behavior and three-dimensional structure (Klouda and Mikos, 2008; Peppas, 2004; Peppas *et al.*, 2006).

Hydrogels are one of the upcoming classes of polymer-based systems that embrace numerous biomedical and pharmaceutical applications, such as tissue engineering, molecular imprinting, wound dressing materials, immunoisolation, drug delivery, etc (Kashyap *et al.*, 2005; Peppas *et al.*, 2006). In addition, hydrogels provide new approaches for culturing mammalian cells *ex vivo*, which are increasingly needed, to study cell and tissue physiology and to grow replacement tissues for regenerative medicine. Two-dimensional culture has been the paradigm for typical *in vitro* cell culture; however, it has been demonstrated that cells behave more "*in vivo*-like" when cultured in three-dimensional environments such as hydrogels scaffolds (Tibbitt and Anseth, 2009).

Among the various tissue engineering scaffolds comprehensively studied, hydrogels remain most appealing candidates due to the controllable and reproducible polymer properties and to the large water uptake, promoting excellent biocompatibility due to low protein adsorption (Peppas, 2004). In addition, hydrogels present mechanical properties and hydrophilicity that resembles those of the extracellular matrix (ECM) of native tissue, tunable viscoelasticity, and high permeability for oxygen and essential nutrients (Jia and Kiick, 2009; Peppas *et al.*, 2000; Tibbitt and Anseth, 2009).
Apart from favorable physico-chemical and mechanical properties, the most important requirement for a hydrogel to be used in medical applications is its biocompatibility, together with the non-cytoxicity of its degradation products. Most of the toxicity associated with hydrogels regards the unreacted monomers, oligomers and initiators that leach out (Del Guerra *et al.*, 1996; Del Guerra *et al.*, 1995; Kirkpatrick, 1992; Ratner, 1997).

A variety of hydrogel materials have been utilized for tissue engineering applications, including reconstituted ECM components or natural proteins and carbohydrates (Long and Tranquillo, 2003; Robinson *et al.*, 2008; Zhong *et al.*, 2005), self-assembling peptides (Collier *et al.*, 2001; Kisiday *et al.*, 2002), and synthetic materials (Hicks *et al.* 2003; Lou *et al.*, 2001). Thus, the selection of the appropriate hydrogel is governed by the chemical and physical properties, the mass transport properties and the biological interaction requirements that are best suited for a given application.
While the chemical properties of hydrogels (such as hydrophilicity) are determined by 1) the polymer backbone, 2) the functional side chain in the monomer unit, and 3) the cross-linking agent; the physical properties (e.g. mechanical strength and swelling ratio) are mainly controlled by the cross-link density. For instance, the amount of water absorbed by hydrogels is limited by their ability of undergoing elastic network expansion, which can be

controlled by controlling the cross-linking degree during the synthesis of chemically cross-linked hydrogels (Byrne and Salian, 2008).

Hydrogels can show variable swelling behavior, depending on changes in the external environment. Some of the factors that can affect the swelling of responsive hydrogels include pH, ionic strength and temperature (Peppas, 2004). Hydrogels can also be made responsive to diverse external stimuli, such as light, electric current, ultrasound, and the presence of a magnetic field or a particular molecule (Watanabe *et al.*, 2004). This unique property of responsiveness has resulted in the development of hydrogel-based sensors (Bashir *et al.*, 2002; Hilt *et al.*, 2003), self-regulated and externally actuated intelligent drug delivery systems (Miyata *et al.*, 1999; Sershen and West, 2002; Sershen *et al.*, 2000; Yoshida *et al.*, 1993) and microfluidic devices (Beebe *et al.*, 2000; Satarkar and Zach Hilt, 2008; Sershen *et al.*, 2005). Physically or chemically cross-linked hydrogels can also be generated in the presence of living cells, allowing *in situ* encapsulation for tissue engineering (Peppas *et al.*, 2006). Furthermore, hydrogels with controlled biodegradation kinetics may be easily designed using natural polymers susceptible to enzymatic degradation or synthetic polymers with hydrolyzable moieties (Watanabe *et al.*, 2004)

Natural biomaterials: Polysaccharide-based materials

By far, the majority of carbohydrate materials in Nature occur in the form of polysaccharides. By definition, polysaccharides include not only those substances composed uniquely of glycosidically linked sugar residues, but also molecules that contain polymeric saccharide structures linked via covalent bonds to amino acids, peptides, proteins, lipids and other structures. Thus, polysaccharides have a large number of reactive groups, a wide range of molecular weight (MW), and different chemical compositions, which contribute to their diversity in structure and in properties (d'Ayala *et al.*, 2008). In nature, polysaccharides from algae (e.g. alginate), plant (e.g. pectin, guar gum), microbial (e.g. dextran, xanthan gum), and animal origin (chitosan, chondroitin) can be found (Coviello *et al.*, 2007).

The most common constituent of polysaccharides is D-glucose, but D-fructose, D-galactose, L-galactose, D-mannose, L-arabinose, and D-xylose are also frequent. Some monosaccharide derivatives found in polysaccharides include the amino sugars (D-

glucosamine and D-galactosamine) as well as their derivatives (N-acetylneuraminic acid and N-acetylmuramic acid), and simple sugar acids (glucuronic and iduronic acids).
Among the numerous macromolecules that can be used for hydrogel formation, polysaccharides are advantageous compared to synthetic polymers. Coming from renewable sources, polysaccharides also have frequently economical advantages over synthetic materials. Polysaccharides are usually non-toxic, biocompatible and show a number of convenient physico-chemical properties (such as hydrophilicity, viscosity and reactive groups) that make them suitable for different applications in drug delivery systems (Coviello *et al.*, 2007). The major disadvantages of natural polymers, when compared with synthetic ones, are the difficulty in controlling their physico-chemical properties, such as molecular weight, strength, degradation time and mechanical properties. However, there are several strategies to overcome these limitations, including the combination with other natural or synthetic polymers. The combination with other natural polymers (e.g., collagen/glycosaminoglycans) or with synthetic polymers (e.g., collagen/PLGA), may improve the biocompatibility of the ensuing scaffolds, by reducing inflammatory response *in vivo* and improving initial cell attachment and differentiation on the material (Flanagan *et al.*, 2006; In Jeong *et al.*, 2007; Liu *et al.*, 2008; Zhong *et al.*, 2005).

Polysaccharides, such as cellulose, starch, chitin/chitosan, alginate, carrageenan, gellan, guar gum, hyaluronic acid, pullulan, dextran, among others, have been used in the formulation of several hydrogels (nanogel, microspheres) (Coviello *et al.*, 2007). In addition, it was also found that water-soluble polysaccharides derivatives – such as carboxymethylcellulose (CMC), carboxymethylstarch (CMS), carboxymethylchitin (CMCT), and carboxymethylchitosan (CMCTS) – lead to the formation of hydrogels at high concentrated aqueous solution (paste-like state) by radiation cross-linking (Yoshii *et al.*, 2003). Table 1 gives examples of several polysaccharides used in hydrogel formulations and their potential biomedical applications.

Table 1 - Example of polysaccharides used in hydrogels formulation for biomedical applications.

Polysaccharide	Application	References
Alginate-based materials (hydrogel, microspheres)	Ocular drug delivery; oral administration of drugs; wound dressings; regional radio-chemotherapy; driving of angiogenesis by diffusion of VEGF; cells encapsulation; bone tissue engineering (repair of osteochondral defects of joints; neurotransplantation)	(Coviello *et al.*, 2007) (Kuo and Ma, 2001) (d'Ayala *et al.*, 2008) (Eiselt *et al.*, 2000) (Novikova *et al.*, 2006)
Dextran-derivates (hydrogels and microgels)	Release of proteins with limited aqueous solubility (such as IL2); tissue engineering bone regeneration (such as BMP release); nasal drug delivery systems; colon drug delivery	(Bos *et al.*, 2004) (Coviello *et al.*, 2007) (Lévesque and Shoichet, 2006)
Chitin and Chitosan-derivates (hydrogels, films and microgels)	Wound treatment; bioadhesive sustained release formulation; tissue engineering (cartilage and bone regeneration); gene delivery; colon drug delivery	(Kim and Chu, 2000) (Felt *et al.*, 1999) (Alsarra *et al.*, 2009) (Sinha *et al.*, 2004) (d'Ayala *et al.*, 2008) (Veerapandian and Yun, 2009)
Gellan-derivates (hydrogels, beads, microspheres)	Ophthalmic formulations; nasal spray pumps; encapsulation of biological components; oral drug delivery; drug release	(Veerapandian and Yun, 2009) (Coviello *et al.*, 2007) (Schwall and Banerjee, 2009)
Carrageenan and Carrageenan-derivates (hidrogels, beads)	Promoter of angiogenesis by diffusion of VEGF; wound and burns dressing application; bone tissue engineering; drug carrier and devilry	(Santo *et al.*, 2009) (Coviello *et al.*, 2007)
Hyaluronic acid and HA-derivates (hydrogels, beads)	Local administration of anti-inflammatory drugs in osteoarthritic knee; immobilization of hydrocortisone; cell encapsulation for cell delivery tissue regeneration; controlled release of vascular endothelial growth factor and basic fibroblast growth factor, promoting neovascularization	(Coviello *et al.*, 2007) (Schwall and Banerjee, 2009)
Guar-derivates (hydrogels)	Prodrugs formulation (as coating material or as hydrogel entrapping dugs inside its network); colon delivery; treatment of open-angle glaucoma	(Coviello *et al.*, 2007) (Schwall and Banerjee, 2009)
Pullulan-derivates (hydrogels, micro and nanogels)	Capacity to bind hydrophobic substances (such as anticancer drugs); imaging of specific sites (such as tumor and ischemic area)	(Coviello *et al.*, 2007)
Xanthan- derivates (hydrogels)	Enzyme immobilization; loading bioactive substances; delivery proteins in nasal cavity	(Andreopoulos and Tarantili, 2001) (Bejenariu *et al.*, 2008) (Coviello *et al.*, 2007)
Xyloglun-derivates (sol-gel transition)	Vehicle for sustained release of percutaneous formulation (non-steroidal anti-inflammatory drugs); release of indomethacin suppositories; orally and intraperitoneal administration of drugs; neural tissue engineering of the spinal cord	(Coviello *et al.*, 2007) (Nisbet *et al.*, 2009) (Nisbet *et al.*, 2006)

Functionalization of polysaccharide-based biomaterials

Biomaterials are expected to fulfill biological functions, such as promoting cell proliferation and differentiation and enhancing the growth of surrounding tissues for defective regeneration. A straightforward method to incorporate these functions is to create hydrogels made up of natural macromolecules or macromolecular blends. Biologically active molecules can also be incorporated into polymer networks (e.g., by physical or chemical entrapment) to produce conjugated biomaterials, in order to design biomimetic scaffolds that can provide biological cues to elicit specific cellular responses and direct new tissue formation.

The surface and bulk modification of materials with peptide sequences can allow for the modulation of cellular functions such as adhesion, proliferation and migration through modulation of the peptide concentration or its spatial distribution. Hydrogels have been synthesized so that they contain functional groups for enhancing cellular adhesion (Burdick *et al.*, 2004; Hern and Hubbell, 1998). In this scheme, the addition of such modalities can dramatically change the properties of the hydrogels. The most common peptides used to modify hydrogels are derived from natural proteins, such as RGD (derived from proteins such as fibronectin, laminin, or collagen), IKVAV, and YIGSR from laminin (Peppas *et al.*, 2006; Tashiro *et al.*, 1989). Using these approaches, PEG (Burdick *et al.*, 2004; Hern and Hubbell, 1998) and other hydrogels, such as alginate (Rowley *et al.*, 1999), have been modified with RGD to enhance cellular adhesion.

Despite the recent advances toward the development of biomimetic materials for tissue engineering applications, several challenges still remain, including the design of adhesion molecules for specific cell types, as required for guided tissue regeneration and the synthesis of materials exhibiting the mechanical responsiveness of living tissues.

In particular, carbohydrate-based hydrogel (such as dextrin and cellulose) may be functionalized, by using recombinant proteins containing a carbohydrate-binding module (CBM) fused to bioactive peptides. CBMs present several specificity and affinities (Boraston *et al.*, 2004; Boraston *et al.*, 2007; Shoseyov *et al.*, 2006b); therefore they may be used to adsorb peptides to a different polysaccharide materials, including starch and cellulose-based materials (Andrade *et al.*, 2009).

Starch-based hydrogels

Among the many applications of starch-based materials, the development of hydrogels for biomedical applications has drawn the attention of several research groups. The biocompatibility and degradability makes starch a suitable component of hydrogels with technological applications in a large number of areas, such as medical, pharmaceutical and biological (Marques *et al.*, 2002).

Starch is a natural mixture of amylose, a linear polymer of D-glucose unites linked to 1,4-α-D-glucosidic linkages, and amylopectin or pullulan, a branched polymer of α-D-Glucose units containing 1,4- α-D-glucosidic linear linkages and 1,6- α-D-glucosidic linkages at the branch points (Figure 2). There are a several hydroxyl groups on starch chains, two secondary hydroxyl groups at C-2 and C-3 of each glucose residue, as well as one primary hydroxyl group at C-6 when it is not linked (Lu *et al.*, 2009). These hydroxyl groups on the starch chains can be oxidized and reduced, and may participate in the formation of hydrogen bonds, ethers and esters (Tomasik and Schilling, 2004).

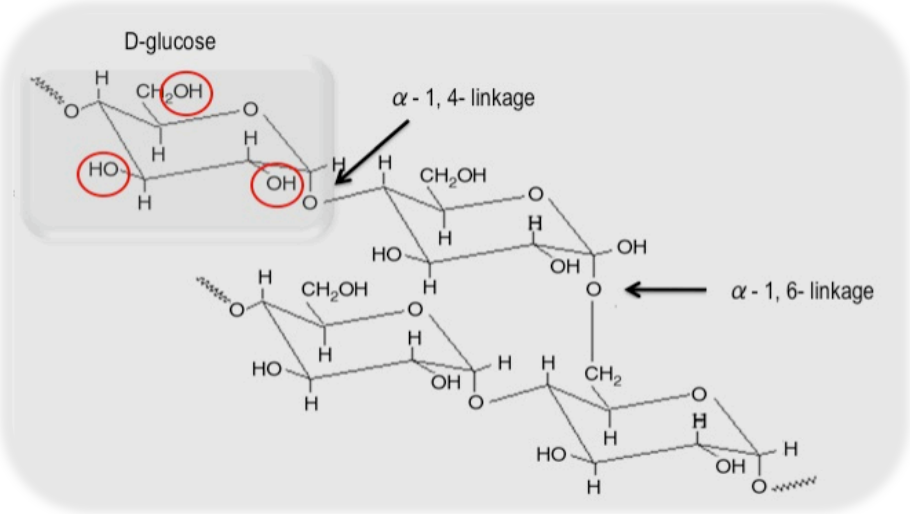


Figure 2 - Schematic representation of molecular structure of starch, showing D-glucose units and their hydroxyl groups, and the α-1,4 glucosidic linear linkages or α-1,6 linkage in the branch points. (Adapted from Lu *et al.*, 2009)

Starch itself is poor in processability, as well as in the dimensional stability and mechanical properties of its end products. In addition, starch is not capable of gelling

naturally; it must be modified by chemical derivatization, in order to introduce functional groups in the raw material, which may then gellify using either of the techniques described bellow. The chemical modification of starch (and the polysaccharides in general) has profound effects on its macroscopic behavior (e.g. solubility, stability and viscosity characteristics). Thus, for extended practical utilization of starch-based products, reinforcement or modification of starch is often essential (Ulrich Riedel, 1999; Vargha and Truter, 2005).

Starch-based hydrogels may be produced by using: 1) free radical polymerization (in one-step or two-step), 2) cross-linking by chemical reaction of complementary groups, 3) radiation-induced polymerization and 4) cross-linking and physical self-assembly.

1. The polymerization using free radicals may be achieved by one-step or two-step synthesis. In the first case, the hydrogel is obtained through free radical copolymerization of low molecular weight hydrophilic vinyl monomers onto the starch substrate, in the presence of polymerizable cross-linking agents (Zhang *et al.*, 2005). In a two-step synthesis, the polysaccharide is first functionalized with reactive double bonds and then cross-linked by free radical polymerization in water. Hydrogels obtained by these processes can combine the advantages of natural and synthetic polymeric hydrogels, and usually bear improved mechanical properties (Coviello *et al.*, 2007; Zhang *et al.*, 2005).

2. Starch-based hydrogels may also be achieved through the reaction of functional groups with complementary reactivity. The cross-linking is possible when specific functional groups (mostly -OH, -COOH) are present along the macromolecular chains of starch and its derivatives, forming covalent bonds (Van Tomme *et al.*, 2008; Zhang *et al.*, 2005).

3. The chemical cross-linking of starch-based hydrogels can also be achieved by using high-energy radiation (especially gamma and electron beams (Zhang *et al.*, 2005). During the irradiation of aqueous starch system, radicals are formed on the polymer chain by homolytic scission of C—H bonds. In addition, the radiolysis of water molecules generates hydroxyl radicals which subtract protons from the polymer chains, resulting in the formation of macroradicals (Zhang *et al.*, 2005). Under an inert atmosphere, the recombination of the macroradicals on different

chains leads to the formation of covalent bonds and finally produces a cross-linked network structure. The advantage of this process is that it can be done in water, under mild conditions (room temperature and physiological pH). However, when these hydrogels are used as matrices for the controlled release of bioactive drug molecules, drug loading is possible only after preparation of the hydrogels, because the radicals formed during irradiation could potentially damage the biologically active substance.

4. For the preparation of physically cross-linked starch-based hydrogels, amphiphilic starch derivatives, generally synthesized by hydrophobic modification of water-soluble polysaccharide, self-assembles in aqueous solution to form physical hydrogels with a micelle-like structure with hydrophobic cores derived from the association of hydrophobic segments and a hydrophilic shell made of the polar groups on the polymer (Janes *et al.*, 2001; Zhang *et al.*, 2005). The major disadvantage of these hydrogels, when compared with chemically cross-linked ones, is the instability of their mechanical properties (Zhang *et al.*, 2005).

Dextrin as a biomaterial

Among starch-based materials, those based on dextrin are widely used in a variety of applications, from adhesives used in food and textile industries (Lazarus, 1983), peritoneal dialysis solution (Hreczuk-Hirst *et al.*, 2001) to the moisture-maintaining component of powders for skin used in the cosmetic industry (Tipson *et al.*, 1989). Recent work reported the ability of dextrin conjugates to exhibit anti-endotoxin activity as well as to regulate the inflammatory response (Davtyan *et al.*, 2007; Davtyan *et al.*, 2009). In another recent work, dextrin-Hydroxyapatite (HAp) complex was used as a bone filling material, with good performance (Asai *et al.*, 2009). In addition, nanoparticle based on dextrin were also described as potential drug carriers (Goncalves *et al.*, 2007), and dextrin-based microspheres were used for encapsulation of the photosensitizer porphyrin, which aggregates in aqueous solutions, allowing its administration in the monomeric form, in photodynamic therapy (Luz *et al.*, 2008).

Hardwicke *et al.* described the development of a bioresponsive polymer–drug conjugate designed specifically to promote wound healing. Dextrin was selected for conjugation with recombinant human epidermal growth factor (rhEGF), as the former is degraded by α-

amylase in wound fluid. Dextrin was used to protect rhEGF from proteolytic attack (which is a major clinical challenge when growth factors are administered topically) and liberate it at a controlled rate (Hardwicke *et al.*, 2008). More recently, a similar strategy was used to reduce the nonspecific neurotoxicity of phospholipase A2 (PLA2) crotoxin (an antitumor protein that appears to act by interaction with epidermal growth factor receptors). According to this concept, the bioresponsive dextrin−PLA2 conjugate will be activated in the tumor interstitium (which presents high levels of α-amylase), through dextrin degradation (Ferguson and Duncan, 2009).

Dextrins are a group of low-molecular-weight carbohydrates produced by partial hydrolysis of starch, which can be accomplished by the use of acid, enzymes, or a combination of both. Dextrin is a glucose-containing saccharide polymer linked by α-1,4 D-glucose units, containing few (< 5%) α-1,6 links, having the same general formula as starch, but smaller and less complex (Carvalho *et al.*, 2007; Hreczuk-Hirst *et al.*, 2001).

Among the many possibilities of starch modification, the creation of reactive double bonds through a transesterification reaction has been exploited recently by several groups to functionalize sugars (in particular dextran and dextrin) (Carvalho *et al.*, 2009a; Carvalho *et al.*, 2007; Carvalho *et al.*, 2008a; De Groot *et al.*, 2001). The final product of the transesterification of vinyl acrylate monomers has side chains (vinylic groups) attached to the polysaccharide backbone and polymerizes through well-established free radical methods (De Graaf *et al.*, 1998; Vargha and Truter, 2005). Depending on the vinyl ester used in the dextrin substitution, and on the degree of substitution, different degradability may be achieved. Thus, using vinyl acrylate (VA) or hydroxyethylmethacrylate (HEMA) the dextrin-based hydrogel obtained is non-degradable or degradable, *in vitro*, respectively (Carvalho *et al.*, 2009b). In addition, in both cases, the degree of substitution (DS), which refers to the average number of substituted polysaccharide hydroxyl groups, is of major importance in determining the properties of the resulting hydrogel, such as the degradation rate or the release profiles (Carvalho *et al.*, 2009; Carvalho *et al.*, 2009b).

In vitro biocompatibility and degradability of dextrin-based hydrogels, namely dextrin-VA and dextrin-HEMA, were already evaluated. It was demonstrated that dextrin-based hydrogels do not present toxicity; moreover, cells adhere to the hydrogel surface and remain viable (Carvalho *et al.*, 2009b). In addition, the release profile of those dextrin-

based hydrogels using a model protein shows its suitability for their application as a delivery system (Carvalho *et al.*, 2009).

Bacterial Cellulose as a biomaterial

Bacterial cellulose (BC) is a polysaccharide produced by the *Acetobacter xylinum* bacteria into long non-aggregated nanofibrils (Backdahl *et al.*, 2006; Brown *et al.*, 1976; Klemm *et al.*, 2001).
The cellulose synthesized by *A. xylinum* is identical to that made by plants in respect to molecular structure. However, the secreted polysaccharide is free of lignin, pectin, and hemicellulose as well as other biogenic products, which are associated with plant cellulose. Additionally, the BC displays many unique properties including high mechanical strength, high water content, high crystallinity and an ultra-fine highly pure nanofibril network structure (Backdahl *et al.*, 2006; Czaja *et al.*, 2007; Lee *et al.*, 2001).

One of the main requirements of any biomedical material is its biocompatibility, which is the ability to remain functional in contact with the living tissue, without causing any toxic or allergic side effects. Studies carried out *in vitro* and *in vivo* have demonstrated the BC biocompatibility. Thus, several applications were described for this material, including micro vessel prosthesis (Klemm *et al.*, 2001), temporary skin substitutes (Fontana *et al.*, 1990), in periodontal treatments and as a replacement for dura mater (the membrane that surrounds brain tissue) (Andrade *et al.*, 2009; Czaja *et al.*, 2007). In addition, BC can be combined in composite materials in order to further improve its characteristics. For instance, Yasuda *et al.* used microbial cellulose immersed in two types of polymer solutions (2-acrylamide-2-methyl-propane sulfonic acid and gelatin), to create a cellulose-based hydrogel with enhanced mechanical toughness, for the replacement of cartilage tissue in damaged joints (Yasuda *et al.*, 2005).

Some researchers have also obtained modified BC by introducing different additives into the culture media. The modification of the bacterial cellulose occurs, in this case, during biosynthesis, by introducing selected bioactive polysaccharides, such as chitosan and derivatives into the culture medium. Such composite materials can be applied in the treatment of burns, bedsores, skin ulcers, hard-to-heal wounds and wounds requiring frequent changes of dressing (Ciechanska, 2004). In addition, polysaccharides

degradable *in vivo*, exhibiting both chitin- and cellulose-like properties and susceptible to lysozyme attack, could be achieved by introducing GlcNac residues into bacterial cellulose (Ogawa and Tokura, 1992).

The formation of networks with distinct architecture and the modification of other molecular features, such as reduction of crystallinity, was also obtained by adding mannan-based polysaccharides to the culture medium (Whitney *et al.*, 1998). Further, it was shown that a range of different cellulose-associated networks could be formed, depending of the levels of glucomannan and galactomannans added. Cellulose with lower cristallinity and a smaller crystallite size was also obtained by adding sodium alginate to the culture medium (Zhou *et al.*, 2007).

BC-Nanofibres toxicology

In the recent years there is an increasing interest in nanomaterials, including metallic nanoparticles (NPs), metal oxide nanoparticles, dendrimers, quantum dots, nanoclusters, nanocrystals, nanowires, fullerenes, fullerene-based derivatives, single- and multi-wall carbon nanotubes, functionalized carbon nanotubes, polymer nanoparticles, carbon black, nano-coatings, among others, and its applications. With the rapid development of nanotechnology and its applications, a wide variety of nano-structured materials are now used in commodities, pharmaceutics, cosmetics, biomedical products, and industries (Ashammakhi *et al.*, 2007; Ma *et al.*, 2005). In particular, BC-based materials, has been described as a promising scaffold in tissue engineering, since it can better mimic the nanostructure of extracellular matrix due to its nanofibrilar structure. Furthermore, BC nanofibres can be combined with other materials in order to improve their characteristics (Grande *et al.*, 2009; Millon *et al.*, 2008; Yoon *et al.*, 2006).

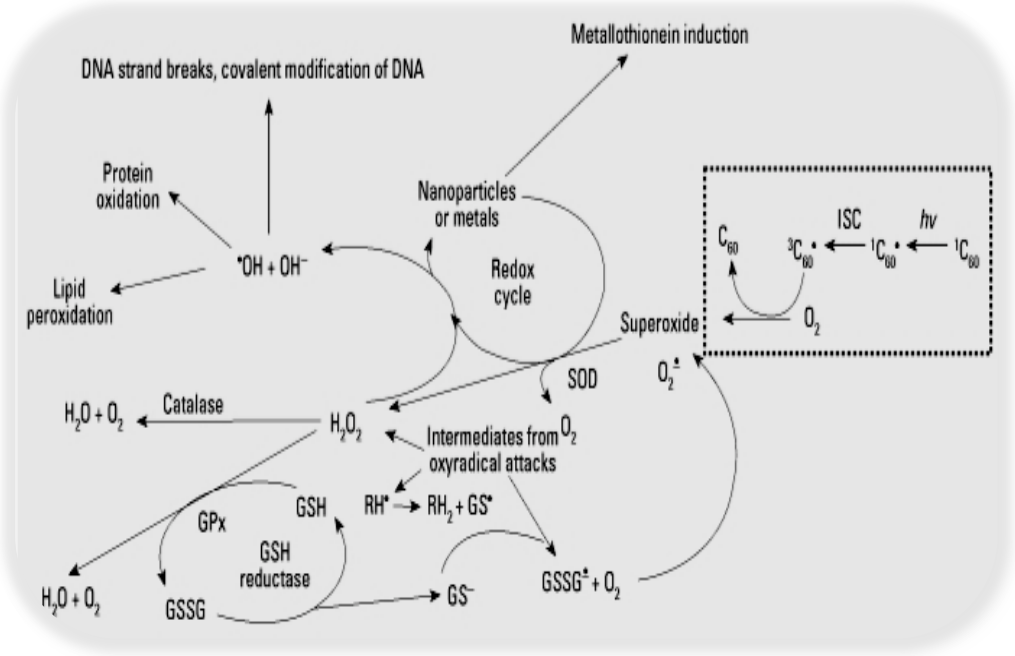


Figure 3 – Schematic representation of possible interaction of oxyradicals release by NPs with the antioxidant defense system (Picture from Oberdorster *et al.*, 2005), Abbreviations: GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; ISC, intersystem crossing; R, any organic molecule; SOD, superoxide dismutase. In addition to fullerenes, metals such as cadmium, iron, nickel quantum dots, or iron from SWNT manufacturing, could also act in Fenton-type reactions. Phase II biotransformation, ascorbic acid, vitamin E, beta-carotene, and other interactions are not shown.

While nanoscale materials possess novel and unique physicochemical properties different from those of bulk materials, they also have an unpredictable impact on human health. The impact of nanomaterials on the human body, their interactions with biological systems, and their risk assessment have generated intense scientific curiosity. Researchers have demonstrated that NPs may exhibit unique biological behavior, even when physical and chemical properties remain unaltered from those observed in large particle. Perhaps the most striking example lies on the fact that the smaller particles size enables nanoscale particles to cross or circumvent barriers that are impenetrable to larger particles (Bernstein *et al.*, 2005; Pan *et al.*, 2007). The greater surface area per mass, compared with larger-sized particles of the same chemistry, renders NPs more active biologically. This activity includes a potential for inflammatory and pro-oxidant, but also antioxidant activity, which can explain early findings showing mixed results in terms of toxicity of NPs to environmentally relevant species. For instance, Zhao *et al.* have used

computer models to predict that C60 molecules can damage DNA if intracellular exposure occurs (Zhao *et al.*, 2005b) (Figure 3) and Lynch *et al.* have hypothesized that protein adsorbed onto nanoparticles may alter their shape, such that normally hidden amino acids residues are exposed as cryptic epitopes-triggering an immune response (Lynch *et al.*, 2006).

The interaction of nanomaterials with biological systems is affected by several factors, such as size, surface area, shape, chemical composition, lattice structure, surface chemistry and charge (Borm *et al.*, 2006; Pan *et al.*, 2007).
Nanomaterials can exhibit various shapes and structures; among them, needle-like nanofibres have been described as a potential toxic. For instance, the long, thin geometry and water insolubility of carbon nanotubes may have the potential to cause effects similar to those arising from inhalation of asbestos fibres (a well-known harmful material for man health), even if the chemical composition is completely different (Donaldson *et al.*, 2006).

As refereed above, there is an increasing interest in nanomaterials-based on BC, and since, BC-NFs also present needle-like shape, it seems important to evaluate their toxicity. Therefore, in this work, several assays were carried out to access BC-NFs toxicology.

1.2

E. coli expression systems towards the production of recombinant proteins

The technology of recombinant DNA allows the production of pure, soluble and functional recombinant proteins for several applications, including immunization, biochemical studies, three-dimensional structural protein analysis, and biotechnological and therapeutic use. Although the advances in a number of different expression systems, including bacteria, yeast, mammalian cells, and even cell-free systems, the bacterial ones still is the first approach used. However, despite the advantages, well recognized, the bacterial expression systems present also several limitations, specially for the production of protein from eukaryotic origin (Demain and Vaishnav, 2009; Sahdev *et al.*, 2008; Schumann and Ferreira, 2004).

Among the distinct strategies described for the production of functional recombinant proteins, the fusion with carbohydrate-binding modules (CBMs) is an attractive strategy towards the functionalization of biomedical materials based on polysaccharides. In the following subchapters the bacterial expression systems for recombinant proteins production, as well as the CBMs functions and applications, will be reviewed.

Recombinant expression systems for the production of heterologues proteins

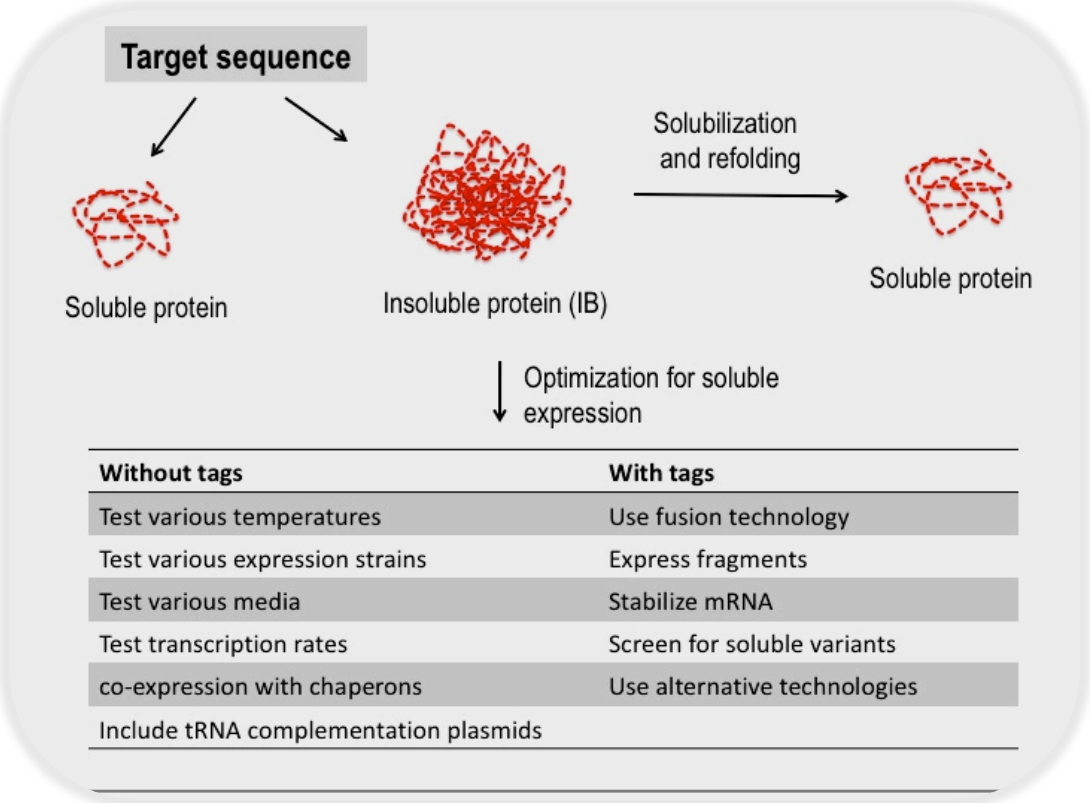
Production of recombinant proteins involves the cloning of the appropriate gene into an expression vector, under the control of a well-known regulable promoter. Bacterial systems remain the most attractive due to low cost, high productivity, well-known genetics and large number of compatible molecular tools available (Sorensen and Mortensen, 2005a; Terpe, 2006). Thus, many successful *Escherichia coli* expression systems have been described and are available, from a variety of academic and commercial sources. But the efficient production of recombinant proteins depends on a variety of factors, such as optimal expression signals (both at the level of transcription and translation), correct protein folding and cell growth conditions. Although high yield may be achieved in the first expression attempt, proteins vary in structural stability, solubility, and toxicity, resulting in different rates of protein degradation, formation of insoluble inclusion bodies (IB), and cell death; thus, optimization of the process is usually required (Doyle, 2005; Schumann and Ferreira, 2004).

Despite the great diversity of expression systems - including expression vectors, host strains, and growth medium, among others - for heterologous protein production (Niiranen *et al.*, 2007) the major drawbacks of *E. coli* as an expression system include: 1) the inability to perform many of the posttranslational modifications found in eukaryotic proteins; 2) the lack of a secretion mechanism for the efficient release of protein into the culture medium; and 3) the limited ability to facilitate extensive disulfide bond formation (Esposito and Chatterjee, 2006; Jana and Deb, 2005; Sahdev *et al.*, 2008).

Under normal cellular conditions, a subset of cytoplasmic proteins are able to fold spontaneously (Anfinsen, 1973). However, many over-produced recombinant proteins in artificial *E. coli* factories accumulate as IB. These aggregated proteins are misfolded or partially-folded intermediates, clustered through inter- or intra-molecular interactions of solvent-exposed, hydrophobic polypeptide stretches (Stampolidis *et al.*, 2009). Their formation is attributed to the inability of the bacterial cell factories to maintain protein quality control during over-expression or thermo-induction of heterologous proteins. Thus, expression conditions must be found which balance heterologous protein production and host physiology, to optimize the overall yield of the active product.

Although no universal approach has been established for the efficient folding of recombinant proteins (Sorensen and Mortensen, 2005 a,b), several factors contributing to protein aggregation must be accounted: 1) lack of sufficient amount of the appropriate catalytic and molecular chaperoning machinery, e.g. trigger factor (Maier *et al.*, 2005), DnaK-DnaJ-GrpE (Martinez-Alonso *et al.*, 2006; MartÌnez-Alonso *et al.*, 2007; Xu and Sigler, 1998) and GroEL-ES or other heat-shock proteins (Gonzalez-Montalban *et al.*, 2008); 2) failure of the cognate co- or post-translational modifying enzymes (Sklar *et al.*, 2007) to operate in an orderly manner or to repair the misfolded structures (Gonzalez-Montalban *et al.*, 2006); 3) failure to maintain precursors in an unfolded state, for their localisation/translocation to extra-cytoplasmic compartments, which may also contribute to the formation of protein clusters (Stampolidis *et al.*, 2009).

Several approaches have been developed with the purpose of avoiding the expression of insoluble protein, including: fusion tags; optimization of expression conditions (e.g., medium, inductor concentration, temperature); expression host; co-expression of chaperons, among others (Figure 4).



Without tags	With tags
Test various temperatures	Use fusion technology
Test various expression strains	Express fragments
Test various media	Stabilize mRNA
Test transcription rates	Screen for soluble variants
co-expression with chaperons	Use alternative technologies
Include tRNA complementation plasmids	

Figure 4 - Summary of strategies to express soluble recombinant proteins (Adapted from Sørensen *et al.* 2005).

Enhancing solubility of recombinant proteins

As shown in figure 4, the insolubility of recombinant proteins may be reduced by optimizing the expression conditions, such as temperature, growth media, induction parameters, promoters and *E. coli* strain (Baneyx, 1999; Demain and Vaishnav, 2009; Hoffmann *et al.*, 2004; Schumann and Ferreira, 2004).

A well-known approach to limit the *in vivo* aggregation of recombinant proteins consists on the cultivation of the bacteria at reduced temperature (Baneyx and Mujacic, 2004; Garg and Quinones, 1997). Lowering the concentration of the inducer, when using expression systems with an inducible promoter, also contributes to enhancing the solubility of recombinant protein produced in *E. coli* (Schein, 1989). Using a lower incubation cell growth temperature and inductor concentration in culture media slows down the rate of protein synthesis, minimizing the saturation of the cellular folding machinery and aggregation (Ellis and Minton, 2006; Rabhi-Essafi *et al.*, 2007). The aggregation reaction is favored at high temperature, because of the strong temperature dependence of hydrophobic interactions (Kiefhaber *et al.*, 1991). It has been reported that the direct consequence of decreasing temperature is the partial elimination of heat-shock proteases, induced during foreign protein over-expression (Chesshyre and Hipkiss, 1989). Moreover, expression and activity of a number of *E. coli* chaperones are increased at lower temperatures (around 30°C), contributing for proper folding of expression proteins (Hartl and Hayer-Hartl, 2009; Mogk *et al.*, 2002; Rabhi-Essafi *et al.*, 2007).
The utilization of different *E. coli* strains (with supplemental tRNA genes) is a strategy often used to overcome the codon-usage problems or the disulfide-bond formation necessary for proper folding (Sorensen and Mortensen, 2005a, b). In addition, expression systems that allow the secretion of recombinant protein for the periplasmatic space, or into the medium, are also available (Zhang and Huang, 2002). However, higher yields are normally obtained in the cytoplasm rather than in the periplasmic space. Cytoplasmic proteins can be exported, to simplify purification and facilitate correct folding. Thus, this must be done whenever proteins need disulfide bonds to fold properly, since the cytoplasm is a reducing environment. To achieve the secretion of these proteins into the periplasm, a fusion is made with a leader peptide at the N-terminus. To get the proteins out of the periplasm and released into the medium, osmotic shock or cell wall permeabilization is used (Demain and Vaishnav, 2009).

Co-expression of chaperons

The co-production of selected chaperones along with the recombinant protein has been largely explored, as a strategy to minimize aggregation of the product, with rather unpredictable and not always consistent results (Martinez-Alonso *et al.*, 2006).

Molecular chaperones are ubiquitous and highly conserved proteins that help other polypeptides reaching a proper conformation, without becoming part of the final structure. They are not true folding catalysts, since they do not accelerate folding rates. Rather, they prevent “off-pathway” aggregation reactions by transiently binding hydrophobic domains in partially folded polypeptides, thereby shielding them from each other and from the solvent. Molecular chaperones also facilitate protein translocation, participate in proteolytic degradation, and help proteins - that have been damaged by heat shock or other types of stress - regaining an active conformation (Baneyx and Palumbo, 2003).

Fusion proteins and Protein tags

A vast number of tags - proteins, domains or peptides - are available that may be fused to the target recombinant proteins, for different proposes (table 2). The advantages of using fusion proteins to facilitate purification and detection of recombinant proteins are well recognized. Nevertheless, it is difficult to choose the right purification system for a specific protein of interest.

Table 2- Examples of tags used to fuse to proteins and their applications.

Tag	Goal			References
	Purification	Solubility enhancer	Detection	
His-tag	X		X	(Goel *et al.*, 2000; Lomas-Lopez *et al.*, 2008; Loughran *et al.*, 2006; Terpe, 2003)
Strep II-tag	X			(Korndörfer and Skerra, 2002; Skerra and Schmidt, 2000)
T7-tag	X			(Arnau *et al.*, 2006; Chatterjee and Esposito, 2006)
FLAG-tag	X	X		(Arnau *et al.*, 2006; Einhauer and Jungbauer, 2001)
S-tag	X		X	(Arnau *et al.*, 2006; Backer *et al.*, 2002)
Hemagglutinin HA-tag	X		X	(Hage, 1999)
cMyc-tag	X		X	(Arnau *et al.*, 2006; Terpe, 2003)
Chitin-binding domain	X			(Humphries *et al.*, 2002)
Calmodulin-binding domain	X		X	(Durst *et al.*, 2008)
Cellulose-binding domain	X			(Terpe, 2003)
Glutathione S-Transferase GST	X	X	X	(Arnau *et al.*, 2006; Braun *et al.*, 2002; Kim and Lee, 2008; Shih *et al.*, 2002)
Maltose-binding protein MBP	X	X		(Nallamsetty and Waugh, 2006; Rachel B. Kapust, 1999; Sachdev and Chirgwin, 1998)
N-utilizing substance A NusA		X		(De Marco *et al.*, 2004; Kim and Lee, 2008; Nallamsetty and Waugh, 2006)
Thioredoxin Trx	X	X	X	(Kim and Lee, 2008; Sachdev and Chirgwin, 1998)
Small ubiquitin-like modifier SUMO		X		(Panavas *et al.*, 2009; Saitoh *et al.*, 2009)
Ubiquitin Ub		X		(Marblestone *et al.*, 2006)
Mistic	X			(Roosild *et al.*, 2005)
Green fluorescent protein GFP	X		X	(Waldo *et al.*, 1999)
Elastin-like polypeptides ELP	X	X		(Araujo *et al.*, 2009; Chow *et al.*, 2006; Ge *et al.*, 2005; Trabbic-Carlson *et al.*, 2004)

Purification Tags

The use of affinity tags to improve protein purification becomes indispensable tools for structural and functional proteomic initiatives (table 2). Much of the protein-purification technology is based on the use of affinity chromatography, in which an expressed recombinant protein is fused to a peptide having high affinity for a particular ligand.
The most popular tag for protein purification, based on the use of immobilized metal affinity chromatography (IMAC), is the polyhistidine-tag, a motif of at least six histidine residues (6xHis), held either at the N- or C-terminus of the protein. In addition, this tag may be used for protein detection, since antibodies recognizing 6xHis are commercial available (Goel *et al.*, 2000; Lomas-Lopez *et al.*, 2008; Loughran *et al.*, 2006). Another common tag is the glutathione S-transferase (GST) tag, for purification on glutathione-based resins. Similarly, Strep-tag II, which consists of a streptavidin-recognizing octapeptide (WSHPQFEK), can be purified by affinity using a matrix with a modified streptavidin, being eluted with a biotin analog.

Another approach is based on antibodies-antigen interactions; for instance, affinity tags like FLAG octapeptide, cMyc, and hemagglutinin (HA) can be purified using the respective antibodies, immobilized on a chromatographic matrix (Arnau *et al.*, 2006; Sorensen and Mortensen, 2005b). Several other affinity tags exist and have been extensively reviewed (Terpe, 2003).

More recently, a new purification tag has been described, based on the transition properties of an Elastin-like polymers (ELP) and their ability to retain this inverse temperature-phase transition when conjugated to other molecules (Chow *et al.*, 2006; Trabbic-Carlson *et al.*, 2004).

Solubility-Enhancing Tags

The enhancement of the recombinant protein solubility may be achieved by fusing a soluble protein or peptide at its N-terminal. The fusion of a soluble partner - at the protein N- terminal - may act as chaperon (Kim *et al.*, 2007). No single fusion tag may be expected to increase the expression and solubility of all target proteins; however, some fusion tags have been more successful than others in increasing the proteins solubility.

Solubility-enhancing tags are generally large peptides, or proteins, that increase the expression and solubility of the partner fusion proteins (Hammarström *et al.*, 2006).
Recently, several comparative studies examined the effect of various fusion partners on the total and soluble protein expression yields (Table 3). Considering the data from these comparative studies, it is clear that the establishment of the best fusion partner for difficult-to-express proteins in *E. coli* remains empirical (Marblestone *et al.*, 2006; Sorensen and Mortensen, 2005a, b).

Table 3- Examples of comparative studies that examine the effects of various fusion partners on heterolougues protein solublility expression yield.

Reference	Proteins	Results
(Braun *et al.*, 2002)	32	GST~MBP>CBP>His6
(De Marco *et al.*, 2004)	3	NusA>GST
(Dyson *et al.*, 2004)	20	Trx~MBP>His10>GST>GFP
(Hammarstrom *et al.*, 2002)	27	Trx~MBP~Gb1>ZZ>NusA>GST> His6
(Marblestone *et al.*, 2006)	3	Trx>SUMO~NusA>Ub~MBP~GST
	3	SUMO~NusA>Ub~GST~MBP~TRX
(Kim and Lee, 2008)	2	Trx> GST>NusA>His
(Shih *et al.*, 2002)	40	NusA~MBP>GST>Trx~His6>Intein~CBP~CAP

The development of new tags for enhanced protein solubility is still ongoing. For instance, Araújo *et al.* suggested that ELP-tags, besides performing as a purification-tag, acts by enhancing the solubility of subtilisinE in *E. coli* (Araujo *et al.*, 2009). Recently, also it has been described that the fusion of chaperones DnaK or GroEL with two target proteins, which are expressed in insoluble form in *E. coli*, resulted in their soluble expression (Kyratsous *et al.*, 2009).

Tags-cleavage

Despite the advantages, the addition of a tag to a fusion protein has also been reported to negatively affect the target protein resulting in e.g., 1) a change in protein conformation (Chant *et al.*, 2005), 2) lower protein yields (Goel *et al.*, 2000), 3) inhibition of enzyme activity (Cadel *et al.*, 2004; Kim *et al.*, 2001), 4) alteration in biological activity (Du *et al.*,

2005; Kenig *et al.*, 2006), 5) undesired flexibility in structural studies (Smyth *et al.*, 2003) and 6) toxicity (Fonda *et al.*, 2002). Due to the somehow unpredictable changes that adding a tag may introduce in a protein and its behavior, it is usually desirable to remove the tag. Tag removal is often achieved utilizing specific endoproteases, to cleave a peptide sequence engineered between the tag and the target protein. The commercially available expression plasmids, with sequences encoding 6xhistidine or other tags, usually also code for a sequence that enables enzymatic removal of the tag. Among the proteases available for this purpose, the most commonly utilized are present in table 4.

Table 4- Examples of enzyme used to cleave tags in fusion protein (adapted from Arnau *et al.*, 2006)

Enzyme	Recognition site	Comments
Enterokinase	DDDDK↓	Secondary sites at other basic amino acid
Thrombin	LVPR↓GS	Secondary sites. Biotin labeled for removal of the protease
Factor Xa	IDGR↓	Secondary sites at GR
3C Protease	ETLFQ↓GP	GST tag for removal of the protease
TEV protease	EQLYFQ↓G	His-tag for removal of the protease
DAPase (TAGZyme)	Exo(di)peptidase	Cleaves N-terminal. His-tag (C-terminal) for purification and removal
Aeromonas aminopeptidase	Exopeptidase	Cleaves N-terminal, effective on M, L. Requires Zn
Aminopeptidase M	Exopeptidase	Cleaves N-terminal, does not cleave X-P
Carboxypeptidase A	Exopeptidase	Cleaves C-terminal. No cleavage at X-R, P
PreScission	LEVLFQ↓GP	GST tag for removal of the protease
SUMO	Conformation	No affinity purification per se (requires His-tag)
Sortase A	LPET↓G	Ca2+-induction of cleavage, requires an additional affinity tag (e.g., his-tag) for on column tag removal
Granzyme B	D↓X, N↓X, M↓N, S↓X	Serine protease. Risk for unspecific cleavage

↓- Cleavage site.

Inclusion bodies

Despite protein expression in the form of inclusion bodies (IB) being often considered undesirable, several examples demonstrate the production of functional proteins through IB solubilization and refolding. Thus, this approach presents some advantages in several cases and it has been most widely used for the commercial production of proteins (Demain and Vaishnav, 2009; Singh and Panda, 2005; Walsh, 2003).

The major advantages associated with the formation of IB are, namely: 1) expression of a very high level of protein, more than 30% of the cellular protein in some cases; 2) easy isolation of the IB from cells due to size and density differences, as compared with cellular contaminants; 3) lower degradation of the expressed protein; 4) resistance to proteolytic attack by cellular proteases; and 5) homogeneity of the protein of interest in IB (fewer contaminants) which helps in reducing the number of purification steps to recover pure protein. Thus, particularly when the expressed proteins have no easily detectable bioactivity (e.g. enzymatic), IB facilitates straightforward purification of the protein of interest (Demain and Vaishnav, 2009; Singh and Panda, 2005; Walsh, 2003).

The production of the pure, active protein, from IB requires the following steps: IB must be removed from the cell lysate, the proteins solubilized by denaturants- unfolding the proteins - and disulfide bonds must be eliminated using reducing agents. Refolding is accomplished by the removal of the denaturant and the reducing agent, followed by renaturation of the protein in a proper environment (Demain and Vaishnav, 2009).

1.3

Carbohydrate-binding modules: fuctions and applications

Adapted from: Moreira, S., Gama, F.M., Carbohydrate Binding Modules: functions and applications. In Carbohydrate Polymers: Development, Properties and Applications, Nova Science Publishers 2009. (Accepted)

The CBM Story

The first CBM was described in 1986 by Tilbeurgh and coworkers (Gilkes *et al.*, 1992; Gilkes *et al.*, 1988; Srisodsuk *et al.*, 1997; van Tilbeurgh *et al.*, 1986). These authors obtained two peptides by treating a cellulase from *Trichoderma reesei* with a protease; the higher molecular weight peptide retained the cellulolytic activity, while the smaller one exhibited cellulose affinity, therefore being designated a cellulose-binding domain. Later on, other CBM with different specificities were described. “Cellulose-binding domain” are thus a kind of a more general class of proteins, the “carbohydrate binding modules” (Boraston *et al.*, 2004; Shoseyov *et al.*, 2006a). CBMs are present in a large variety of enzymes, with different functions and substrate affinities, crossing a wide range of species, from archea, bacteria and virus to eukaryotic organisms, including fungi, plant and mammalian. The CBM specificities include crystalline cellulose, non-crystalline cellulose, chitin, β-1,3-glucans and β-1,3-1,4-mixed linkage glucans, xylan, mannan, galactan and starch (Boraston *et al.*, 2007). Furthermore, some CBMs display ‘lectin-like’ specificity, binding to a variety of cell-surface glycans (Boraston *et al.*, 2000). The number of CBM families is still growing and since the last review on the subject (Boraston *et al.*, 2007) another 10 families were described. Recent findings establish a connection between CBMs and host-pathogen interactions (Ficko-Blean and Boraston, 2006), *N*-gycosylation in eukaryotic organisms (Schallus *et al.*, 2008), cell energy balance (McBride

et al., 2009), among other functions. The knowledge of the CBM structures, elucidating their function and role in nature, may give rise to new biotechnological applications.

CBM classification

CBM Families

More than 300 proteins are currently classified in the CAZy database, including glycoside hydrolases (GHs), glicosyltransferases (GTs), polysaccharide lyases (PLs) and carbohydrate esterases (CEs). Usually, CAZymes present a modular structure, nearly 7% of the enzymes having at least one carbohydrate-binding module (Cantarel *et al.*, 2009). CBMs are also classified in families based on sequences similarities. Presently, 54 CBM families are described in the CAZy database (http://www.cazy.org/). There is considerable heterogeneity in binding specificity, towards crystalline, amorphous and soluble polysaccharides, both between and within the families (Boraston *et al.*, 2004; Boraston *et al.*, 2001; Boraston *et al.*, 2007; Hachem *et al.*, 2000). A CBM is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme, with a discreet fold having carbohydrate-binding activity (Shoseyov *et al.*, 2006a). CBMs contain from 30 to about 200 amino acids and exist as a single, double, or triple protein domains. The location within the parental protein can be either C- or N-terminal; occasionally, the CBM is centrally positioned within the polypeptide chain (Wang *et al.*, 2002; Zverlov *et al.*, 2001). A few exceptions include 1) CBMs that integrate the cellulosomal scaffoldin proteins and 2) those not associated with catalytic domains (rare instances of independent putative CBMs have been described) (Boraston *et al.*, 2007; Gilbert, 2003; Gilbert *et al.*, 2008).

Besides having modular architecture with independent structure and function, in the more general case integrating a protein with catalytic activity, CBMs are distinguishable from other non-catalytic sugar binding proteins (such as lectins and sugar transport proteins) by the scarcity of hydrogen bonds between CBMs and their target ligands; instead, binding is dominated by hydrophobic interactions (Boraston *et al.*, 2007).

The three-dimensional (3D) structures indicate that CBMs from different families share structural similarity. The carbohydrate binding capacity can be attributed, at least in part, to several aromatic amino acids defining an hydrophobic surface (Bayer *et al.*, 1998; Shoseyov *et al.*, 2006a; Vandermarliere *et al.*, 2009). Other features are also important for CBMs or CAZymes activity, namely the electrostatic environment (pH, ion strength) and

the presence of a linker (Nielsen *et al.*, 2001; Receveur *et al.*, 2002). For instance, the enzymatic activity of many different cellulolytic enzymes is affected by the deletion, shortening or lengthening of the linker region bridging the CBM and catalytic modules (Howard *et al.*, 2004; Receveur *et al.*, 2002; Rixon *et al.*, 1996; Shen *et al.*, 1991; von Ossowski *et al.*, 2005). Such findings suggest that the two domains act in concert on the cellulose surface during catalysis, and that a flexible linker is needed for full cellulolytic activity.

Cellulosomes

Cellulosomes are extracellular multiprotein complexes, first identified in early 1980s on the thermophilic anaerobic bacteria *Clostridium thermocellum*. Since then, several other cellulolytic bacteria and fungi have been reported to produce cellulosomes (Ding *et al.*, 2008b). In 1999, a cellulosome holding a GH in the scaffoldin subunity was described; later on Xu and colleagues (2004) reported another scaffoldin protein, from a *Bacteroides cellulosolvens* cellulosome that includes a cellulase (Ding *et al.*, 1999; Ding *et al.*, 2008b; Doi and Kosugi, 2004; Rincon *et al.*, 2005; Xu *et al.*, 2004a; Xu *et al.*, 2004b).

In general, two major types of subunit compose cellulosomes: the noncatalytic scaffoldin(s) and the catalytically active components (Figure 5). Each of these structures may be quite complex. The assembly of the cellulosome is facilitated by the high-affinity recognition between the scaffoldin cohesin and the enzymes dockerin modules. The scaffoldin often contains multiple cohesin modules, thereby enabling numerous different enzymes to be assembled into the cellulosome complex. In addition, in some species, such as *Acetivibrio cellulolyticus*, the cellulosomes present multiple scaffoldins with different cohesins (Xu *et al.*, 2004a). The interaction cohesin-dockerins is type and specie-specific.

Another important cellulosomal component is the cellulose-specific binding module, the major determinant of substrate recognition. Only a few enzymes in cellulosomes contain a CBM; this is normally present in the scaffoldin protein (Bayer *et al.*, 1998; Ding *et al.*, 2008b; Doi and Kosugi, 2004; Mingardon *et al.*, 2007). As shown for the first time by Goldstein and colleagues, the cellulose-binding protein A (CbpA) from *C. cellulovorans*, is a functionally independent domain of the scaffoldin protein (Goldstein *et al.*, 1993). Later on, Fierobe and coworkers, using a recombinant engineered cellulosome, showed that the

proximity of the cellulosomal enzymes and the presence of the CBM3 in the scaffoldin is responsible for the synergy among the components, resulting in the efficient degradation of the native substrate (Fierobe *et al.*, 2002).

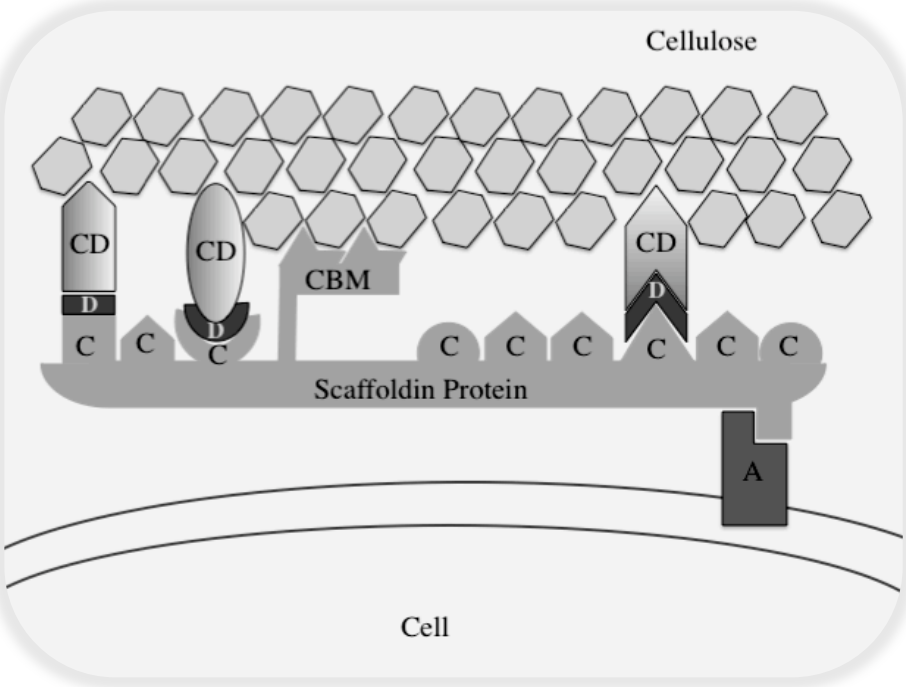


Figure 5 - Schematic representation of a cellulosome attached to the cell membrane. CD- catalytic domain; D- dockerin; C- cohesin, A- anchoring protein.

CBM types

The CBMs may also be classified according to the topology of the binding sites, reflecting the macromolecular structure of the target ligand (Boraston *et al.*, 2004; Boraston *et al.*, 2007). Despite the large variability of carbohydrate structures, three types of binding topologies have been identified. This classification is based on both structural and functional similarities. Although the 3D structure of a number of CBMs has been solved, most have not been functionally characterized as yet. Furthermore, the binding pattern of CBMs determined so far vary widely, even within each family. However, it was shown that the modules are composed almost exclusively of β-strands arranged in a "jelly roll" motif, whose topography reflects the macroscopic nature of the target substrate. CBMs with this fold recognize several polysaccharides: crystalline and non-crystalline cellulose, chitin, β-

1-3-glucans and β-(1-3)-(1-4)-mixed glucans, xylan, mannan, galactan, and starch. Some CBMs display 'lectin-like' specificity, binding to a variety of cell-surface glycans (Boraston *et al.*, 2004; Boraston *et al.*, 2007; Ding *et al.*, 2006). Other families of β-sandwich CBMs are beginning to emerge with more complex glycan-binding specificities (Abbott *et al.*, 2008; Gregg *et al.*, 2008; Hashimoto, 2006; McDonough *et al.*, 2004).

Based on the macroscopic nature of the target ligand, a classification of CBMs in three types, A, B or C, has been proposed. Table 5 presents the CBM families and corresponding type, A B or C. It should be noted that CBMs with type A and B-topology are found in the same family, while other still remains to be classified.

Table 5- CBM types and families.

CBMs Types	A	B	C	Unknown
Families	1, 2, 3, 5, 10 49	4, 6, 11, 15, 17, 22, 27, 28, 29, 30, 35, 36, 44 (?) 2, 20, 25, 26, 34	9, 13, 14, 18, 32, 40, 42	8, 12, 16, 19, 21, 23, 24, 31, 33, 37, 38, 39, 41, 43

Type A CBMs

Type A CBMs, with affinity for crystalline cellulose and chitin, display aromatic amino acid residues forming a planar hydrophobic surface that interacts with the glucosyl-pyranose ring of the substrate. These CBMs recognize multiple cellulose chains and strongly prefer insoluble microfibrils, such as cellulose or chitin, to soluble polysaccharide molecules (Bolam *et al.*, 1998; Boraston *et al.*, 2004; Boraston *et al.*, 2007; Shoseyov *et al.*, 2006a).

Type B CBMs

Type B, the commonest class of CBMs, bind less-ordered plant structural polysaccharides such as amorphous cellulose, mannan, or xylan (Boraston *et al.*, 2007). Conversely to

type A, type B CBMs have a cleft that accommodates a single chain of the poly/oligosaccharide ligand (Notenboom *et al.*, 2001), comprising several sub-sites able to interact with the individual sugar units of the polymeric ligand. Although ligands are recognized by aromatic side chains, similarly to type A CBMs, the side chains of tryptophan and tyrosine – and less commonly phenylalanine - form planar, twisted or sandwich platforms for ligand binding (Hashimoto, 2006).

Type C CBMs

Type C CBMs have a solvent-exposed binding pocket or blind canyon, small binding sites which interact with mono or disaccharides. Thus, these CBMs are lectin-like, lacking the extended binding site grooves present in type B CBMs (Boraston *et al.*, 2007). Indeed, type C proteins (i.e. CBM13, 14 and 18 families) were initially identified as lectins. Indeed, both kinds of protein are thought to share similar evolutionary origins. They are involved in toxin delivery, oligosaccharide synthesis, and in host-microbe interaction processes (Boraston *et al.*, 2007; Gunnarsson *et al.*, 2007).

CBM at work

In general, CBMs are linked to GHs that degrade insoluble polysaccharides. Although many of these modules target components of the plant cell wall or insoluble storage polysaccharides (cellulose, starch, glycogen), CBMs also bind soluble oligosaccharides such as malto-oligosaccharide (Boraston *et al.*, 2007). Indeed, the non-catalytic CBMs are recognized as an essential component of several CAZymes and are thought to have three primary functions: proximity effects, substrate targeting and microcrystallite disruption (Bolam *et al.*, 1998; Boraston *et al.*, 2004; Eriksson *et al.*, 2005; Henshaw *et al.*, 2006). More recently, multivalency was also described for tandem CBMs (Boraston *et al.*, 2002; Vaaje-Kolstad *et al.*, 2005). These functions are important in several biological mechanisms, such as substrate binding, mediation of protein-protein interactions or cell surface anchoring. Recently, putative cellulose-binding modules that do not bind cellulose were described. Three homologous CBM3b modules from A. cellulolyticus and *C. thermocellum* were over-expressed, and surprisingly none bound to cellulosic substrates (Jindou *et al.*, 2006). These results raise fundamental questions concerning the possible role(s) of the newly described CBMs. Phylogenetic analysis and preliminary site-directed

mutagenesis studies suggest that the status of the family-3 CBMs and of the family-9 GHs is much more intricate and diverse than hitherto considered (Jindou *et al.*, 2006).

The proximity effect

CBMs promote the association of the enzyme with the substrate (Figure 6), insuring a prolonged contact, and thereby increasing their effective concentration (proximity effect) (Bolam *et al.*, 1998; Reinikainen *et al.*, 1992). In fact, several studies show that enzymes fail to effectively perform when the CBM is removed by proteolysis or by recombinant DNA technology (Boraston *et al.*, 2007; Gilkes *et al.*, 1991; Levy *et al.*, 2002a; Linder *et al.*, 1998; Shoseyov *et al.*, 2006a; Tomme *et al.*, 1995). This effect is observed mostly in enzymes that act on insoluble substrates and in cellulosomes.

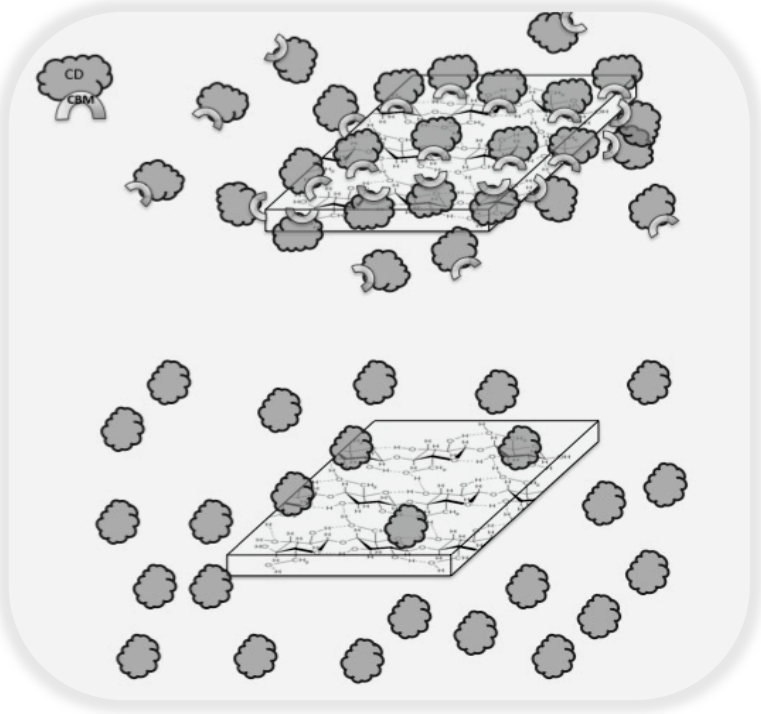


Figure 6 – Schematic representation of the CBM mediated proximity effect. CAZymes with CBMs are able to bind to the insoluble substrates (such as crystalline cellulose) increasing the effective concentration of enzyme on substrate.

The targeting effect

CBMs have been shown to have selective substrate affinity (Figure 7), distinguishing different crystalline, amorphous, soluble and non-soluble polysaccharides (targeting function) (Ganesh *et al.*, 2004; Lamed *et al.*, 1994; Linder and Teeri, 1997; Tomme *et al.*, 1995; Wang *et al.*, 2002). In 2004, Boraston *et al.* reviewed the mechanisms of polysaccharide recognition (Boraston *et al.*, 2004). Since then, other CBMs with novel specificities were described (Gregg *et al.*, 2008; Henshaw *et al.*, 2006). The data suggest that CBMs have fine specificity for polysaccharide substructures. Thus, CBMs may be highly specific, subtle structural differences leading to diverse ligand specificity. This makes them an attractive system for biotechnological applications, namely as tools for the elucidation of protein-carbohydrate interaction mechanisms and as probes to identify different polysaccharides in plant cell-walls (McCartney *et al.*, 2004).

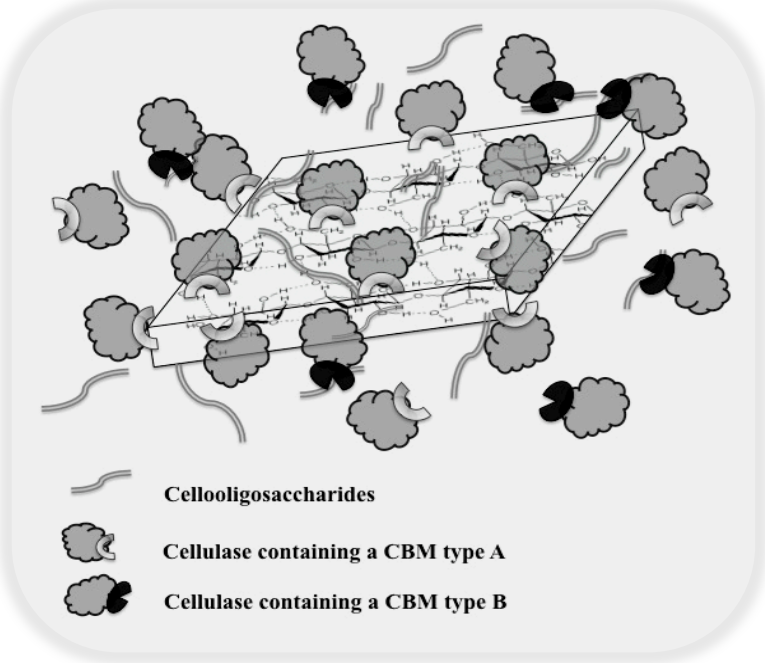


Figure 7– Schematic representation of the targeting effect of CBMs, showing the specificity of the CBM type A for insoluble subtract (such as crystalline cellulose) and CBM type B for soluble derivates of cellulose (such as cellooligosaccharides)

The disruptive effect

The concept of the CBM disrupting function (Figure 8), rendering the substrate more susceptible to enzymatic hydrolysis, emerged several years ago (Boraston *et al.*, 2004; Din *et al.*, 1994). It was first demonstrated in 1991, by Din *et al.* The non-catalytic cellulose-binding domain, isolated by these authors from endoglucanase A (*Cellulomonas fimi*), was able to disrupt the cellulose fibers, releasing small particles. Further, it was showed that the isolated catalytic domain did not disrupt the fibril structure, rather polishing the fibers surface (Din *et al.*, 1991). Other cellulase-associated CBMs with similar effect on cellulose fibers have been described (Gao *et al.*, 2001). Recently, it was showed that the CBM from CBHI (cellobiohydrolase I from *T. pseudokoningii* S-38) not only addresses the enzyme to the cellulose fibrils, but it also is involved in the structural disruption of the cellulose fiber surface (LuShan *et al.*, 2008).

The disruption effect was also reported for starch-binding modules (Giardina *et al.*, 2001) and for expansins, which have significant sequence identity with microbial cellulases (Cosgrove, 2000; Levy *et al.*, 2002b).

Recently, Vaaje-Kolstad and colleagues demonstrated that also chitin-binding modules have similar disruption ability. They showed that crystalline chitin is disrupted by a non-catalytic protein, leading to an increase in substrate access for a range of chitinases (Vaaje-Kolstad *et al.*, 2005). The modification of cellulose fibers with CBMs may lead to improved properties of textile and paper pulps (Pinto *et al.*, 2004).

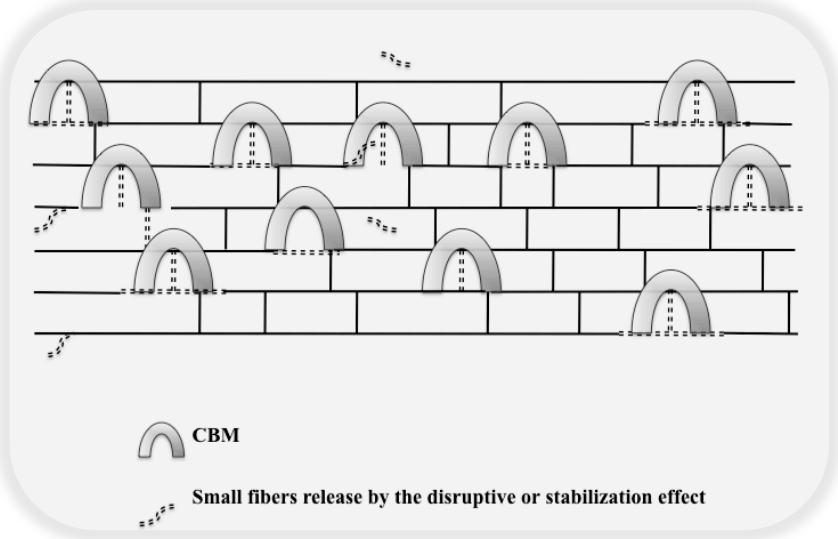


Figure 8 – Schematic representation of the disruptive effect of the CBMs on polysaccharide fibers.

The avidity effect

CBMs can be present in single, tandem or multiples copies within the enzymes architecture (Flament *et al.*, 2007; Shoseyov *et al.*, 2006a). It has been shown that they can bind their specific glycan targets when isolated from the parent molecule, behaving in a cooperative manner when organized in tandem (Boraston *et al.*, 2002; Crennell *et al.*, 1994).

Boraston and coworkers identified a family 6 CBM present as a triplet in *C. stercorarium*. The multiple modules act cooperatively in the binding process. It has been suggested that the duplication or triplication of CBMs may, evolutionary, balance the loss of binding affinity of thermophilic GHs at higher temperatures (Boraston *et al.*, 2002).

The analysis of CAZymes showed that the same enzyme may be linked to several CBMs (CBM multimodularity), with similar or dissimilar binding specificity. The authors speculate that the homogenous multimodularity increases the avidity of the CAZyme for the substrate, while heterogeneous multimodularity allows the enzyme to bind heterogeneous substrates (Abbott *et al.*, 2008; Gregg *et al.*, 2008).

Recently, a recombinant protein containing tandem repeats of the CBM40 from a *V. cholerae* sialidase was constructed. Identical copies of CBM40 can be fused and manipulated in order to enhance its affinity through avidity (Connaris *et al.*, 2009). This approach may be used for the creation of high affinity, multivalent CBMs, that may have broad application in glycobiology.

CBMs and physiologic function

Protein-carbohydrate recognition plays a pivotal role in key biological processes. These macromolecular interactions are central in host-pathogen recognition events, cell–cell communication, cellular defense mechanisms, protein trafficking, and on carbon recycling through the degradation of the plant cell wall.

Although CBMs are described to play a role in several biological processes, such as: 1) protein turn over (Glenn *et al.*, 2008; Schallus *et al.*, 2008); 2) energy balance and diseases (Burnaugh *et al.*, 2008; Hurtado-Guerrero *et al.*, 2009; Langley *et al.*, 2008; McBride *et al.*, 2009; Minassian *et al.*, 2000; Sheldon *et al.*, 2006; Treviño *et al.*, 2008; Wang *et al.*, 2002); 3) plant growth, defense and degradation (Boraston *et al.*, 2004;

Brotman *et al.*, 2008; Catala and Bennett, 1998; Obembe *et al.*, 2007; Safra-Dassa *et al.*, 2006; Turner *et al.*, 2007; Urbanowicz *et al.*, 2007; Valdez *et al.*, 2008; Zhang *et al.*, 2008); only the CBM applications will be reviewed.

CBM applications

The production of CBMs by enzymatic hydrolysis of the enzyme-containing CBMs was described by several authors (Lemos *et al.*, 2000; Pinto *et al.*, 2006; Tilbeurgh *et al.*, 1986). However, the recombinant DNA technology allows for several structural and functional studies, and also for different applications using recombinant proteins fused with CBMs. The utilization of recombinants CBMs for different applications have been described: the improvement of fibers in textile and paper industry; as tags in recombinant proteins for solubilization, purification and immobilization; as probes for protein-carbohydrate interaction and microarrays; CBMs may also find applications in the modification of physical and chemical properties of composite materials, allowing the creation of new materials with improved properties. The CBMs expression *in vivo* may be also a valuable tool to modify plant characteristics, as discussed above (Levy *et al.*, 2002b). Several studies have shown the potential of CBMs for modifying the characteristics of several enzymes. The basic approach in CBM engineering consist in the addition or substitution of a CBM in order to improve the enzyme stability or hydrolytic activity (Ding *et al.*, 2008a).

CBMs in the paper industry

Since CBMs, which do not have catalytic activity, can modify the polymer structure of cellulose and starch materials, they have been also tested in the papermaking process. Several strategies were developed using CBMs or CBMs-conjugates (Kenealy and Jeffries, 2003; Kitaoka and Tanaka, 2001; Yokota *et al.*, 2009).

Recently, Machado *et al.* (2009) demonstrated that a recombinant CBM from *C. thermocellum* conjugated with polyethilenoglycol (PEG) effectively improves the pulps drainability, without significant effects on the strength parameters. Furthermore, the authors showed that the CBM alone does not modify the pulp properties, suggesting that the improved pulp drainability, reported by several researchers, is indeed a strictly

interfacial effect, surface hydration playing a key role. Taken together, the results suggest that CBM technology may have valuable applications in paper recycling.

Recombinant CBMs were also used to improve paper properties. The dry strength of a 3D cellulose fiber network depends on the strength of the individual fibers, of the inter fiber bonds, and on the number and distribution of interfiber bonds. Inter-fiber bonding, which improves the stress transfer between the fibers under tensile deformation, is one of the most important factors affecting the overall stress development in the fiber web. Dry strength additives improve the bonding between the fibers, thus resulting in a marked increase in the dry strength (Levy *et al.*, 2004). Levy *et al.* constructed a bifunctional protein, containing two-fused cellulose-binding modules (CBM3 from *C. cellulovorans*), able to mimic the chemistry of cellulose cross-linking (Levy *et al.*, 2002a), thus increasing the dry strength of paper. Interestingly, applying a single CBM to the paper also improved its mechanical properties, although to a lower extent. In addition, paper sheets treated with the fusion protein became more hydrophobic and demonstrated water-repellent properties (Levy *et al.*, 2002a). Later on, the same authors constructed another bifunctional protein containing a cellulose and a starch-binding module. The treatment of paper fibers with the recombinant protein, together with corn-starch, improved the paper dry strength (Levy *et al.*, 2004). The significant improvement in the mechanical and surface properties of paper by CBMs-containing molecules demonstrates great potential for the bioengineering of novel paper-modification reagents (Levy *et al.*, 2002a).

CBMs in the textile industry

The textile industry requires large amounts of water, energy, and auxiliary chemicals (Feitkenhauer and Meyer, 2001). The search for environmental-friendly methods has lead to the utilization of enzymes. Several enzymes have been used in textile processes in order to achieve improved and fashionable fabric properties. Among other enzymes for textile processing, amylases (used for desizing), cellulases (denim finishing), laccase (decolourization of textile effluents and textile bleaching) are commercially available (Araújo *et al.*, 2008; Saravanan *et al.*, 2009).

Textile fabric may also be treated with isolated CBMs or CBMs fused with other molecules or enzymes. Banka *et al.* demonstrated that a fibril-forming protein from *T. reesei* causes non-hydrolytic disruption of cotton fibers (Banka *et al.*, 1998). Lee *et al.* obtained images,

by atomic force microscopy, of holes left in cotton fibers treated with inactivated CBH I. The holes are attributed to the penetration of fibers by the binding domain (Lee *et al.*, 2000). It has been shown that the surface of ramie cotton is roughened by treatments with CBM2 from *C. fimi*. Gilkes *et al.* proposed that the treatment of cellulosic fibers with CBMs could be used in order to alter the dyeing characteristics of cellulose fibers (Gilkes *et al.*, 1998). Indeed, it was showed that CBM treatment increased the dye affinity of cotton fibers, especially in the case of acid dyes (Cavaco-Paulo *et al.*, 1999).

Fukuda *et al.* used a new approach for the enzymatic desizing of starched cotton cloth. Sizing is required to prevent abrasion, fluffiness, and cutting of the warp during the weaving process. Among the several desizing methods, the use of enzymes (e.g.amylase) is well known as an environmental-friendly technology (Murai *et al.*, 1997). Instead of using an enzyme for desizing, Fukuda *et al.* constructed a yeast strain that codisplayed glucoamylase and CBMs on the cell surface. The yeast cell acquired specific binding ability to cotton-cloth with glucoamylase activity. Furthermore, the codisplaying strain showed greater activity than a strain displaying only glucoamylase activity (Fukuda *et al.*, 2008).

The development of biotechnological tools for the modification of cellulose fibers may be achieved by combining CBMs, specially cellulose binding modules, with catalytic domains of enzymes that do not normally act on insoluble substrates (e.g. laccase, pectinase or lipase), or with other functional proteins/polypeptides (e.g. hydrophobic or chemically reactive) suitable for the modification of the textile surfaces. Since several CBMs belong to enzymes that act in extreme conditions, the CBM fusion proteins may also improve enzyme stability (Charnock *et al.*, 2000; Ding *et al.*, 2008a; Kim *et al.*, 1998; Zhao *et al.*, 2005a). Further, CBMs can be fused with bioactive molecules in order to functionalize the fabric tissue.

CBMs in the food industry

Enzymes have been used for more than 20 years in poultry feed, mainly to improve the digestibility of cereals with high soluble non-starch-polysaccharide (NSP) levels, such as wheat, barley, oats and rye (Wanga *et al.*, 2009; Yua *et al.*, 2007). It is well established that the inclusion of cell wall hydrolases in wheat, barley and rye-based diets of single-

stomach animals, improves the efficiency of feed utilization, enhances growth and contributes to a better use of low-cost feed ingredients (Chesson, 1993; Ribeiro *et al.*, 2008). Several studies on the potential application of CBMs for animals feeding are available (Fontes *et al.*, 2004; Guerreiro *et al.*, 2008a).
Recently, Ribeiro *et al.* studied the effect of supplementing a barley-based diet with a family 11 β-glucan-binding domain, fused to a recombinant cellulase from *C. thermocellum*. The results showed that birds fed on diets supplemented with the recombinant proteins, containing the CBM11 or the commercial enzyme mixture, have improved performance when compared to birds fed with diets without the enzyme supplement (Ribeiro *et al.*, 2008).

Recombinant enzymes containing CBM may also find applicability on the food industry, for instance for the production of soy sauce using soybeans and optionally other vegetable ingredients, such as wheat and rice. During the production process, starch and other carbohydrates are degraded into sugars, used for aroma development by fermentation. It has been found that amylolytic enzymes comprising a CBM leads to an increased rate of starch hydrolysis, as compared to amylolytic enzymes without CBM, under conditions relevant for soy sauce production (Nielsen and Viksoe-Nielsen, 2007).

CBMs as a microarray and probing tool

Several strategies are described to immobilize the probe on the support, including adsorption, physical entrapment or covalent binding. The CBM based microarray technology described by some authors offer fundamental advantages over current non-DNA microarray technology, such as retention of protein functionality after immobilization, ease of fabrication, extended stability of the printed microarray, integrated test for quality control (QC) and the capacity to print test proteins without a purification step (Filonova *et al.*, 2007a; Filonova *et al.*, 2007b; Ofir *et al.*, 2005). These features, together with the intrinsic specificity of CBMs for individual carbohydrates and the facile modification with peptides and fluorescent molecules, allow for efficient production of protein and peptide microarrays. These can be used in a variety of potential applications technically impractical via conventional microarray technologies (Filonova *et al.*, 2007a).

Ofir and colleagues developed a microarray system using an affinity-based probe immobilization strategy. They fused the exceptionally stable family-3a CBM, from the cellulosome of *C. thermocellum*, with antibodies or peptides. The recombinant proteins were immobilized on cellulose surfaces by specific adsorption and used for serodiagnosis of human immunodeficiency virus patients (Cretich *et al.*, 2006; Ofir *et al.*, 2005).

The plant cell wall biology studies require more sensitive and specific probes to target individual wall components. Traditionally, antibodies have been the primary workhorses for the spatial localization of cell wall polysaccharides. Currently, nearly 30 monoclonal antibodies directed toward specific arabinan, galactan, xylan, galacturonan, fucosylated xyloglucan, and cell wall glycoprotein epitopes are available, from academic and commercial sources (Gunnarsson *et al.*, 2006). Nevertheless, CBMs may be used for this purpose, since they present intrinsic specificity for individual carbohydrates.

A quantitative fluorimetric method for the analysis of crystalline cellulose on fiber surfaces was developed. This method quantitatively shows differences in crystalline cellulose binding sites of differently processed pulp fibers. The results indicated that CBMs provide useful, novel tools for monitoring changes in carbohydrate content of non uniform substrate surfaces, for example, during wood or pulping processes and possibly also during fiber biosynthesis (Filonova *et al.*, 2007b).

The CBM4-2 from xylanase of *Rhodothermus marinus* was synthesized and utilized *in vivo* as a xylan-specific protein, for the analysis of hemicelluloses in wood and fibrous materials. It is well known that the CBMs specificity may be altered by genetic engineering; in particular, the CBM4-2 was modified through direct mutagenesis. Variants with specificity for two other polysaccharides were identified using phage display technology (Cicortas Gunnarsson *et al.*, 2004).

CBMs as a protein solubilization, purification and immobilization tool

Several works describe the use of CBMs as a tag for recombinant protein purification (Boraston *et al.*, 2001; Ito *et al.*, 2004; Kavoosi *et al.*, 2007a; Kavoosi *et al.*, 2004; Kavoosi *et al.*, 2007b; Rodriguez *et al.*, 2004; Shpigel *et al.*, 2000) and enzyme immobilization (Ong *et al.*, 1989; Richins *et al.*, 2000; Xu and Foong, 2008). Depending on the binding reversibility, different applications may be envisioned; CBMs with 'irreversible' binding has

limited usefulness as an affinity tag for protein purification, because desorption may require strongly denaturing conditions. In turn, such a CBM may be a very useful tag for enzyme immobilization (Kwan *et al.*, 2005).

An obvious extension of the CBM-fusion technology is to enable a single-step purification and immobilization of fusion proteins by generating active CBM–Protein. Moreover, the utilization of the carbohydrate affinity system, such as cellulose, is attractive because it does not require a derivatized matrix, and cellulose is available in a variety of inexpensive forms, such as preformed microporous beads, highly adsorbent sponges or cloth and microcrystalline powders (Richins *et al.*, 2000).

In fact, several CBMs were already commercialized as protein expression systems (Xu and Foong, 2008). A cellulose-binding module from *C. cellulovorans* scaffoldin CbpA protein has been well characterized and commercialized as a fusion domain for protein purification, using a cellulose matrix (Novagen). In such applications, the use of CBMs offers many industrially attractive advantages. Since CBMs adsorb spontaneously to cellulose, very little or no pretreatment of the samples is required prior to immobilization (Linder *et al.*, 1998; Morassutti *et al.*, 2002). In addition, some CBMs seem to enhance the solubility of recombinant protein (Murashima *et al.*, 2003; Yeh *et al.*, 2005).

Craig and colleagues described the design and application of a recombinant fusion protein containing a cellulose-binding domain (from *C. cellulovorans*) and an antibody-binding domain (protein LG), for direct immobilization of antibodies and cells onto regenerated cellulose hollow fiber membranes. Hollow fiber affinity cell separation is a monoclonal antibody based cell separation process. Cells are bound directly or indirectly via surface epitopes by monoclonal antibody or secondary ligand immobilized on the lumen side of hollow fibers. Deposited cells are fractionated, on the basis of adhesion strength, using the uniform shear field generated by the culture medium flowing through the hollow fiber modules with well-defined header geometry (Craig *et al.*, 2007). With this strategy, several problems associated to covalent binding are avoided: low coupling yield, random orientation of antibody, possible alteration of the structural properties of the hollow fiber membrane resulting from chemical cross-linking or protein degradation.

CBM as bioremediation tool

Another field for CBM application is bioremediation. Richins *et al.* produced a bifunctional fusion protein, consisting of an organophosphate hydrolase (OPH) linked to a *Clostridium*-derived cellulose-binding module. The recombinant hydrolase is highly effective in degrading organophosphate compounds. Furthermore, the CBM enable the purification and immobilization onto different cellulosic materials, in a single step (Richins *et al.*, 2000). In this manner, OPH-activated cellulose materials are generated for a variety of relatively low cost applications, such as reactors with immobilized enzyme for the detoxification of hazardous organophosphates (Richins *et al.*, 2000).
In another study, Xu *et al.* presented a strategy to remove heavy metals from contaminated waters. They reported the cloning and expression of a bifunctional fusion protein, consisting of a synthetic phytochelatin linked to a *Clostridium*-derived cellulose-binding domain. Once again, the CBM enabled purification and immobilization of the fusions onto different cellulose materials, in a single step. The immobilized sorbents were shown to be highly effective in removing cadmium present in parts per million levels (Xu *et al.*, 2002).

CBM as biomedical tool

Cellulose is a chemically inert matrix that has stable physical properties, as well as low affinity for non-specific protein binding. It is pharmaceutically safe and relatively inexpensive. The binding of biomolecules to cellulose through a cellulose-binding domain further enhances its potential as a scaffold or carrier material.

Maurice *et al.* fused an antigen protein (from *Aeromonas salmonicida*) with a CBM (from *C. cellulovorans*), in order to develop a vaccine suitable for fish immunization. Vaccines vary in their efficacy depending on the antigen composition and accompanying adjuvant. Studies have shown that soluble immunogens rarely induce high titers of antibodies, unless strong adjuvants are used (Maurice *et al.*, 2003). Surprisingly, binding Orbicell cellulose beads to a recombinant protein, Maurice and colleagues obtained a significant adjuvant effect. In addition, Orbicell cellulose beads were well tolerated by the fish and no deleterious response reactions were detected (Maurice *et al.*, 2003).

Guerreiro *et al.* recently described the expression of antimicrobial peptides (AMPs) fused with a CBM3 from *C. thermocellum* in a bacterial host. AMPs are cationic molecules with a wide range of antimicrobial activities. The authors suggested CBM3 as a good candidate to overcome difficulties related to the expression of these molecules, namely associated to the small size and potential toxicity for host (Guerreiro *et al.*, 2008b). Furthermore the authors suggested the possible use of the fusion CBM-AMP to confer antimicrobial properties to cellulosic materials.

CBMs were also described as a tool to adsorb bioactive peptides to carbohydrate-based materials (Andrade *et al.*, 2008; Carvalho *et al.*, 2008b). Bacterial cellulose is being studied as a biocompatible scaffold for the engineering of cartilage and blood vessels, wound dressing, guided tissue regeneration, among other applications (Svensson *et al.*, 2005). Andrade *et al.* cloned and expressed a recombinant protein containing a cellulose-binding module (CBM3 from *C. thermocellum* cellulosome) fused with a tripeptide of Arg-Gly-Asp (RGD sequence is a ligand for integrin-mediated cell adhesion), showing that the bifunctional protein improved the fibroblast adhesion and spreading on bacterial cellulose (Andrade *et al.*, 2008).

The utilization of a recombinant CBM (a domain from the Celk gene from *C. thermocellum*) to stabilize single-walled carbon nanotubes (SWNTs) in water was recently described (Xu *et al.*, 2009). After production of SWNTs, the strong non-covalent interactions give rise to aggregated material. Functional molecules including surfactants, polymers, carbohydrates, nucleic acids and peptides or proteins have been reported to debundle and suspend SWNTs via a non-covalent adsorption. A family 4 CBM, cloned and over-expressed in *E. coli*, was successfully used to stabilize SWNTs. However, the mechanism of SWNTs - protein interaction has not been explained. Moreover, another recombinant CBM belonging to family 3 (type A) was also tested, but it did not show binding affinity for SWNTs. The authors suggested that, beside aromatic residues, higher-order protein structure could also play a key role (Xu *et al.*, 2009).

Future perspectives

Although the functions of CBMs were firstly related with cellulase and other enzymes activity, the current research and development in the CBMs field heads in different

directions. In recent years, besides the utilization in textile or paper industry, the CBMs are seen as tools for biomedical application. CBMs are involved in anabolic processes (such as oligosaccharide synthesis), host-microbe interaction, toxin delivery, recognition of complex glycan present on eukaryotic cell surface and extracellular matrix. CBMs may thus be used as tools to elucidate several carbohydrate-protein interactions and targets for the modulation of those processes.

The determination of the 3D structures and mechanism of action of protein modules, such as CBM from family 6 or 2a (Michel *et al.*, 2009) is still ongoing. The finding of new 3D structures may help elucidating the evolution of CBMs.

The combined effect of CBMs from GHs in the recognition of host glycans by bacteria for pathogenesis, colonization, as a nutritional source, and evading the host immune system, defines a new avenue of CBM research, apart from plant cell wall recognition. Future studies might also reveal new avenues for biotechnology applications, such as design of antibacterial or anti-carcinogenic drugs, functionalization of biomaterials or cloth.

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Chapter 2

Functional expression of the human CBM from Laforin

ABSTRACT

The goal of this work was the development of a methodology for the expression and purification of a recombinant Carbohydrate-Binding Module (CBM) from the human protein Laforin. When fused with the bioactive peptides, CBMs may dramatically improve the biocompatibility and interaction with cells of biomaterials, and therefore, in the long run, our aim is to improve the functional properties of biomaterials based on polysaccharides. Laforin is a human protein associated with glycogen metabolism, composed of two structural and functionally independent domains: a phosphatase and a substrate-binding module with glycogen and starch affinity. The Laforin's CBM sequence was originally cloned by PCR from a human muscle cDNA library. CBM was fused to the tri-peptide Arg-Gly-Asp (RGD), which plays a role on cell adhesion. Commercially heterologous expression systems of *Escherichia coli* (pET 29a, pET 25b and pGEXT4-1) were used in order to obtain high levels of soluble protein, that could be purified by affinity chromatography using 6xHis-tag or GST-tag.

With pGEXT4-1 expression system, CBM was fused with the GST protein at the N-terminal, which in theory increases solubility, but the amount of recombinant protein obtained was very low and the CBM did not present starch adsorption. Using pET25b, in the presence of arginine and CHAPS in the lyses buffer, the amount of soluble protein purified was higher. However, it formed aggregates and again the CBM did not present starch affinity. When expressed using the vector pET29a, the CBM was obtained in inclusion bodies and, after solubilization and refolding the soluble and functional protein was recovered. This is the first report on the expression of the functional CBM from the human protein Laforin.

INTRODUCTION

Laforin is a human dual specific phosphatase (DSP) involved in the glycogen metabolism and related with the human disorder Lafora disease (Fernandez-Sanchez *et al.*, 2003; Ganesh *et al.*, 2004a; Girard *et al.*, 2005). Laforin presents the modular structure frequently found in the CAZymes (enzymes that act on carbohydrates) (Cantarel *et al.*, 2009a), containing a carbohydrate-binding module (CBM) at the N terminal connected by a linker to a catalytic module, at the C terminal (Wang *et al.*, 2002a). Since each module performs and folds independently, several applications have been described using CBM as tags in fusion proteins, namely for protein purification and solubilization (Shoseyov *et al.*, 2006a). CBM are classified in families according to the sequence homology. The laforin's CBM belongs to family 20 (http://www.cazy.org/). It has been shown that this CBM binds complex carbohydrates *in vivo* and *in vitro* (Wang *et al.*, 2002a), and the DSP motif can hydrolyze phosphotyrosine and phosphoserine/threonine substrates *in vitro* (Ganesh *et al.*, 2000; Girard *et al.*, 2006). The main function of CBMs is to target the enzyme to the substrate, increasing its activity, particularly in the case of insoluble carbohydrates (Shoseyov *et al.*, 2006a). Accordingly, the CBM of laforin targets the enzyme to Lafora bodies (LB), which are composed of dense aggregates of polyglucosan fibrils, more similar to starch rather than glycogen (Chan *et al.*, 2004). Therefore, it may be expected that it would bind starch-based biomaterials. In this context, bioactive peptides may be adsorbed to those biomaterials, through the fusion with CBM, in order to achieve their functionalization for biomedical applications.

Several CBMs were already used for protein targeting (Andrade *et al.*, 2009; Guerreiro *et al.*, 2008a), including CBM from family 20 (α-amylase) with starch affinity, which was used to functionalize starch-based materials, by fusing it with a bioactive tripeptide Arg-Gly-Asp (RGD) (Moreira *et al.*, 2008b). The RGD sequence, present in several proteins from the extra-cellular matrix (ECM), is a ligand for integrin-mediated cell adhesion; this sequence was recognized as a major functional group responsible for cellular adhesion (Mann and West, 2002; Ruoslahti, 2003).

Laforin has been expressed using in *in vivo* and *in vitro* systems, namely mammalian cells fused with various epitopes for cellular localization. It was expressed as a fusion

protein (with GST, 6x-His) in *Echerichiea coli* and showed phosphatase activity on model substrate and affinity for glycogen and starch (Fernandez-Sanchez *et al.*, 2003; Ganesh *et al.*, 2000; Girard *et al.*, 2006; Minassian *et al.*, 2001; Wang *et al.*, 2002a; Wang and Roach, 2004). It is known that, *in vivo*, laforin dimerization is essential for its phosphatase activity and the CBM is involved in the dimerization process (Liu *et al.*, 2006). Although laforin has been cloned and purified, the application of laforin-CBM as a target partner has never been described; indeed, so far there are no reports on the successful expression of the functional expression of this unique human-CBM. Therefore, in this study, several strategies were employed to express, purify and functionally characterize a recombinant protein containing the human-laforin CBM fused to RGD tripeptide in *E. coli*.

Heterologous bacterial expression systems remain the most attractive ones due to the low cost, the high productivity, the well-known genetics and the large number of compatible molecular tools available (Sorensen and Mortensen, 2005b; Terpe, 2006). In general, overexpressed recombinant proteins accumulate either in the cytoplasm and/or in the periplasmic space. However, overexpression of recombinant proteins in bacterial hosts frequently results in a missfold structure with no biological activity that associates into amorphous protein granules termed inclusion bodies (IB) (Sorensen and Mortensen, 2005a, b; Thomas and Baneyx, 1996; Villaverde and Carrio, 2003). The IB formation frequently occurs when overexpressing mammalian proteins, since the posttranslation modification processes are often required for their correct folding and functionality (Dyson *et al.*, 2004; Esposito and Chatterjee, 2006; Jana and Deb, 2005). Refolding from IB is, in many cases, considered undesirable due to the poor recovery yields, the requirement for optimization of refolding conditions for each target protein and the possibility that the resolubilization procedures could affect the integrity of refolded proteins (Dyson *et al.*, 2004; Sorensen and Mortensen, 2005b). Therefore, several approaches were developed in order to overcome IB formation such as: the fusion of the target protein to a soluble peptide, the use of host strains with specific characteristics, and the optimization of fermentation conditions, among others (Dyson *et al.*, 2004; Shih *et al.*, 2002; Wagner *et al.*, 2008). In this study, several strategies were tested in order to produce functional recombinant human-laforin CBM, which was achieved only by solubilization and refolding from IB.

MATERIALS and METHODS

Reagents and strains

All reagents used were laboratory grade reagents from Sigma-Aldrich (St. Louis, USA), unless stated otherwise. *E. coli* strain XL1 Blue, from Stratagene (Carlsbad, CA, USA) was used has bacterial host for DNA cloning. For protein expression *E. coli* BL21 (DE3), Origami, and Tuner strains and the T7 plasmids (pET25b (+), pET 29a and pGEX) were purchased from Novagen (Madison, USA) and GE Healthcare (Piscataway, USA). The oligonucleotides (0.01 and 0.05 μmol scale) presented in table 1 were purchased from MWG Biotech (Germany). The restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostics GmbH (Penzberg, Germany). The Pfu DNA polymerase used was from Stratagene, and the MasterAmp 10X PCR Enhancer from EPICENTRE Biotechnologies. The Thrombin protease and IPTG (Isopropyl β-D-1-thiogalactopyranoside) were from GE Healthcare. The theoretical molecular masses of recombinant proteins were calculated using the Compute pI/Mw application from Expasy (http://www.expasy.ch/tools).

Gene cloning

The DNA coding sequence of the glycogen-binding module from Laforin was amplified using a human muscle cDNA library (CLONTECH). This sequence was used as template to clone SBM and RGD-SBM codifying sequences by PCR. The PCR reactions were performed using the Pfu DNA polymerase (2.5U), 0.5 mM of each primer (forward and reverse according to table 1), 1.2 mM $MgSO_4$, 0.24 mM dNTP, 1.2x enzyme buffer, and 1.2x of PCR enhancer solution. The PCR conditions were: denaturation at 95ºC, annealing at 56ºC and extension at 72ºC, all steps for 45 seconds (this cycle was repeated 30 times).

The DNA coding sequences were cloned in different expression system, which allowed the fusion of recombinant proteins with a hexa-histidine tag (6xHis) on the C terminal (pET expression systems) or GST on the N terminal (pGEX expression system), for

purification. The nucleotide sequences of the cloned genes were verified by sequencing. The *E. coli* XL1 Blue was used as cloning strain and expression was carried out in *E. coli* BL21 (DE3), Origami or Tuner. In all cases, negative control (cells carrying the plasmids without CBM coding sequence) was used.

Table 1- Primers utilized to amplify the coding sequence in the different expression systems. The sequences recognized by the restriction enzymes are in bold. The RGD coding sequence is underlined.

Plasmid	Primer (5'→3')	Restriction enzyme
pET25b and pET29a	For CATG**CCATGG**GGATGCGCTTCCGCTTTGGGG	*Nco*I
	Rev **GGAATT**CATGGCTTGGTGGCCTGC	*EcoR*I
	Rev CCG**CTCGAG**ATCACCTCTCATGGTTGGTGGCCTGC	*Xho*I
pGEX 4T1	For **GGATCC**ATGCGCTTCCGCTTTGGGG	*BamH*I
	Rev **GGAATT**CATGGCTTGGTGGCCTGC	*EcoR*I
	Rev CCG**CTCGAG**ATCACCTCTCATGGTTGGTGGCCTGC	*Xho*I

The integrity of cloned PCR products was verified by DNA sequencing (Sanger *et al.* 1977) using ABI PRISM310 Genetic Analyser.

Fermentation conditions

Several fermentation conditions were used in order to achieve soluble recombinant protein in cytoplasmatic (pET29a, pGEX4T1) or periplasmatic space (pET25b). The optimization of fermentative conditions was performed changing fermentation medium (Luria broth, M9 medium, Overnight Express™ Autoinduction System from Novagen), supplemented with the respective antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin), induction time (from 3 h until 48 h), IPTG concentration (from 0.1 to 1 mM), and temperature (20, 30 or 37ºC).

Table 2– *E. coli* strains, expression vectors, growth and induction condition used for recombinant protein expression.

E. coli Strains and vectors	Growth temp/ºC	Induction temp/ºC	IPTG concentration/ mM	Induction time/h
BL21(DE3)/pET29	37	37	0.5	3
BL21 (DE3) Origami Tuner	37	4 20 37	0.0 – 1.0	20
pET25b or pGEX	30	30	0.3	20 48

Expression of 6xHis-tagged recombinant protein in periplasmic space

The pET25-unmodified, pET25-CBM or pET25-CBM-RGD were used to transform *E. coli* expression host (BL21(DE3), Origami, Tuner) and cells were cultivated according to the condition presented in table 2. At the end of fermentation, cells were harvested and the periplasmatic proteins recovered using the protocol previous described (Sroga and Dordick, 2002). Briefly, the cells were resuspended in osmotic solution (OS) I (20 mM Tris-HCl, 2.5 mM EDTA, 2 mM $CaCl_2$, 20% (w/v) sucrose, pH 8) at A600 of 0.5. The suspension was incubated in OS I for 10 min on ice, and then centrifuged (4 000g, 15 min, 4ºC). The pellet was resuspended in OS II (same as OS I without sucrose) and then the cells were incubated on ice, for 20 min, and centrifuged again. The supernatant containing periplasmatic protein and the cellular pellet were analysed by SDS-PAGE.

Expression and purification of citoplasmatic 6xHis-tagged recombinant proteins

The pET25b-CBM, pET25-CBM-RGD and the respective unmodified plasmids were introduced into *E. coli* host and the expression of recombinant proteins was performed according to conditions on table 2. After fermentation, the cells were harvested, resuspended in buffer A (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.1% β-mercaptoethanol, 1 mM PMSF) and lysed by sonication.

After sonication, 0.6 M arginine and 1% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) were added to the lysate. The mixture was incubated at 4ºC, with gentle agitation for 16 h and centrifuged (30 min, 15 000 rpm, 4ºC). The recombinant protein in the supernatant was purified by immobilized metal ion affinity chromatography (IMAC), using 5 mL Niquel Hi-Trap Columns (GE Health). Briefly, imidazole was added to the cell lysate (40 mM final concentration), the pH adjusted to 7.4 before its application on the column, that was previously charged with 0.1 M $NiSO_4$ and equilibrated with washing buffer (20 mM Na_3PO_4, 500 mM NaCl, 40 mM Imidazole, pH 7.4). The elution was carried out using different imidazole concentration (200, 300 and 500 mM). Purified protein was stored in buffer A containing 10% glycerol (v/v), at -20ºC.

Expression and purification of GST-tagged recombinant protein

E. coli host strains (BL21 (DE3), Origami, Tuner) transformed with pGEX-CBM, pGEX-CBM-RGD or pGEX vectors were cultivated as indicated in table 1 and the proteins purified according to the conditions on table 2. Cells grown at 37 °C in LB medium (A_{600} 0.7), were induced by decreasing temperature to 20ºC for 1 h followed by IPTG addition (0.1 mM final concentration). After 20 h, cells were harvested, resuspended in lysis buffer B (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) and incubated with deoxyribonuclease I (100 µg/ml) and $MgCl_2$ (100 mM). After DNA digestion, 100 µl of Triton X-100 was added and the supernatant incubated with Glutathione-sepharose CL 4B (GE Healthcare). Protein elution was performed using Tris buffer (50 mM Tris-HCl, pH 8.0) containing 10 mM reduced glutathione. Recombinant protein was treated with thrombin protease according to the manufacturer's instructions (GE Healthcare). During the purification and thrombin treatment, samples were collected for SDS-PAGE analysis.

Recombinant protein expression in inclusion bodies, refolding, and purification

Expression of CBM-RGD was achieved in *E. coli* BL21 (DE3) transformed with pET29a-CBM-RGD after 3 h induction with IPTG (0.5 mM final concentration) when cell culture at A600 reached 0.6 at 37 °C in LB medium. The cells were harvested, resuspended in

buffer C (50 mM Tris-HCl, 50 mM NaCl, pH 7.4), and lysed by adding lysozyme (100 µg/ml). After freezing and thawing, deoxyribonuclease I (100 µg/ml) and $MgCl_2$ (100 mM) were added and incubated at 4°C for 1 h. The IB were then washed for 3 h with buffer C, centrifuged (at 10 000 g for 20 min at 4 °C), and then washed again for another 3 h with buffer C containing 0.1% Triton X-100 (v/v). Upon centrifugation (10 000 g for 20 min at 4 °C), the purified IB were dissolved in 8 M urea containing 100 mM β-mercaptoethanol. The protein was refolded by rapid dilution (20-fold) into 20 mM Tris-base, and the pH was slowly adjusted to pH 8.0. The recombinant protein was then concentrated in a tangential flow ultrafiltration system (Pellicon 2; Millipore, Billerica, MA), ultracentrifuged (50 000 g for 20 min at 4 °C), and the supernatant was applied onto a Superdex 200 gel filtration chromatographic column (GE Healthcare) equilibrated in 20 mM Tris-HCl, 0.4 M urea, pH 8.0 buffer. The fractions corresponding to the second protein peak, which corresponds to the non-aggregated forms of recombinant protein, were then combined and further purified by ion exchange chromatography on a Mono Q column (GE Healthcare) using the same buffer as for the Superdex 200 chromatographic experiment with a gradient of NaCl (0 – 0.5 M).

Recombinant protein analysis

Recombinant proteins were analysed by 12% SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) and the aggregation was evaluated by 10% native PAGE and visualized using standard protocols of Coomassie blue or silver staining. Protein samples were mixed with SDS–PAGE loading buffer and heated at 95 °C for 5 min. For Western-blot analysis, the protein samples separated by SDS–PAGEwere electro-transferred (wet transfer) to a nitrocellulose membrane at 100 V for 60 min. The membrane was blocked with 2% (w/v) BSA in PBS-T buffer (PBS, 0.05% Tween 20) and incubated with peroxidase conjugated anti-His anti-body (Sigma) diluted 1:2000. Image detection and analysis was performed by ChemiDoc XRS and Quantity One software (BioRad).

Adsorption assay

To evaluate the human-laforin CBM substrate affinity and specificity, adsorption assays using starch (positive control) and cellulose (negative control) were carried out. The

purified protein samples (0.25 mg/ml) were mixed with 50 mg of starch or cellulose (previously washed with buffer A for 1 h, at 4ºC. Then, the mixture was centrifuged (13 000 rpm, 10 min, 4ºC) and supernatants were analysed by SDS-PAGE. The recombinant CBM was eluted from starch with 5 mg/ml glycogen solution (0.3 ml, at 4ºC for 1 h).

RESULTS

Expression and purification of recombinant proteins using pET25b (+) expression system

The DNA coding sequences were successfully cloned in pET25b expression vector, and cells carrying the vectors pET25-CBM, pET25-CBM-RGD or pET25 (unmodified vector) were grown in LB or M9 medium, supplemented with ampicilin (leucine was added to M9 medium for Origami fermentation). The molecular weight expected for recombinant proteins was 20.5 KDa for CBM-RGD and 21.2 KDa for CBM, assuming that the pelB leader sequence is correctly processed; otherwise, proteins will have 22.5 and 23.2 KDa, respectively.

The transformed *E. coli* strains were grown in LB or M9 medium at 37ºC and induced with 1 mM IPTG (37ºC, 3 h). Under these conditions, the recombinant proteins were expressed in the insoluble fraction (pellet). It was assumed that combining a low temperature with low concentration of inducer would prevent overloading the *E. coli* periplasmic transport system and the recombinant proteins would be able to fold properly. Therefore, the cells were grown under low temperature (at 30 and 20°C) and the induction phase was performed with low concentrations of IPTG (0, 0.1, 0.3 and 0.5 mM) at low incubation temperatures (30, 20 and 4 °C). Figure 1 shows representative results of all conditions tested using two different *E. coli* strains transformed with pET25b or pET25b-CBM-RGD, grown in M9 medium at 30ºC, and induced with IPTG (0.3 mM) at 20ºC for 20 h. Among all the conditions tested recombinant proteins CBM and CBM-RGD were expressed, except for IPTG concentrations lower than 0.3 mM. However, none of the recombinant proteins were detected in the periplasmic space, irrespectively to the strain and fermentative conditions utilized, which indicates that the pET25b (+) pelB leader sequence is not a suitable signal sequence to export these particular

proteins, in the set of conditions tested. Moreover, recombinant proteins were not detected by SDS-PAGE analysis in soluble fraction under all the conditions utilized.

Figure 1 presents the SDS-PAGE analysis of the soluble fraction obtained by Tuner lysis using buffer A before and after purification by affinity chromatography. IPTG (0.3 mM final concentration) was added to the cells grown at 30°C in M9 medium, when culture reached A600 of 0.4. Although no protein was detected in soluble fraction by SDS-PAGE analysis, it was still possible to purifyit using IMAC system. Nevertheless, the production by this method was very inefficient not only due to the low amount of soluble protein present (figure 1), but also due to the protein precipitation after buffer exchange (PD10 column).

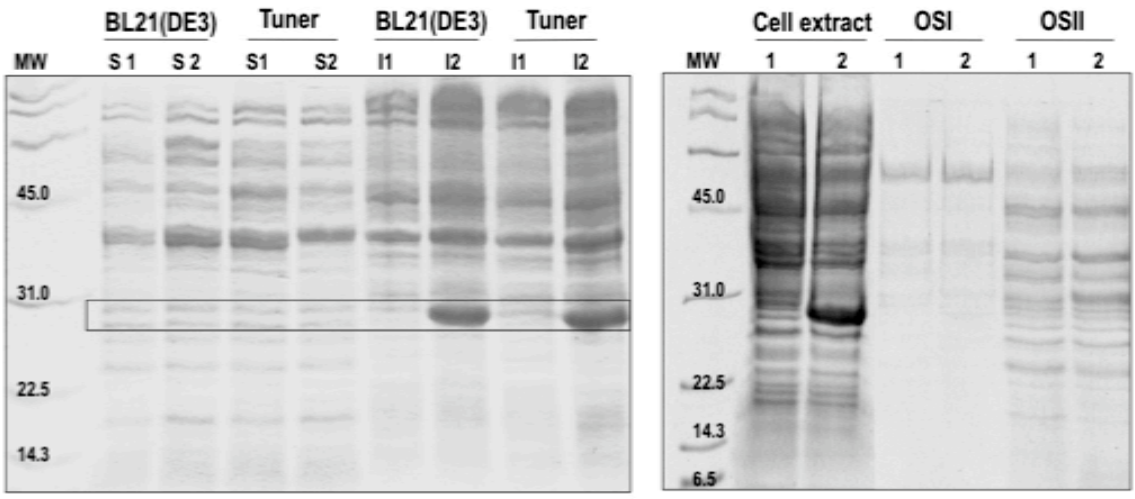

Figure 1 – SDS-PAGE (Coomassie staining) of protein expressed using different *E. coli* strains grown at 30 ºC, induced with IPTG 0.3 mM at 20ºC (left pannel). 1- pET25b; 2- pET25b-CBMRGD; S-soluble fraction; I-insoluble fraction; MW- molecular weight (Biorad). SDS-PAGE analysis of protein from *E. coli* Tuner cells treated with osmotic solution I and II (right pannel).

The addition of 0.6 M of arginine to the Tuner cell lysates increased the recombinant protein in soluble fraction; however, it was not possible to obtain pure recombinant protein after His-Trap affinity chromatography (Figure 2). The purification was achieved by incubation of cell lysate with arginine and CHAPS. The soluble fraction obtained was then passed through nickel column and the pure recombinant protein His-tagged CBM-RGD was eluted with imidazole (300 mM) as confirmed by the Western-blot analysis. Eluted protein was stable in buffer A, however it did not show starch affinity (data not

shown). Furthermore, the analysis of purified protein by native electrophoresis revealed the production of CBM-RGD aggregates (Figure 7). All these data point to the non-functionality of the protein due to its incorrect folding.

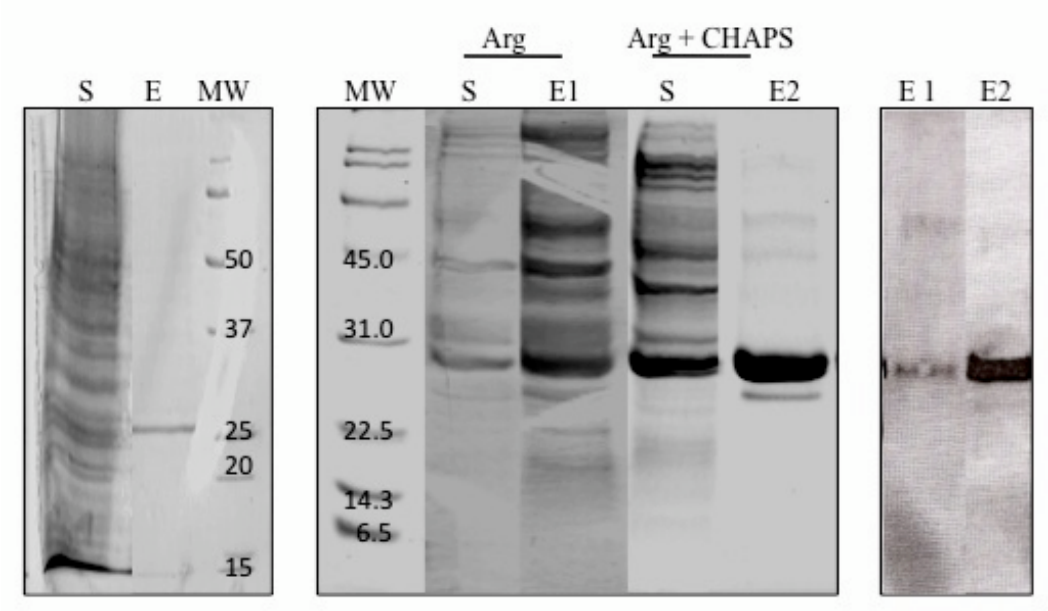

Figure 2 – Coomassie stained SDS-PAGE of the protein CBM-RGD detected in cells lysates (left panel) of *E. coli* Tuner in M9 medium, at 30ºC, induced with IPTG 0.3 mM, at 18ºC, 48 h. The cell lysates were also treated with arginine, with or woth CHAPS before centrifugation (central panel) for soluble fraction recover (S); E-fraction eluted with imidazole during protein purification, using affinity chromatography. Identical results were obtained with *E. coli* BL21 (DE3), and Origami strains (data not shown). On the right panel: Western-blot analysis of E1 and E2 samples, using anti-His antiboby (Sigma).

Expression and purification of recombinant proteins using pGEX expression system

The *E. coli* (BL21 (DE3), Origami, Tuner) cells transformed with the different expression vectors (pGEX-CBM, pGEX-CBM-RGD or pGEX), were grown and induced under several conditions. The recombinant GST and GST-CBM-RGD with 26 KDa and 46 KDa, respectively, were found in soluble fraction of cell lysates that were grown at low temperature culture conditions and 0.1 mM IPTG (Figure 3). The same results were obtained in cells grown in autoinductive medium at 20ºC or 30ºC for 20 h, however when fermentation was carried out at 37ºC, the recombinant proteins were expressed in the insoluble fraction (data not shown).

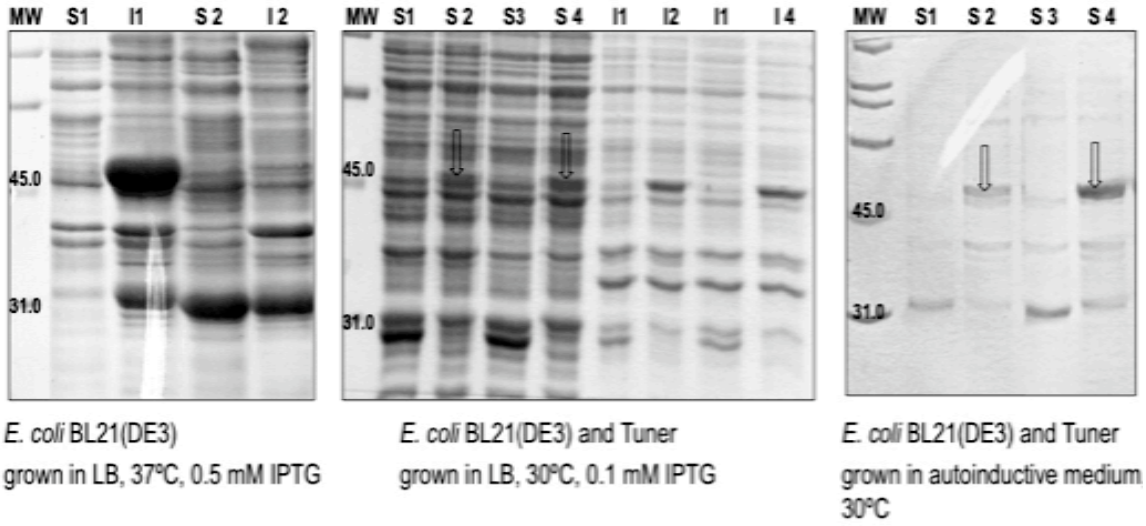

Figure 3– Coomassie Blue stained SDS-PAGE obtained from cell lysates of *E. coli* strains (1 and 2 BL21; 3 and 4 Tuner) transformed with pGEX (1 and 3) or pGEX-CBM-RGD (2 and 4) under different growth and induction conditions. Recombinant GST-CBM-RGD expressed in soluble fraction (arrows). S – soluble fraction; I – insoluble fraction; MW – molecular weight (Biorad).

For GST-CBM-RGD purification the soluble fraction of *E. coli* BL21 (DE3) grown in LB medium, induced with 0.1 mM IPTG, 20ºC for 20 h was passed through Glutathione sepharose column, dialyzed and treated with thrombin to cleave the GST tag. Figure 4 shows the results of the time course analysis of thrombin cleavage. Thrombin cleaves on LVPRGS sequence releasing two peptides: GST with 26 KDa and CBM-RGD with 20 KDa. However, this recombinant protein presents a second sequence potentially recognized by thrombin (WEPRGA). A peptide with 15 KDa is also observed in the gel on Figure 4, revealing that the second site is also cleaved, for longer digestion times. By limiting the hydrolysis at 20 min it was possible to obtain the CBM-RGD with the correct sequence (20 KDa).

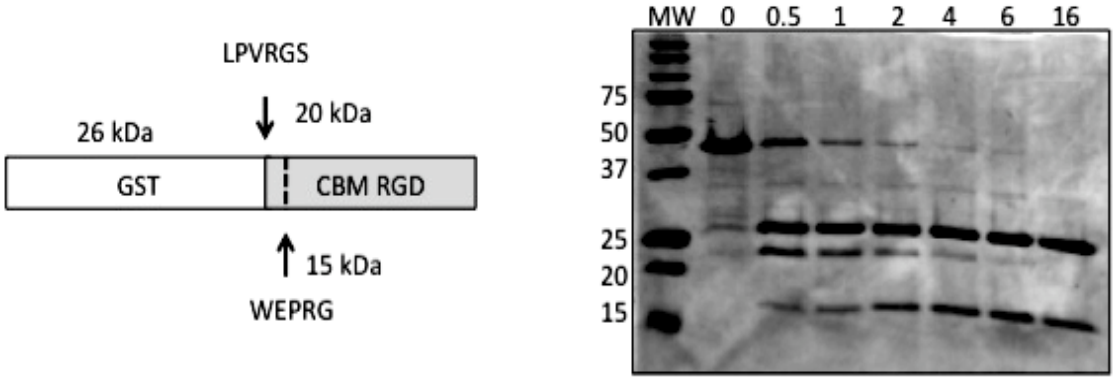

Figure 4 – Schematic representation of recombinant protein and thrombin cleavages sites. Time course analysis by SDS-PAGE silver stained of GST-CBM-RGD protein during thrombin cleavage (0-16 hours).

The adsorption assay carried out with unpurified protein showed that recombinant protein adsorbed both to starch and to cellulose, suggesting that CBM was not functional. Furthermore, protein did not desorbed from starch when incubated with glycogen, confirming that the unspecific adsorption reaction (Figure 5).

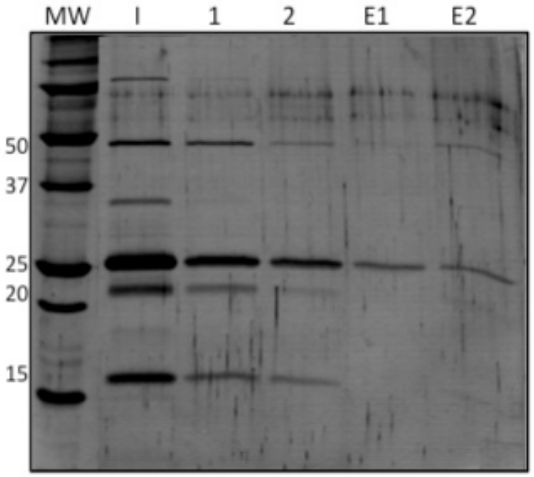

Figure 5 – Silver stained SDS-PAGE (of adsorption assay. Initial protein (I); protein non-adsorbed to starch (1) or cellulose (2); Elution fraction (E) with buffer containing glycogen after cellulose and starch washing.

Expression and purification of recombinant proteins using pET29a expression system

E. coli BL21 (DE3) transformed with pET29a or pET29a-CBM-RGD expression vectors was grown in LB medium, at 37ºC and induced with 0.5 mM IPTG for 3 h. Protein was expressed, with the expected molecular weigh (22 KDa), in insoluble fraction as inclusion bodies. After solubilization and refolding, the recombinant protein was passed through Superdex 200 gel filtration chromatographic column. The fractions corresponding to the second protein peak, which corresponds to the non-aggregated forms of CBM-RGD, were then combined and further purified by ion exchange chromatography on a Mono Q column. Native PAGE analysis showed that the refolded protein was dimeric (44 KDa); on the contrary, protein obtained with pET25b expression system, although soluble, was highly aggregated (Figure 6).

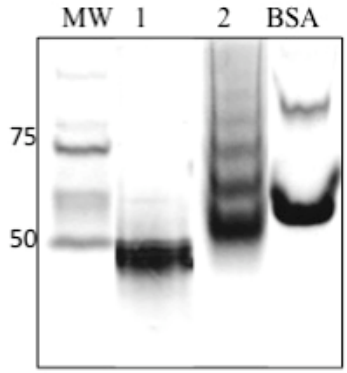

Figure 6 – Native PAGE stained with Coomassie Blue of CBM-RGD obtained by pET29a (1) and pET25b expression system; Bovine serum albumin (BSA) with 66 kDa was used as MW marker.

The functionality of the recombinant protein was evaluated by an adsorption assay on starch and cellulose. It was observed that only a 10% of initial CBM-RGD adsorbed to starch, and surprisingly almost 100% adsorbed to the cellulose. The starch and cellulose particles with protein adsorbed were washed out before protein elution using buffer containing glycogen. Protein was eluted only from starch, indicating that protein was functional (Figure 7).

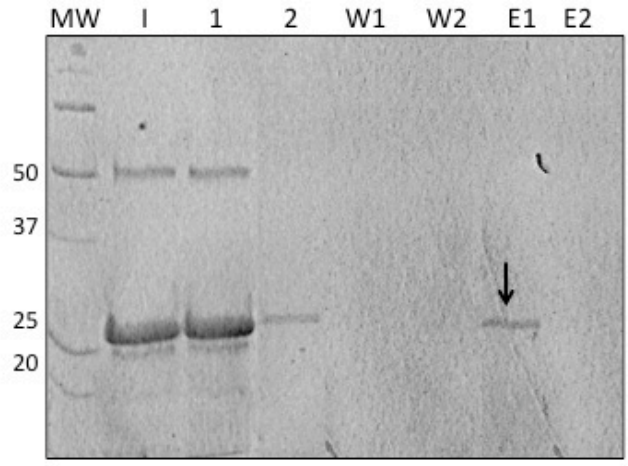

Figure 7– Analysis of CBM-RGD adsorption by SDS-PAGE (Coomassie staining). Initial protein (I); protein non-adsorbed to starch (1) or cellulose (2); Washing fraction (W); Elution fraction of CBM-RGD with buffer containing glycogen. Protein eluted from starch (arrow).

DISCUSSION

Expression of soluble and functional proteins in heterologous system may be a difficult task, especially for mammalian proteins (Dyson *et al.*, 2004). There are several tags described for protein expression and purification, among them, GST has been described as a useful tag to enhance the solubility of recombinant proteins (Braun *et al.*, 2002; Hammarstrom *et al.*, 2002; Shih *et al.*, 2002). In addition, it was referred that Laforin presents different solubility depending on the tag fused (Girard *et al.*, 2006), namely, 6xHIS-tagged Laforin showed to be less soluble then GST-Laforin.

In order to express soluble recombinant human-laforin CBM and CBM-RGD, several conditions were tested, including vector systems, bacterial hosts, fermentative medium, cell culture temperature, and induction and lysis conditions (Figure 8). Since the results obtained for CBM and CBM-RGD expression were similar, only the results from CBM-RGD were presented.

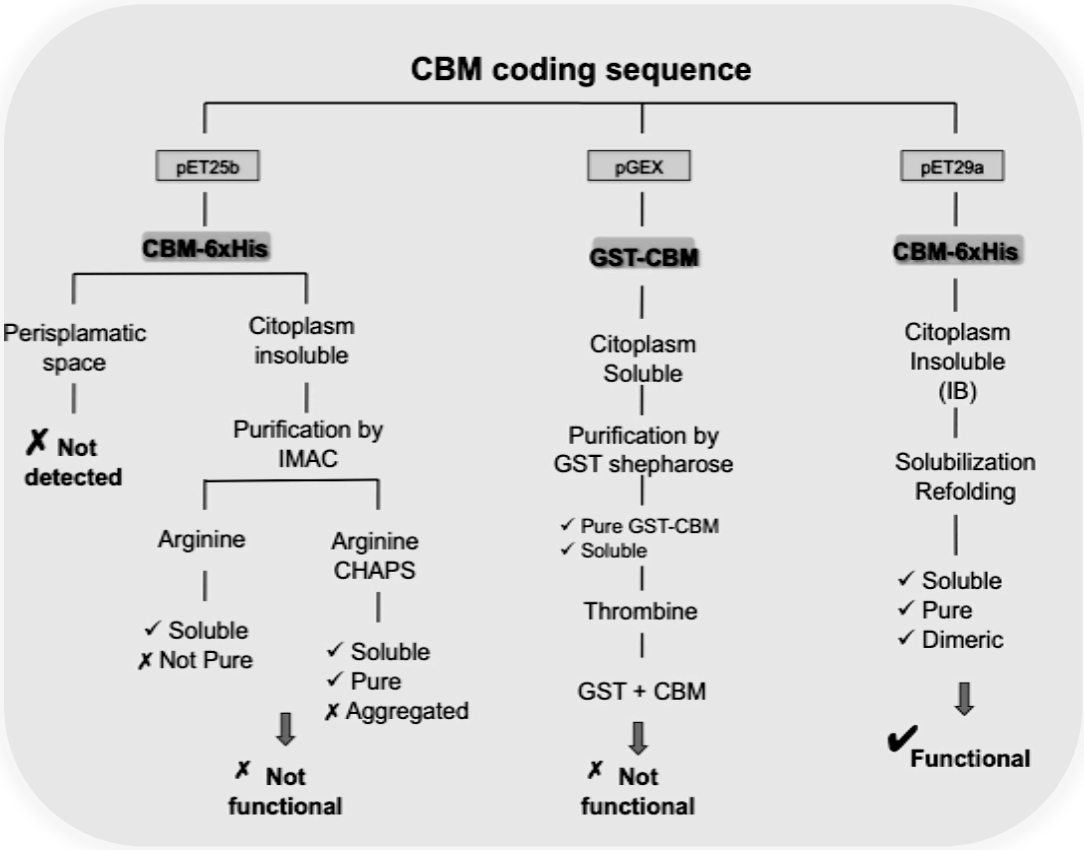

Figure 8- Schematic summary of strategies and results.

The DNA coding sequence of CBM and CBM-RGD were successfully cloned in the different expression systems (pET25b, pET29a and pGEXT1-4). However, the stability and functionality of recombinant proteins were different depending on the expression system used, as previously described for recombinant Laforin expressed in *E. coli*. In addition, the proteins expressed showed higher molecular weigh than theoretically expected in SDS-PAGE analysis, again, as described for recombinant Laforin (Wang and Roach, 2004).

In this work, the CBM was fused to 6xHis-tag at the C-terminal and a pleB leader sequence at the N-ternimal. The pelB leader signal peptide directs the recombinant protein to the periplasmic space, where it was expected to be mostly soluble and correctly processed. However, independently of the host strain and fermentation conditions used, the recombinant proteins were never detected in the periplasmic space. It is well established that lowering the expression may enhance the solubility, folding and

activity of difficult target proteins (Dyson *et al.*, 2004; Winograd *et al.*, 1993). Therefore, the temperature used for cell growth and heterologous protein induction was a parameter analysed. In addition, expression was carried out using Tuner cells, which enable adjustable levels of protein expression throughout the culture by adjusting the concentration of IPTG. When recombinant proteins were expressed with pET25b system using Tuner strain, cultivated under low temperature, M9 minimal medium, and low IPTG concentration, it was not possible to obtain a clear separation of the soluble and insoluble fractions. Instead, the cell lysate originated two phases hard to separate by centrifugation (30 m, 15 000 rpm). When supernatant was applied to nickel column, a few amount of purified recombinant protein was obtained. However, a high concentration of imidazole (500 mM) was required for protein elution. Moreover, the purified protein was not stable after desalting. To overcome the insolubility of expressed protein arginine was added to the lysis buffer. Arginine has been described as effective in suppressing aggregation of proteins and several works show its beneficial effect when included during purification processes (Arakawa *et al.*, 2007a; Arakawa *et al.*, 2007b; Tsumoto *et al.*, 2004). The addition of arginine to the cell lysate increased the soluble protein fraction, but purification through affinity chromatography was again not efficient. It has been reported that laforin is a plasma membrane and ER - associated protein (Ianzano *et al.*, 2003; Minassian *et al.*, 2001), therefore it was speculated that the presence of a detergent may increase its solubility. In addition, in a previous work, CHAPS (a zwitterionic detergent) was required to solubilize His-tagged laforin (Girard *et al.*, 2006). When CHAPS was added to cell lysate, the recombinant protein was solubilized and purified by affinity chromatography using 300 mM of imidazole. After purification, recombinant proteins remained stable in solution. Despite solubility and stability of purified proteins, the CBM did not adsorb to starch, indicating that it was not functional. Furthermore, the native PAGE analysis revealed protein aggregation.

In a second approach, CBM was expressed fused with GST at the N-terminal and after optimization of fermentation conditions, soluble GST-fused proteins were expressed under low temperature (20ºC) and inducer concentration (0.1 mM). With this expression system, similar results were obtained using *E. coli* BL21(DE3), Origami or Tuner strains. Recombinant proteins were purified and GST was cleaved by thrombin hydrolysis. Thrombin cleaved GST-CBM in two sites; however, by limiting the proteolysis to 20 min, the integral CBM could be recovered. Girard *et al.* reported that GST-laforin was resistant to thrombin cleavage; therefore, those authors inserted a 5-glycine linker

between GST and laforin. In addition, same authors showed that laforin purification requires an extra-purification by hydroxyapatite chromatography to remove the GroEL (*E. coli* chaperon with 63 KDa) that was not removed by Glutathione-Sepharose (Girard *et al.*, 2006). In the case of recombinant GST-CBM, it was not necessary to introduce a glycine linker to enable proteolytic cleavage and GroEL was not observed after purification with Glutathione-Sepharose. Since CBM did not show starch affinity, the purification protocol of GST-CBM protein was not further optimized.

Commonly, the strategies to express and purify recombinant proteins avoid the protein expression in IB. In our work, the recombinant CBM was obtained functional only through IB solubilization and refolding. Moreover, the protein obtained by this method was highly pure, even without any affinity chromatography, dimeric and stable at 4ºC, in buffer containing 0.4 mM urea, for up to 2 months. Unexpectedly, CBM adsorbed to cellulose, most likely through unspecific adsorption; indeed, the CBM was only desorbed using glycogen from starch, indicating that CBM was removed from cellulose during the washing steps. Furthermore, this result indicating that starch may be used, as a final purification step, to select functional CBM-containing proteins. Laforin was described as aggregating easily, and that the same applies to the CBM (Girard *et al.*, 2006; Girard *et al.*, 2005). Moreover, results using chaperon co-expression and *Pichia pastoris* expression systems (unpublished results) also lead to aggregate and no functional recombinant CBM. Laforin and its CBM are therefore proteins very difficult to express in soluble fraction in prokaryotic and fungal expression systems. The production of laforin in inclusion bodies was never described, and CBM obtained by this approach showed high purity without affinity chromatography, stable and functional.

Since CBM of laforin binds to starch it may be used as tag molecule for adsorb small bioactive peptides to starch-based biomedical materials. The future work includes assays using CBM-RGD on dextrin hydrogel, as described in a previous work for bacterial CBM (Moreira *et al.*, 2008b).

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Chapter 3

Development of a strategy to functionalize a dextrin-based hydrogel for animal cell cultures using a starch-binding module fused to RGD sequence

Results published on BMC biotechnology

Moreira, S., Andrade, F., Domingues, L., Gama, M., 2008. Development of a strategy to functionalize a dextrin-based hydrogel for animal cell cultures using a starch-binding module fused to RGD sequence. BMC Biotechnology 8, 78.

ABSTRACT

Several approaches can be used to functionalize biomaterials, such as hydrogels, for biomedical applications. One of the molecules often used to improve cells adhesion is the peptide Arg-Gly-Asp (RGD). The RGD sequence, present in several proteins from the extra-cellular matrix (ECM), is a ligand for integrin-mediated cell adhesion; this sequence was recognized as a major functional group responsible for cellular adhesion. In this work a bi-functional recombinant protein, containing a starch binding module (SBM) and RGD sequence was used to functionalize a dextrin-based hydrogel. The SBM, which belongs to an α-amylase from *Bacillus sp.* TS-23, has starch (and dextrin, depolymerized starch) affinity, acting as a binding molecule to adsorb the RGD sequence to the hydrogel surface.

The recombinant proteins SBM and RGD-SBM were cloned, expressed, purified and tested in *in vitro* assays. The evaluation of cell attachment, spreading and proliferation on the dextrin-based hydrogel surface activated with recombinant proteins were performed using mouse embryo fibroblasts 3T3. A polystyrene cell culture plate was used as control. The results showed that the RGD-SBM recombinant protein improved, by more than 30%, the adhesion of fibroblasts to dextrin-based hydrogel. In fact, cell spreading on the hydrogel surface was observed only in the presence of the RGD-SBM.

The fusion protein RGD-SBM provides an efficient way to functionalize the dextrin-based hydrogel. Many proteins in nature that hold a RGD sequence are not cell adhesive, probably due to the conformation/accessibility of the peptide. We therefore emphasise the successful expression of a bi-functional protein with potential for different applications.

INTRODUCTION

Hydrogels are a class of water-swollen polymeric materials, capable of maintaining a distinct three-dimensional structure (Kopecek, 2007; Wang *et al.*, 2006), which can be used as scaffolds in tissue engineering, as wound dressing, and drug delivery systems, among other applications (Carvalho *et al.*, 2007). Several approaches have been developed to produce hydrogels from different synthetic and natural polymers (Peppas *et al.*, 2000). Among them, the starch-based hydrogels have appealing characteristics in the perspective of biomedical application. They are biocompatible, have convenient degradation kinetics and release profiles and also present appropriate mechanical properties (Marques *et al.*, 2002; Wong and Mooney, 1997; Zhang *et al.*, 2005b). Despite its wide and successful application, the resistance of the hydrogel surfaces to cell adhesion and differentiation might represent a considerable limitation. In this context, the hydrogel functionalization, through the incorporation of adhesive molecules, emerges as a promising approach to overcome these limitations.

Several molecules, namely proteins of the ECM (extra-cellular matrix), poly-L-lysine (PLL) and a natural adhesive protein extracted from mussel (MAP) (Hwang *et al.*, 2007) have been successfully applied in promoting cell adhesion and proliferation (Dai and Saltzman, 1996; Hwang *et al.*, 2007; Mann and West, 2002; Schraa *et al.*, 2002; Tan *et al.*, 2005). In addition, the Arg-Gly-Asp (RGD) motif – found in ECM proteins and in the blood, such as fibronectin, vitronectin, osteopontin, collagens, thrombospondin, fibrinogen, and von Willebrand factor – was described as the major functional group responsible for cellular adhesion (Hwang *et al.*, 2007; Ruoslahti, 2003; Ruoslahti and Pierschbacher, 1987). Various strategies of surface functionalization, which include the coupling or grafting of the RGD peptide, have been already reported. Most of these involve complex chemical reactions, to activate the chemical groups in the polymer or in the RGD containing sequence, to allow for the covalent binding (Hersel *et al.*, 2003; Li *et al.*, 2008; Massia and Stark, 2001). In this study, a new approach of RGD-activation of dextrin-hydrogel (a depolymerized starch) is proposed. It has been reported that RGD bioactivity can be conserved in fusion proteins (Wang *et al.*, 2006; Wierzba *et al.*, 1995). Likewise, a recombinant protein containing a starch-binding module (SBM) and a RGD sequence was used in this work. Several enzymes that metabolize carbohydrates have a modular structure with two independent domains, a catalytic domain and a substrate-binding

domain, generically designated as carbohydrate-binding module (CBM). A CBM is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme with a discrete fold having carbohydrate-binding activity. The CBM used in this work, is a starch-binding module (SBM), belonging to a α-amylase from *Bacillus sp*. strain TS-23 (Lin *et al.*, 1994), which specifically binds to starch (Lin *et al.*, 1997).

The present work shows the successful functionalization of a dextrin-based hydrogel, using a fusion protein containing a C-terminal SBM and a N-terminal RGD sequence. Viability and microscopic evaluation of the protein-activated hydrogels, revealed an effective improvement of cellular adhesion and spreading.

MATERIALS and METHODS

Reagents and strains

All reagents used were laboratory grade reagents from Sigma-Aldrich, St. Louis, USA, unless stated otherwise.

The bacterial hosts used for cloning and expression of the fusion proteins were *Escherichia coli* strain XL1 Blue [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacIqZΔM15 Tn10 (Tetr)] (Stratagene) and strain BL21 (DE3) [F- ompT hsdSB (r-B mB-) gal dem Δ(srl-recA) 306::Tn10(DE3)](Novagen), respectively. The pET 29a(+) (Novagen) was used as expression vector. The restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostics GmbH (Penzberg, Germany). The Vent DNA polymerase used was from New England Biolabs.

In vitro assays were performed using mouse embryo fibroblasts 3T3 (ATCC CCL-164), grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% newborn calf serum (Invitrogen) and penicillin/streptomycin (1 µg/mL) (Sigma-Aldrich, St. Louis, USA), at 37°C, in a fully modified air containing 5% CO_2.

Gene cloning

The DNA coding sequence of the starch-binding module from *Bacillus sp*. strain TS-23 was synthesized (Epoch Biolabs, Missouri City, USA). This sequence was used as template to clone SBM and RGD-SBM codifying sequences by PCR. In the case of RGD-SBM peptide, 10 amino acids were cloned in the N-terminal between SBM and RGD, to act as a linker and allow some mobility of the RGD. Briefly, SBM and RGD-SBM sequences were cloned using the forward primers 5'-GGGAATTCCATATGACGTCAAACGTCACATTTAC-3' and 5'-GGGAATTCCATATG**AGAGGTGAT**GGAGGCTCCGTTTCGATTTGG-3', respectively, and the reverse primer 5'-CCGCTCGAGTGGCACATTCCAGCTCGC-3', the underline sequences are the restriction sites for the *Nde* I and *Xho* I, and the bold sequence codify the RGD. The PCR reactions were performed using the Vent DNA polymerase and the PCR conditions were: denaturation at 95°C, annealing at 53°C and extension at 72°C, all steps for 45 seconds (this cycle was repeated 30 times).

Both DNA coding sequences were cloned in pET29a (+) expression system, which allows the fusion of recombinant proteins with a hexa-histidine tag on the C-terminal, for purification. The nucleotide sequences of cloned genes were verified by sequencing. The *E. coli* XL1 Blue was used as cloning strain and expression was carried out in *E. coli* BL21 (DE3).

Production and purification of recombinant proteins

For the production of the recombinant proteins, the *E. coli* BL21 (DE3) cells transformed with the expression vectors, pET29a(+)-SBM and pET29a-RGD-SBD were grown at 37°C, in LB medium supplemented with Kanamycin (50 µg/ml). Cultures were induced with IsoPropyl β-D-1-ThioGalactopyranoside (IPTG, Invitrogen) at 1 mM. Five hours after induction, the cells were separated from the culture medium by centrifugation (13 000 g, 10 min) and resuspended in buffer A (20 mM Tris, 20 mM NaCl, 5 mM $CaCl_2$, pH 7.4 and PMSF 0.1 mM) and then lysed by sonication. The soluble and insoluble fractions were separated by centrifugation (15 000 g, 4°C, 30 min). The purification was made by affinity chromatography, using a HisTrapTM HP (GE health care). For that, imidazole was added to the cell lysated (40 mM final concentration) and the pH was adjusted to 7.4 before its

application on the nickel column. After purification, proteins were dialyzed against the buffer A, sterilized by filtration (0.22 µm) and stored at -20°C, prior to use.

SBM adsorptions assays

To evaluate the SBM starch affinity and specificity, an adsorption assay using starch (positive control) and cellulose (negative control). The protein of the soluble fraction (0.5 mL) obtained from the cells lyses (0.5 mg/mL) was mixed with 50 mg of starch or cellulose, for 1 h, at 4°C. Then, the mixture was centrifuged (13 000 rpm, 10 min, 4°C) and the total protein in supernatant was quantified by the Bradford assay (BioRad), using BSA as standards, and analysed by SDS-PAGE. The recombinant SBM was eluted from starch with 2% β-cyclodextrin solution (0.5 mL, 4°C, 1 h).

Effect of the recombinant proteins on the adhesion and spreading of fibroblasts on the tissue culture polystyrene plate (TCPP)

The cell viability was determined by the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS, Promega) assay, a colorimetric assay that gives a measure of the mitochondrial metabolic activity. The fusion proteins were added to the 96-well TCPP (0.05 µg of protein per well) to allow adsorption (4°C, overnight). The unbound protein was washed out with phosphate buffer saline (PBS); then 200 µL of fibroblast suspension was plated in each well, yielding a final density of 5×10^3 cells. After 1 h the wells were washed with PBS and the culture medium refreshed. The MTS assay and microscope observations of the attachment and spreading of fibroblasts were carried out at 1, 5, 24 and 48 h after the addition of the cells.

Effect of the recombinant proteins on the adhesion and spreading of fibroblasts on the dextrin-based hydrogel

In a second test, the recombinant proteins were added to the hydrogel. For this propose hydrogels were prepared in a 96-well polypropylene plate (autoclavable), as described by Carvalho *et al.* (2007). Briefly, 30 µL of the dextrin-based solution (300 mg/mL in PBS)

was placed on the bottom of each well and the initiators were added to allow the polymerization. After sterilization (20 min, at 121°C and 1 atm) the hydrogel was washed with PBS and then the recombinant proteins were added (0.25 µg of protein per well). Afterwards, plates were incubated overnight at 4°C. Unbound protein was removed and analysed by SDS – PAGE and the hydrogel was washed out with PBS before cells seeding. A fibroblasts suspension was plated into each well, to yield a final density of 2×10^4 cells. The plates were incubated and after 4 h, the wells were washed with PBS and the medium refreshed. The MTS assays were carried out on the non-adherent cells. Microscope observations of the attachment and spreading of 3T3 fibroblasts was carried out at 4, 24 and 48 h after the addition of the cells and then trypsinized from the hydrogel before MTS analysis. The results expressed as cell proliferation inhibition index (CPII) were calculated as CPII = 100 - ($OD_{490\ nm}$ of test culture/$OD_{490\ nm}$ of control culture) × 100.

RESULTS and DISCUSSION

Expression and purification of recombinant proteins

As shown in figure 1, the SBM and RGD-SBM recombinant proteins were successfully expressed using the pET29a(+) expression system and *E. coli* BL21(DE3) cells. The proteins were presented in the soluble fraction, exhibiting the expected MW, 12.0 and 13.3 KDa respectively. Previous works used another expression system (pQE, Quiagen) and host (*E. coli* M15) to produce recombinant proteins fused to this SBM (Hua *et al.*, 2005; Hua *et al.*, 2004; Lin *et al.*, 2003). However, in the present work the amount of the recombinant protein obtained in the soluble fraction was much higher (40%), indicating that this expression system is preferable.

The SBM functionality was analysed through a starch adsorption assay, confirming that the binding module is functional. The SBM specificity was evaluated using starch (positive control) and cellulose (negative control). The SBM adsorbed only to starch and it could be eluted using soluble β-cyclodextrin (Figure 1).

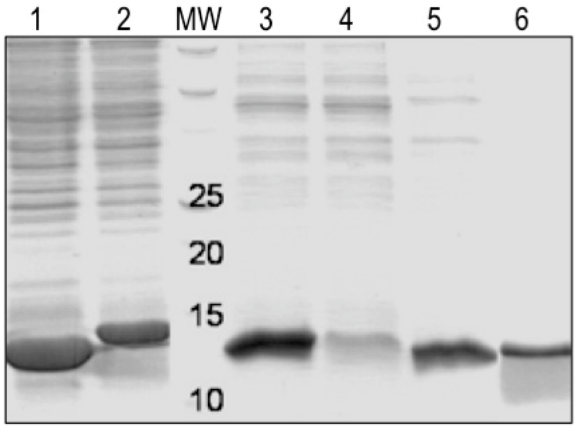

Figure 1- Analysis of protein expression (A) and starch specificity (B) by SDS-PAGE. A-Soluble protein extract obtained from lyses of *E. coli* BL 21(DE3) carried pET29a(+)-SBD (1) and pET29a(+)-RGD-SBD (2) vectors. B-Total soluble protein extract (containing SBM) used in adsorption assays (3); supernatant obtained after starch (4) and cellulose adsorption (5), supernatant obtained after protein elution of starch with β-cyclodextrin (6). (MW – molecular weight, KDa).

Attachment of fibroblasts to the recombinant protein-coated TCPP

The TCPP was used as a first approach to observe the effect of the presence of protein coating the material on the adhesion of cells. Thus, both polystyrene and fibroblasts are here considered as a model system. The actual applications envisaged involve the use of dextrin made materials (as described ahead) and other cell lines (not in the scope of this work). The microscopic analysis and MTS results showed that, when the polystyrene plate was coated with RGD-SBM, fibroblast adhesion was improved, as compared to the uncoated wells or the ones treated with SBM (controls). In fact, after 1 h adhesion, the MTS results showed that fewer cells adsorb to the plate coated with SBM, as compared to the ones coated with RGD-SBM (figure 2). Furthermore, the wells with or without SBM adsorbed exhibited the same amount of adherent cells. It seems, thus, reasonable to conclude that SBM did not affect cell adhesion, being the RGD sequence the responsible for the improvement of the CPII. Indeed, a decrease in CPII of about 30 to 35% was achieved in the presence of the RGD-SBM, the same trend being also observed in the assays carried out for longer periods of times (5, 24, 48 h). Regarding morphology, the cells cultivated in different conditions were similar.

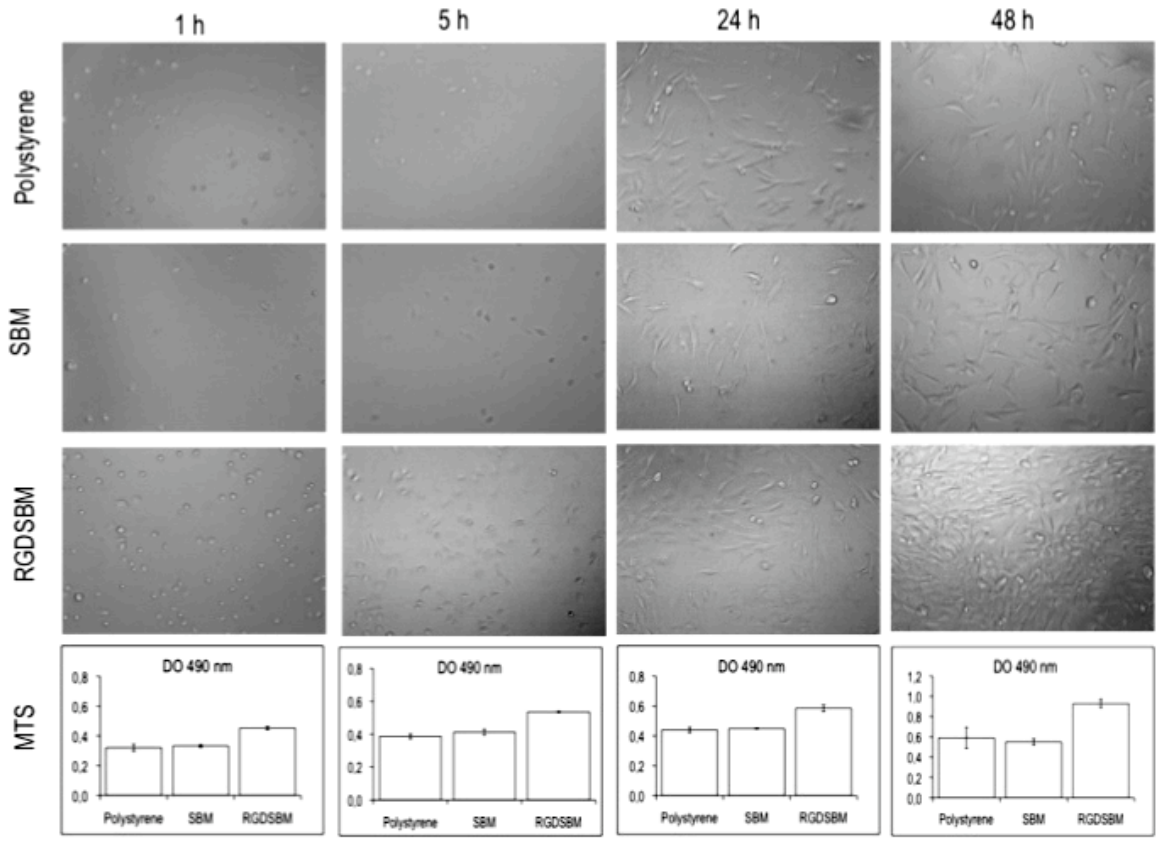

Figure 2 - Microscopic observation and MTS analysis of the cells attached to the polystyrene plate and polystyrene plate coated with SBM or RGD-SBM peptides, at different times (MTS results were performed in triplicate). The MTS assay shows the optical density at 490 nm under different conditions.

Attachment of fibroblasts on dextrin-based hydrogel surface

The recombinant proteins were added to the hydrogel and left adsorbing overnight. Before the addition of cells, the hydrogel was washed out with PBS, in order to remove the unbound proteins. The supernatant containing the unbound proteins was analysed by SDS-PAGE (figure 3), confirming that the SBM successfully adsorbs to the hydrogel and the amount of recombinant proteins used was enough as to saturate the hydrogel. A TCPP was used as a control, for comparison of cell adhesion and morphology. The specificity and stability of the adhesive proteins present in the surface of the biomaterial have been referred as a critical factor for the cells attachment and behaviour (Neff *et al.*, 1999). The SBM used in this study belongs to the α-amylase secreted by the *Bacillus sp*. TS-23, a thermophilic and alcaliphilic bacteria (Lin *et al.*, 1997). This enzyme is functional

under extreme conditions, and it was described as rather stable in a large range of temperatures and pH. Furthermore, it should be remarked that CBM's have been used in the development of commercial systems for recombinant protein purification (Shoseyov *et al.*, 2006a). Therefore, the adsorption of the SBM on the hydrogel surface may be expected to be stable and specific, allowing its successful application to functionalize the hydrogel surface.

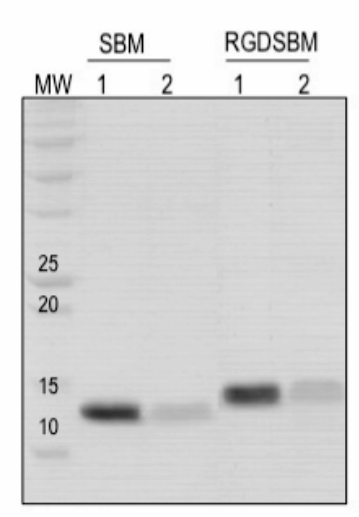

Figure 3 - SDS-PAGE analysis of the recombinant proteins adsorbed to the dextrin-based hydrogel. Recombinant proteins SBM and RGD-SBM, purified by affinity chromatography, before (1) and after (2) adsorption on the hydrogel. (MW – molecular weight, KDa).

The fibroblasts were added to the hydrogel treated with SBM or RGD-SBM and incubated for 4 h (polystyrene and non treated hydrogel were used as controls). Then, the non-adherent cells were washed out and fresh medium was added. To evaluate the cellular adhesion to the hydrogel, MTS assays were performed, both with the non-adherent cells and the ones trypsinized from the hydrogel. The results obtained using the two cell samples (adherent; non adherent) are in good agreement. The MTS results showed that, in the case of the polystyrene, 100% of the cells adhered after 4 h of incubation. In the case of the hydrogel coated with RGD-SBM, about 80% of the total cells were present in the adherent fraction and only 50% of the cells adhered to the hydrogel controls (untreated hydrogel or containing SBM peptide) (figure 4). These results demonstrated that the RGD sequence was able to significantly increase the adhesion of fibroblasts to the hydrogel surface, as compared to the controls. Previous work reported that the CPII of the starch-based hydrogels were in the range 50% – 60% when compared to polystyrene

(Carvalho *et al.*, 2008a). Herein, the same range of values was obtained for hydrogel controls. Nevertheless, when the hydrogel was treated with RGD-SBM, it was possible to reduce the CPII to 17%, which represents an improvement on cell adhesion of more than 30% (the values reported are the average of 2 different assays, each one performed in triplicate).

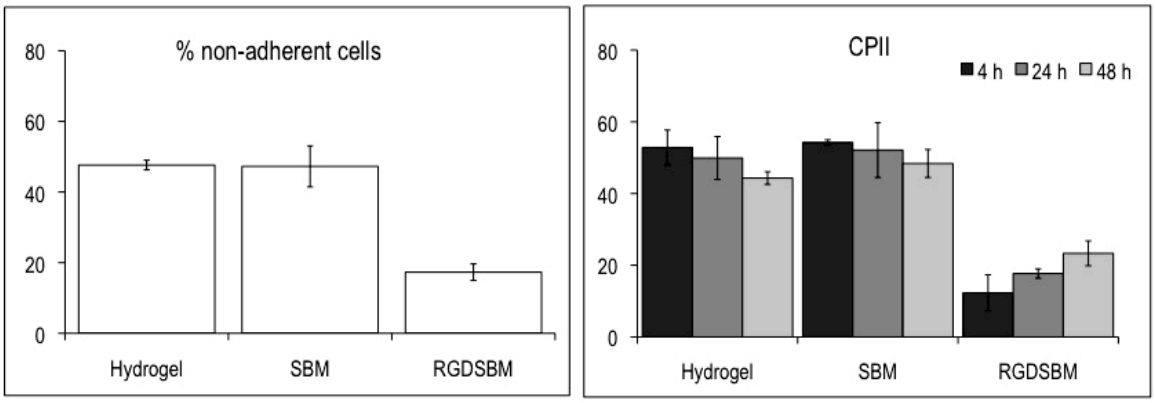

Figure 4 - MTS assays from non-adherent cells to the hydrogel and hydrogel coated with recombinant proteins after 4 h of adhesion. CPII of hydrogel with different treatments compared to the polystyrene plate at 4, 24 and 48 h of incubation after fibroblasts seeding.

The ability of the biomaterials to promote cell attachment is an important factor for tissue engineering applications. However, other important factors for the survival of the cells must be considered, namely cell spreading, migration, proliferation and matrix proteins production (Galbraith *et al.*, 2002; Mann and West, 2002). The cellular spreading on the hydrogel was evaluated by microscopic observation. Previous studies on starch-based hydrogels have shown differences in the morphology of the cells growing on the hydrogel surface (Ferreira *et al.*, 2004; Massia and Stark, 2001). Although viable, cells appear rounded and clustered when grown in the hydrogels. In contrast, in the presence of RGD sequence the cells are uniformly distributed on the hydrogel, exhibiting the characteristic fibroblast morphology (figure 5). The cellular spreading was only achieved under these conditions and the confluence was reached after 48 h of incubation.

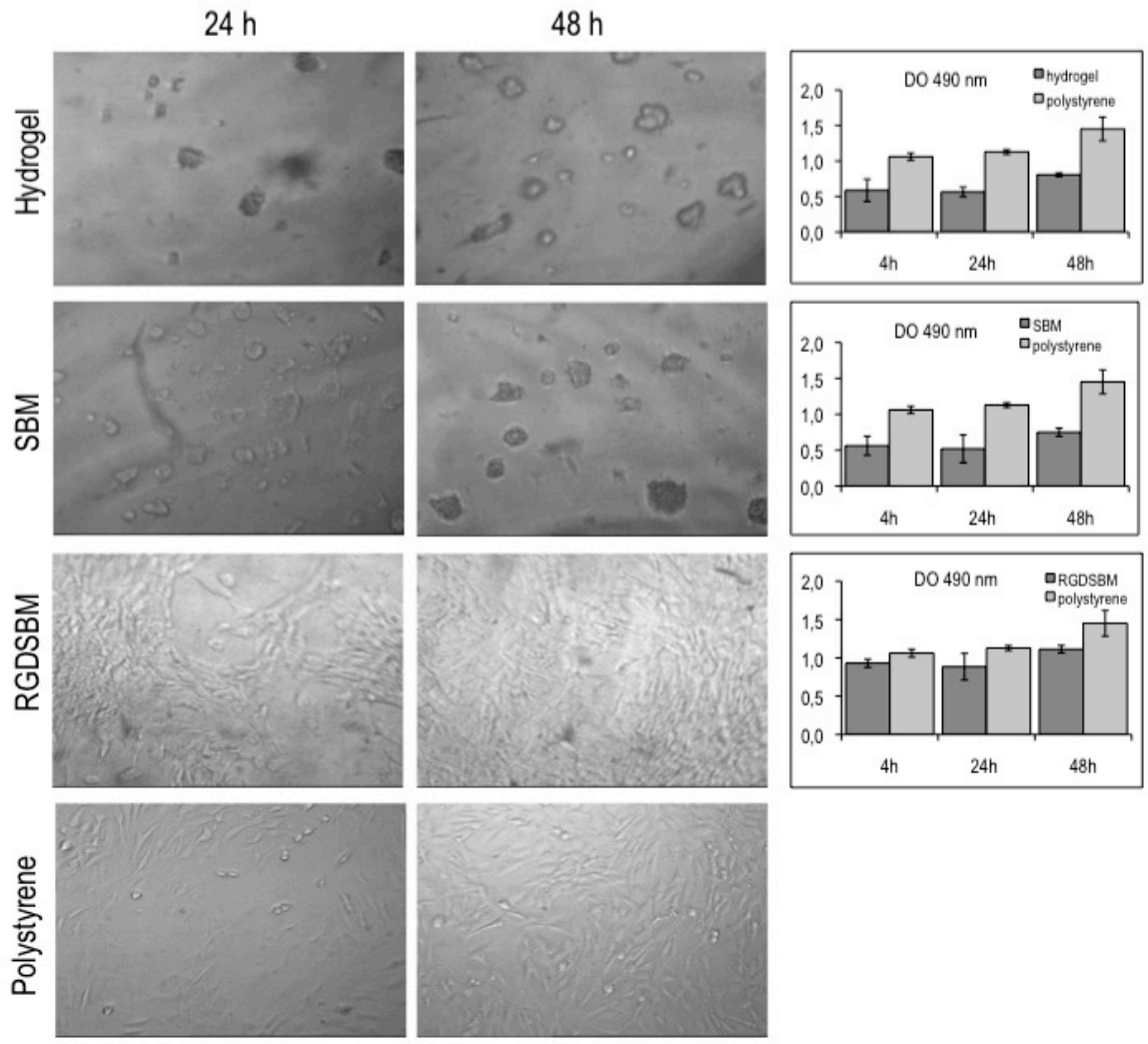

Figure 5 - Microscopic analysis and MTS assays of the fibroblasts cultivated on hydrogel without recombinant proteins, hydrogel coated with SBM or RGD-SBM; and cultivated on polystyrene plate, at different incubation times. The MTS assay compares the optical density at 490 nm between hydrogel with the different pre-treatments and the polystyrene plate at 4, 24 and 48 h of incubation after fibroblasts seeding.

The effect of the RGD-SBM peptide on proliferation was also evaluated. The MTS results suggested that the cell proliferation was only moderate irrespective of the hydrogel activation, as can be seen by the evolution of the non-normalized absorbance values (figure 5). These results are in good agreement with previous reports (Carvalho *et al.*, 2009b; Ferreira *et al.*, 2004) of a moderate cell growth on similar non-activated hydrogels. It could be expected that the presence of RGD-SBM peptide would increase the proliferation rate. However, despite the relevant effect on cell morphology, the presence of the RGD-SBM does not lead to a significant increase in the rate of proliferation. It is well

known that the fibroblast migration and proliferation on a biomaterial surface, coated with adhesive peptides, is dependent on the peptides density (Mann and West, 2002; Neff *et al.*, 1999; Tan *et al.*, 2005). Neff *et al.* (1999) found that fibroblast migration and proliferation decreased with the increasing of the adhesion peptide concentration. These authors found that for a maximum fibroblast proliferation, the adhesive peptide density should be intermediate. A similar effect was observed for other cells lines, namely, murine melanoma cells (Burgess *et al.*, 2000) and smooth muscle cells (DiMilla *et al.*, 1993). In this work, the effect of the RGD-SBM concentration on both cells migration and proliferation was not evaluated. Likewise, it is possible that the peptide concentration should be under or over the optimum, which would explain the proliferation rate observed. Thus, future work should address the optimization of the peptide concentration in order to maximize cell proliferation on the dextrin-based hydrogel (Mann and West, 2002).

CONCLUSIONS

In this work, a new approach was applied to functionalize a dextrin-based hydrogel: a recombinant protein, with a C-terminal starch-binding module and a N-terminal RGD sequence was cloned, expressed and successfully used to improve fibroblast adhesion and spreading on the hydrogel surface. The recombinant DNA techniques allow the fusion of different peptides in order to obtain chimeric proteins with specific functionality. However, the fusion of two peptides individually functional does not necessarily lead to a bi-functional fusion protein, according to our own experience. The loss of peptide functionally in the recombinant fusion protein may be a result of conformational changes that interfere with substrate accessibility or cell interaction. This is not the case with the protein produced in this work. The RGD-SBM recombinant protein improved by more than 30% the adhesion and spreading of fibroblast on the starch-based hydrogel.

The major advantages of the approach developed in this work may be summarized as follows: 1) the RGD sequence is expressed in *E. coli* fused to the SBM, not chemically synthesized; normally, the RGD sequence obtained chemically is attached to a linker and/or a reactive amino acids, and thus are expensive products. 2) The fusion protein strongly and specifically binds dextrin or starch-based materials, without the need for complex chemistry, toxic chemicals, etc. The specific affinity of the SBM may be used also

for the purification of the protein. 3) This approach, here demonstrated with the RGD case study, may be adopted to a wide range of peptides, particularly to short peptides like SBM, which may easily be produced using this expression system. 4) Several applications may be envisaged for this system: the production of biomimetic materials for the development of cell culture medium, the functionalization of materials for cell immobilization or even biomedical applications (addressing multifunctional nanoparticles made of starch to neoplastic tissue by RGD active targeting, for instance). Preliminary results show that the foreign body reaction to hydrogel implants is not affected by the presence of RGD-SBM.

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Chapter 4

Dextrin - based hydrogels: *in vivo* biocompatibility and biodegradability

Results accepted for publication on JBCP

Moreira, S., Costa, C., Guardão, L., Gärtner, F., Vilanova, M., Gama, M., 2009. Dextrin-based hydrogel; *in vivo* biocompatibility and biodegradability.

ABSTRACT

In vivo biocompatibility studies of dextrin hydrogels, obtained by radical polymerization of dextrin-hydroxyethylmethacrylate (dextrin-HEMA) and dextrin-vinyl acrylate (dextrin-VA), are reported in this work. The histological analysis of subcutaneous implants of these hydrogels, featuring inflammatory and reabsorption events, was carried out over a period of 16 weeks, in mice. While dextrin-HEMA hydrogel was quickly and completely degraded and reabsorbed, dextrin-VA degradation occurred slowly, apparently through an erosion controlled process. Indeed, although amylase is present in human plasma, the degradation of dextrin-VA hydrogel was very limited. A thin fibrous capsule was observed 16 weeks post-implantation, surrounding the non-degradable hydrogel. In the case of the degradable material, only a mild inflammatory reaction was observed, a few foamy macrophages being detected around the implant. This reaction was followed by complete resorption, with no signs of capsule formation or fibrosis associated with the implants. Altogether, these results strongly suggest that the dextrin hydrogels are fully biocompatible, since no toxicity on the tissues surrounding the implants was found. Moreover, it may be speculated that a controlled degradation rate of the hydrogels may be obtained, using dextrin with grafted HEMA and VA in different proportions.

INTRODUCTION

Hydrogels are water-swollen polymeric materials, with three-dimensional stable structure (Kopecek, 2007) providing good scaffolds for tissue engineering and replacement, wound dressings, drug delivery systems, among other applications. Hydrogels have great potential for drug delivery purposes. They can be used to encapsulate cells that produce therapeutic agents, such as growth factors, to be released in the surrounding tissues (Carvalho *et al.*, 2007; Hardwicke *et al.*, 2008; Hoffman, 2002; Langer and Peppas, 2003). Hydrogels must meet several strict requirements to make it eligible for biomedical applications. Among them, biocompatibility is an essential issue, together with the non-toxicity of the leachable and degradation products. Therefore, the comprehensive characterization of the degradation processes and the biological effects of the by-products are crucial for the long-term success of the hydrogel applications (Del Guerra *et al.*, 1996; Del Guerra *et al.*, 1995; Kirkpatrick, 1992; Ratner, 1997)

Natural polymeric materials, modified using different strategies, are an important source of hydrogels (Cadée *et al.*, 2000; Draye *et al.*, 1998; Hasırcı *et al.*, 2001; Vieira *et al.*, 2008). Our group recently developed hydrogels made of dextrin, a polysaccharide obtained by partial hydrolysis of starch, composed of $\alpha(1\rightarrow4)$ linked D-glucose residues. In a previous study, it was shown that the dextrin-based hydrogels have distinct degradation profiles, *in vitro*, depending on the acrylate ester used to functionalize the polysaccharide: dextrin-vinyl acrylate (VA) originates a non-degradable hydrogel, while polymerized dextrin-hydroxyethylmethacrylate (HEMA) is a degradable one (Carvalho *et al.*, 2009a). Moreover, the degree substitution (DS) of the polymer was shown to influence the degradation profile.

Although similar to previously developed dextran-VA and dextran-HEMA materials (Cadée *et al.*, 2000), the use of dextrin made hydrogels represents, in our view, a better choice for biomedical applications. Dextrin, although already used in clinical treatments, namely in end-stage renal failure patients, as peritoneal dialysis solution, and as a carrier for the anticancer agent 5-fluorouracil (Frampton and Plosker, 2003; Hardwicke *et al.*, 2008; Hosie *et al.*, 2003; Hreczuk-Hirst *et al.*, 2001), is relatively unexploited in the biomedical field. However, it presents excellent properties: biocompatibility, non-immunogenicity and *in vivo* degradability by α-amylases, yielding maltose and isomaltose. Dextrin has

appropriate molecular weight to ensure renal elimination, excluding the threat of progressive accumulation, after repeated administration (Carvalho *et al.*, 2009b; Carvalho *et al.*, 2007; Garcia *et al.*, 2008; Hreczuk-Hirst *et al.*, 2001). Indeed, it has a very low molecular weight, and is likely to be degraded to glucose by the amylases present in the human fluids. Thus, it is expected to be completely metabolized or removed by renal filtration. According to *in vitro* studies, the dextrin made hydrogels are not cytotoxic (Carvalho *et al.*, 2009a).

Biodegradability and resorption are highly desirable for tissue engineering and also for other biomedical applications where a temporary use of a medical device is envisaged, avoiding a second surgery for its extraction. Therefore, the extent of hydrogel degradation, under a set of conditions, must be characterized in order to determine the suitability of the material for a given application. This work describes the *in vivo* biocompatibility and degradability of two hydrogels, composed of dextrin-HEMA and dextrin-VA, respectively *in vitro* degradable and non-degradable.

MATERIAL AND METHODS

Reagents and Animals

Dextrin - Koldex 60 starch was a generous gift from Tate & Lyle (Decatur, IL, USA). All chemicals used in the preparation of the hydrogels, including Vinyl acrylate (VA), 2-hydroxyethylmethacrylate (HEMA), N,N,N',N'-tetramethylenethylenediamine (TEMED), ammonium persulphate (APS) were laboratory grade from Sigma-Aldrich, St. Louis, USA.

The *in vivo* biocompatibility studies were performed using male BALB/c mice (8 weeks old) purchased from Charles River (Barcelona, Spain). Animals were kept at the animal facilities of the Institute Abel Salazar during the experiments. Procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92).

Preparation of Dextrin-based Hydrogels

The functionalization of the dextrin with vinyl acrylate (VA) or hydroxyethylmethacrylate (HEMA) was achieved as described by Carvalho *et al.* (Carvalho *et al.*, 2007; Carvalho *et al.*, 2009a).

Dextrin-VA and dextrin-HEMA monomers were synthesized from dextrin in DMSO in the presence of different amounts of the VA or HEMA monomers and the degree of substitution (DS) was determined by proton nuclear resonance spectroscopy (^{1}H-NMR) in D_2O as previously described (Carvalho *et al.*, 2007; Carvalho *et al.*, 2009a). The hydrogel slabs with different DS were prepared by radical polymerization of aqueous solution of either dextrin-VA or dextrin-HEMA. The hydrogel discs for implantation were prepared dissolving 300 mg of dextrin-VA (degree of substitution, DS 20 or 70) in 1 ml PBS (phosphate buffered saline), and a volume of 30 μl of this solution was transferred into the circular casts (5 mm diameter and 5 mm height). Following the radicalar polymerization, started by adding 3 μl TEMED (13.3 %, v/v) and 3 μl APS (80%, w/v) to the dextrin solution, the discs were transferred into an eppendorf with PBS buffer and sterilized (121ºC, 1 atm, 20 min). The dextrin-HEMA hydrogel with DS 20 was prepared using two concentrations, 300 mg/ml and 150 mg/ml.

MALDI-TOF Analysis

The molecular weight of dextrin used in the hydrogels preparation was evaluated by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF), using a 4700 MALDI-TOF Proteomics Analyser (Applied Biosystems) and a matrix of 2, 5-dihydroxybenzoic acid.

Cryo-SEM Analysis

The cryo-scanning electron microscopy (cryo-SEM) analysis of polymerized hydrogels was performed using a JEOL JSM 6301F/ Oxford INCA Energy 350 / Gatan Alto 2500, 15kV, WD 15 mm.

Subcutaneous Implantation

The hydrogels were implanted subcutaneously, by surgery, on the back of mice, each one receiving two implants. Mice were anaesthetised by intramuscular injection of a mixture of ketamine (Imalgene 1000, Material) and Bompun 2% (Bayer Healthcare), at a 50 mg/Kg and 10 mg/Kg dose, respectively. Then, hair was shaved and two small incisions were made in the skin, to prepare bilateral subcutaneous pockets along the backbone where the hydrogel discs were implanted. Wounds were closed by stitches. The animals used as control received no implant. Two animals, each one holding two implants, were used for each post-implantation period analysed.

Histological Analysis

The aspect of the wound and the presence of oedema were observed before extraction of the implants. Two animals, each one containing two implants, were sacrificed sequentially at 1, 2, 4, 8 and 16 weeks after implantation. The implantations sites were completely excised for histological analysis. Samples were fixed in 10% neutral buffered formalin for 24 h and paraffin-embedded. Sections 4 μm-thick were obtained and used for haematoxylin-eosin (H&E), Schiff's periodic acid (PAS) and Masson's trichrome stains. Slides were examined under a light microscope (Nikon E600) and photographs were obtained using a digital camera (Nikon DS-5M).

RESULTS

Dextrin hydrogels developed in our lab were previously described; details on the structure, rheology and *in vitro* biocompatibility has been published elsewhere (Carvalho *et al.*, 2007; Carvalho *et al.*, 2009a; Garcia *et al.*, 2008). Further characterization is provided in this work. The dextrin MALDI-TOF mass spectrum and the chemical structures of dextrin-VA, dextrin-HEMA and of the corresponding hydrogels are presented in Figure 1.

The dextrin mass spectrum confirms previous results, obtained by Gas Chromatography methylation analysis: the polymer has very low molecular weight and high polydispersity.

The MALDI-TOF analysis is not quantitative, the lower molecular weight oligomers ionizing more easily and consequently giving rise to peaks with higher intensity. However, peaks corresponding to a degree of polymerization of 15 are detected, and the average degree of polymerization, as evaluated previously, is probably of about 13. This is a relevant finding, since the dextrin low molecular weight will probably contribute to the efficient clearance of the hydrogel degradation products.

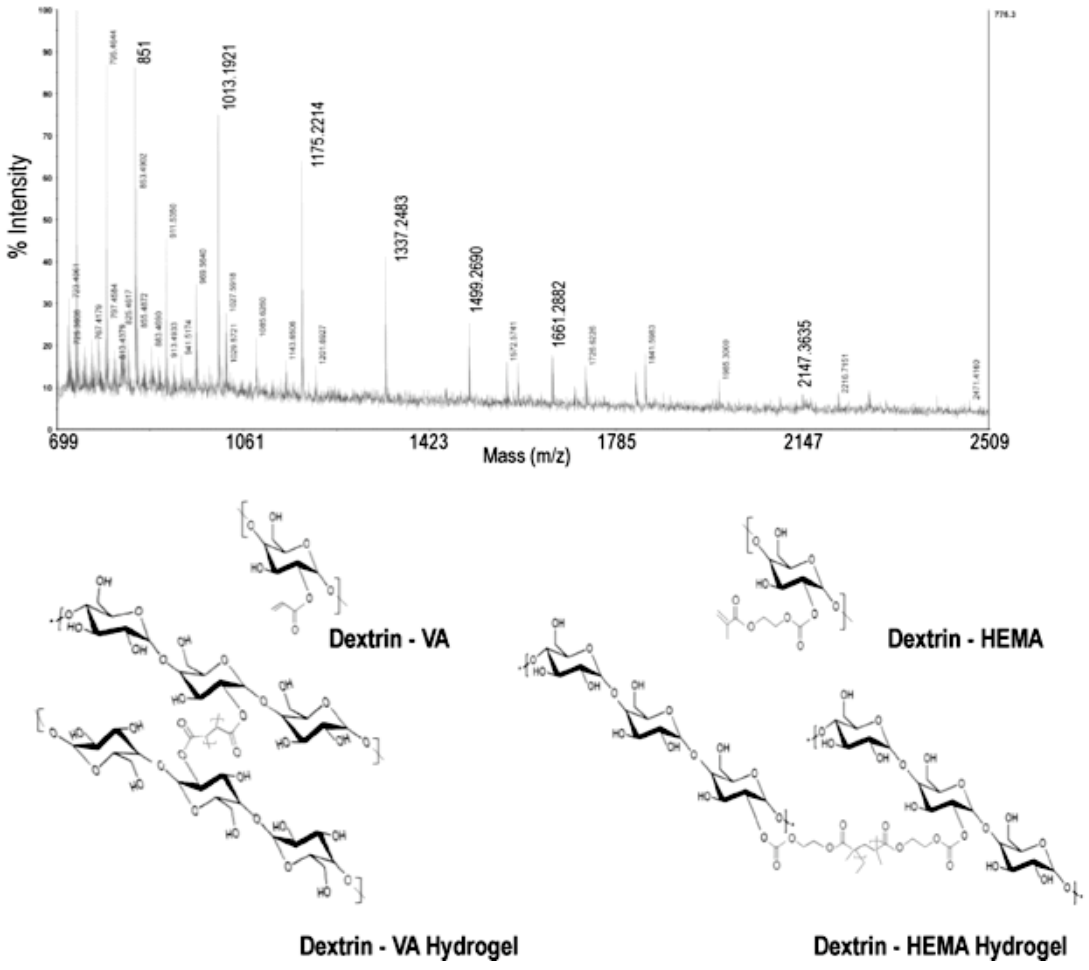

Figure 1- Dextrin MALDI-TOF mass spectra and chemical structure of dextrin substituted with VA or HEMA and its structure following polymerization.

In both cases, the polymer was functionalized by transesterification with acrylate esters, yielding the water-soluble functionalized dextrin-VA or dextrin-HEMA. Gelation is achieved through free radical polymerization of the reactive double bonds cross-links, in the presence of TEMED and APS. In figure 1 is the schematic representation of the dextrin substituted with VA or HEMA as well as their structures after polymerization, based on the dextran-HEMA and dextran-VA structures described in previous works (van Dijk-Wolthuis *et al.*, 1997; Van Tomme *et al.*, 2006). The ester bonds obtained using VA or HEMA are different: the VA cross-links are highly resistant to hydrolysis, while HEMA are hydrolysable in an enzyme free system (shown elsewhere Carvalho *et al.*, 2009a).

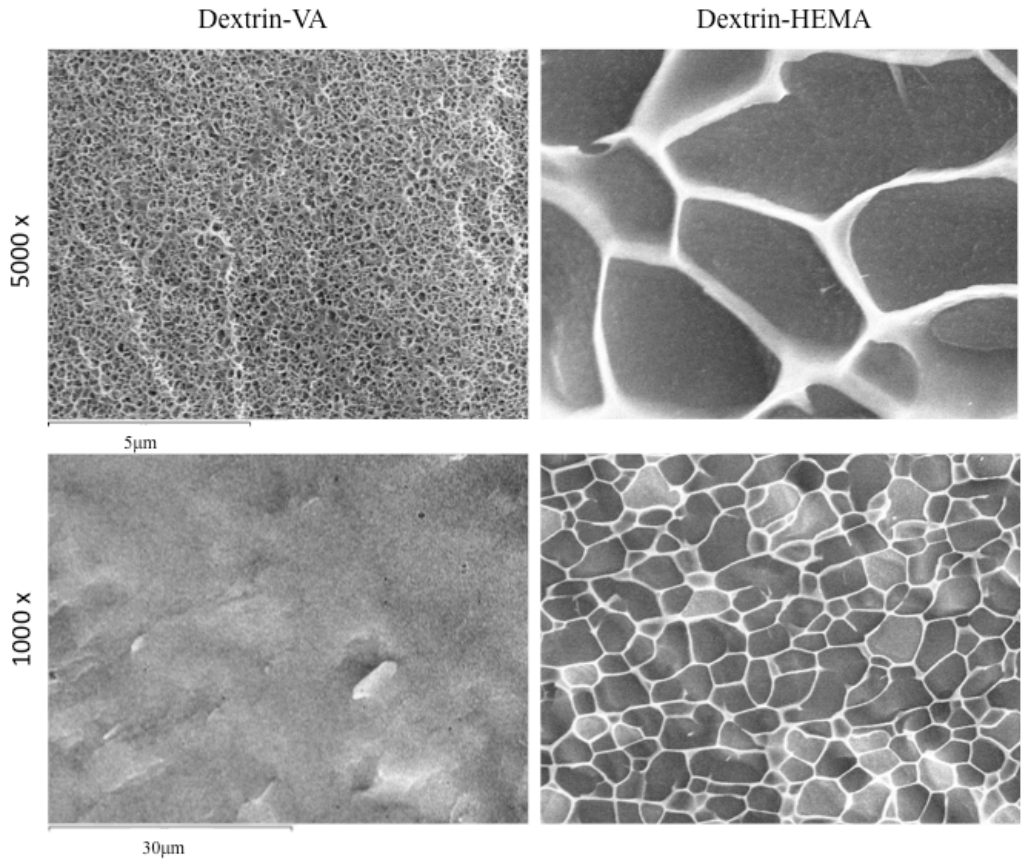

Figure 2- Cryo-SEM analysis of polymerized hydrogels: dextrin-VA and dextrin-HEMA (DS 20%; 300 mg/ml). Analysis performed at 15kV, Amp 5000X and 1000X.

Figure 2 reveals the structural pattern of the hydrogels, as obtained by cryo-SEM analysis. The dextrin-VA and dextrin-HEMA hydrogels exhibit rather distinct porosities, the former being much more compact and less porous, for the same DS (20%) and concentration

(300 mg/ml). This is an unexpected finding, since the hydrogels are obtained from dextrin with similar DS. However, it must also be remarked that the hydrogels present rather distinct mechanical properties (Carvalho *et al.*, 2009a), differences that are also noticeable by visual evaluation.

In order to evaluate the biocompatibility of dextrin-based hydrogels and its degradation behaviour *in vivo,* BALB/c mice were implanted subcutaneously with dextrin-VA or dextrin-HEMA hydrogels. The implanted tissue was removed at different times and histological analyses were carried out.

Dextrin–VA is a Non-degradable Hydrogel *in vivo*

After 1 week post-implantation, a sub-acute, mild to moderate, focal inflammatory response was present on surgical sites, on both controls and implanted animals. Both groups showed marked vascular congestion and interstitial oedema, with moderate, mixed infiltration of neutrophils, a few macrophages and lymphocytes, together with proliferation of fibroblastic and endothelial cells (Figure 3). This was interpreted as a reaction to surgical trauma rather than to the implants, since treated and control animals reacted identically.

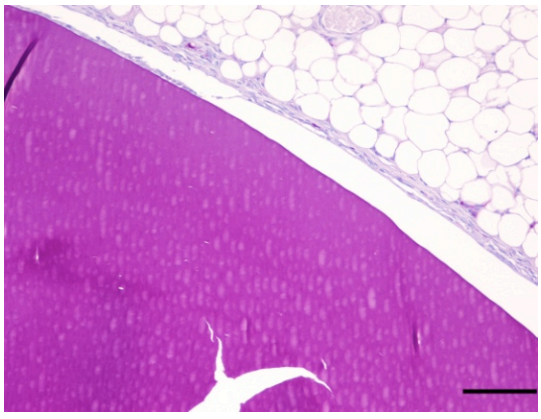

Figure 3- DS 20 dextrin-VA implant, 1 week post-implantation. The implant (*) is intact (PAS, bar = 50μm).

From 2 to 4 weeks post-implantation, the vascular phenomena decreased in magnitude. Macrophages progressively became predominant within the cellular infiltrate and

concentrated around the implanted material. Smaller hydrogel particles were often observed while surrounded by foamy macrophages, which showed moderate amounts of intracytoplasmic PAS-positive hydrogel material. This was more noticeable with DS 20 hydrogels, possibly because these were softer and more easily fragmented during surgery.

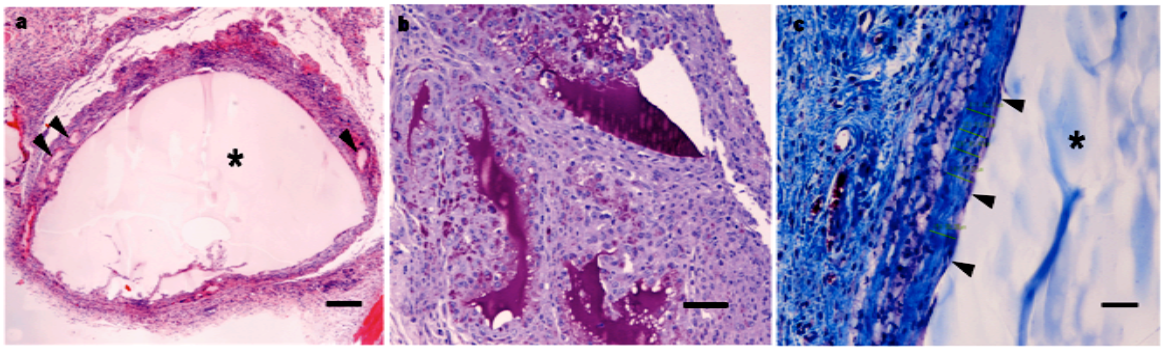

Figure 4- DS 20 dextrin-VA implant, 16 weeks post-implantation. The implant (*) is generally intact. a) Note a ring of macrophages around the implant and a few scattered fragments (arrows) in its vicinity. (H&E, bar = 200μm). b) Small DS 20 dextrin-VA fragments surrounded by numerous macrophages showing small amounts of intracytoplasmic PAS-positive material. (PAS, bar = 50μm). c) The implant (*) is surrounded by a fibrous capsule (arrows), showing 5 consecutive measurements (Masson's trichrome stain, bar = 20μm).

By week 16 post-implantation, the inflammatory reaction had subsided, with only a thin macrophagic ring surrounding the implants. While small hydrogel fragments seemed to be amenable to phagocytosis, the main bulk of the implants showed no signs of resorption (Figures 4a and b). Using Masson's trichrome stain, a thin fibrous capsule could be demonstrated, at 16 weeks post-implantation, around both DS 20 (30.78±1.79μm) and DS 70 (34.78±2.64μm) implants (Figure 4c).

Dextrin-HEMA is a Degradable Hydrogel *in vivo*

After 1 week post-implantation, while control animals showed identical changes to those described for dextrin-VA, implanted animals presented a sub-acute, moderate to intense, inflammatory response, focused around the implanted material. There was moderate vascular congestion and mild interstitial oedema, with moderate to intense, mixed

infiltration of neutrophils and less macrophages. Both 150 mg/ml and 300 mg/ml implants showed mild signs of resorption, with a few foamy macrophages exhibiting intracytoplasmic PAS-positive material (Figure 5).

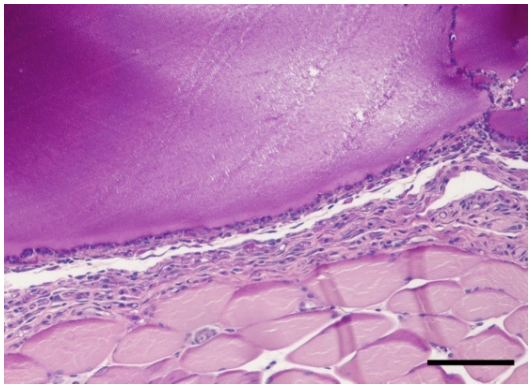

Figure 5- 300 mg/ml dextrin-HEMA implant, 1 week post-implantation. The implant is generally intact, however, is surrounded by macrophages with PAS-positive material (PAS, bar = 10μm).

From weeks 2 to 4 the number of neutrophils progressively decreased and foamy macrophages became largely predominant. At week 4, there were no signs of extracellular 150 mg/ml material left, but variably sized accumulations of foamy macrophages laden with PAS-positive material were present, forming a subdermal band (Figures 6a and b). Fragments of the 300 mg/ml implants were still partially visible (Figure 6c). By week 8 post-implantation, both groups already showed complete implant resorption and neither group presented any signs of capsule formation or, indeed, of fibrosis associated with the implants (data not shown).

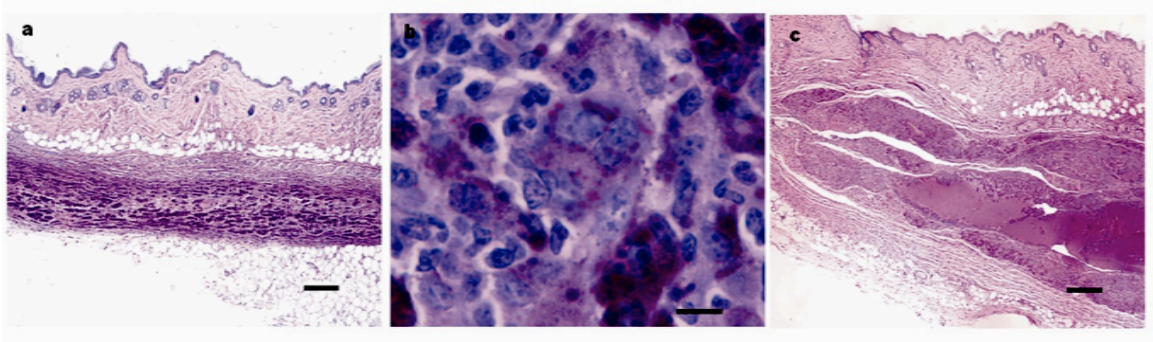

Figure 6- 150 mg/ml (a and b) or 300 mg/ml (c) dextrin-HEMA implant, 4 weeks post-implantation. a) There are no visible extracellular fragments of the implant. Numerous macrophages containing abundant intracellular PAS-positive material form a subdermal band located at the implantation site (PAS, bar = 200μm). b) Variably abundant, intracytoplasmic, globular, PAS-positive material is present in macrophages (PAS, bar = 10μm). c) Large, extracellular implant fragments remain in the deep dermis, surrounded by a thick macrophagic ring (PAS, bar = 200μm).

DISCUSSION

Hydrogels may be used implanted or otherwise in touch with body fluids and tissues, to assist or substitute the function of organs and tissues. Therefore, the characterization of the interaction with the living organism is of paramount importance. In spite of dextrin being a biodegradable polymer, the degradation profile of a polymeric material could be affected by its chemical modification and also by the degree of substitution (Hreczuk-Hirst *et al.*, 2001; Vercauteren *et al.*, 1990). Therefore, the evaluation of its toxicity and degradation behavior *in vivo* was analysed in this work. The results from cryo-SEM showed that, although both hydrogel are made of dextrin, the structures of the polymerized hydrogels depended deeply of the molecule used to functionalize it. As showed in Figure 2 the dextrin-VA have smaller porous than dextrin-HEMA for the same DS (20%) and concentration (300 mg/ml).

The results obtained with dextrin-VA hydrogels indicate its apparent non-degradability *in vivo*, irrespective of the DS of the polymer used. Previous results showed that *in vitro* the degradation of the dextrin–VA hydrogels in phosphate saline buffer is very slow. Still, the hydrogel could be rendered degradable, through the incorporation of amylase; indeed, this approach proved to be an effective route to modulated the gel degradation and

release of entrapped molecules (Carvalho *et al.*, 2009b). It could thus be expected that α-amylase present *in vivo* could promote the hydrogel hydrolysis. However, a significant degradation was not observed for the dextrin-VA hydrogels used in the time frame analysed. This indicates that the hydrogels do not undergo enzymatic degradation *in vivo*, as described for dextran-MA (methacrylated) hydrogels (Cadée *et al.*, 2000). In the vicinity of the implanted DS 20 hydrogel, macrophages positively stained with PAS were observed. However, the bulk material remained intact. This suggests that, while the smaller hydrogel fragments (probably resulting from mechanical fragmentation during implantation) could be reabsorbed, the hydrogel is basically non-degradable. As a matter of fact, similar findings were described for dextran-base hydrogels (Cadée *et al.*, 2000; De Groot *et al.*, 2001).

Regarding the dextrin-VA DS 70 hydrogel, although several paraffin section were observed, the implanted material was always lost during the preparation, due to its high stiffness. The use of a harder inclusion medium would be necessary to allow a proper sectioning of the highly substituted, very hard material. Nevertheless, as a fibrous capsule could be observed, this allowed the identification of the implant site (data not shown). Indeed, the formation of fibrous capsules was observed to occur using either of the dextrin-VA hydrogels. However, following the sub-acute focal response due to the surgical procedure, the inflammatory response decreased all along the time course of the experiment. By 16 weeks after placing the implant only a thin macrophagic ring surrounding the implants was observed, together with the fibrous capsule. Since the material did not induced detectable necrosis, immunotoxicity, and damage to muscle tissue, it can be assumed that dextrin-VA hydrogels presented biocompatible behaviour *in vivo* (Rihova, 1996).

The other hydrogel used, dextrin-HEMA, was previously shown to be degradable *in vitro,* in PBS, its degradability rate depending on the polymer's DS (Carvalho *et al.*, 2009a). The dextrin-HEMA degradation observed *in vivo* was therefore expected. The histological analysis showed that at 1 week post-implantation, besides the inflammatory response resulting from implantation, it was also possible to observe macrophages positively stained with PAS, indicating that resorption occurred. The resorption process increased during the time course of the experiment, until the complete degradation of the hydrogels was accomplished by 4 weeks pos-implantation, for the 150 mg/ml hydrogels. The hydrogel with higher concentration (300 mg/ml) required more time for complete

resorption. Similarly to dextrin-VA, the dextrin-HEMA hydrogels presented biocompatible behaviour. Moreover these findings also showed that its degradation rate could be controlled through selection of the polymer concentration.

CONCLUSION

Work currently ongoing in our lab explores the potential of the dextrin hydrogels as delivery systems and tissue engineering scaffolds. Carvalho *et al.* showed that, *in vitro*, dextrin-hydrogels effectively operate as a protein depot, the release being controlled through the use of amylase, selection of the polymer concentration and DS (Carvalho *et al.*, 2009a). Furthermore, it was also shown that these hydrogels may be easily functionalized, using recombinant proteins containing a starch binding module fused with bioactive molecules, in order to improve cell adhesion and spreading (Moreira *et al.*, 2008). In this work two dextrin-hydrogels were subcutaneously implanted in mice; both presented biocompatible behaviour *in vivo*, since none induced necrosis, immunotoxicity, or damage to muscle tissue.

The degradation rate of the biomaterial is an important issue depending on its application. While in some applications, such as a pacemaker or breast implants, maintenance of the physical integrity and mechanical properties are required, in other, such as surgical suture, rapid biodegradability is required. The control of the rate and extent of degradability of a biomaterial is therefore critical for its assigned function. Here we presented two biocompatible hydrogels that can be used in different applications once their degradation profile can be controlled by selecting the acrylate ester used in the dextrin functionalization, the polymer DS and its concentration. Based on the results obtained, may be speculated that dextrin hydrogel with mixed reticulation chemistry, either HEMA or VA, in different proportions, may be used to fine-tune the degradation rate.

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Chapter 5

BC nanofibres: *in vitro* study of genotoxicity and cell proliferation

Results published on Toxicology Letters

Moreira, S., Silva, N., Almeida-Lima, J., Rocha, H., Medeiros, S., Alves, C., Gama, M., 2009. BC nanofibres: *in vitro* study of genotoxicity and cell proliferation. Toxicology Letters 189, 235–241

ABSTRACT

Nanomaterials have unusual properties not found in the bulk materials, which can be exploited in numerous applications such as biosensing, electronics, scaffolds for tissue engineering, diagnostics and drug delivery. However, research in the past few years has turned up a range of potential health hazards, which has given birth to the new discipline of nanotoxicology. Bacterial cellulose (BC) is a promising material for biomedical applications, namely due to its biocompatibility. Although BC has been shown not to be cytotoxic or genotoxic, the properties of isolated BC nanofibres (NFs) on cells and tissues has never been analysed. Considering the toxicity associated to other fibre-shaped nanoparticles, it seems crucial to evaluate the toxicity associated to the BC-NFs.

In this work, nanofibres were produced from bacterial cellulose by a combination of acid and ultrasonic treatment. The genotoxicity of nanofibres from bacterial cellulose was analysed *in vitro*, using techniques previously demonstrated to detect the genotoxicity of fibrous nanoparticles. The results from single cell gel electrophoresis (also known as comet assay) and the *Salmonella* reversion assays showed that NFs are not genotoxic under the conditions tested. A proliferation assay using fibroblasts and CHO cells reveals a slight reduction in the proliferation rate, although no modification in the cell morphology is observed.

INTRODUCTION

The development of artificial materials with biomimetic behavior is essential for tissue engineering purposes. Scaffolds based on nanofibres mimic the natural extracellular matrix and its nanoscale fibrous structure. Several approaches have been described in order to achieve materials based on nanofibres from synthetic or natural polymers (Ashammakhi *et al.*, 2007; Ma *et al.*, 2005).

Bacterial cellulose (BC), secreted by *Gluconacetobacter xylinus,* has been presented as a biocompatible scaffold for the engineering of cartilage and blood vessels, wound dressing, guided tissue regeneration, among other applications (Andrade *et al.*, 2008; Astley *et al.*, 2003; Backdahl *et al.*, 2008; Czaja *et al.*, 2007; Entcheva *et al.*, 2004; Maneerung *et al.*, 2008; Rambo *et al.*, 2008; Svensson *et al.*, 2005; Tabuchi and Baba, 2005; Teeri *et al.*, 2007). BC has unique characteristics including high purity, high crystallinity and remarkable mechanical properties, due to the uniform ultrafine-fiber network structure, the high planar orientation of the ribbon-like fibres when compressed into sheets, the good chemical stability, and the high water holding capacity (Svensson *et al.*, 2005). Several materials based on bacterial cellulose, recognized as non genotoxic and non cytotoxic, have been commercialized (Jonas and Farah, 1998; Schmitt *et al.*, 1991).

Since nanomaterials have unusual properties, not found in the bulk material, such as high surface reactivity and ability to cross cell membranes, concerns about their safety and toxicology have emerged. The impact of nanostructural features in the interaction of a material with cells and tissues is dependent on the size, chemical composition, surface structure, solubility, shape, and on the supramolecular structural organization (Barnes *et al.*, 2008). A major concern with fibres is their carcinogenic potential. There is sufficient evidence that all forms of asbestos (generic term for a group of six naturally occurring fibrous silicate minerals) are carcinogenic and co-carcinogen to man (Dopp *et al.*, 2005; Speit, 2002). Moreover, recent studies described the toxicity of materials associated to size or shape; namely, the toxicity of carbon nanotubes (Donaldson *et al.*, 2006; Poland *et al.*, 2008) and the size-dependence toxicity of gold or ferric oxide nanoparticles was reported (Backdahl *et al.*, 2008; Pan *et al.*, 2007; Wang *et al.*, 2009).

The toxicity associated with inhaled fibres such as asbestos has been described. Inhaled fibres may be toxic, particularly when they are "long, thin and durable" (Donaldson *et al.*,

2006). Asbestos fibres are dangerous because the fibres split lengthwise, producing thin fibres that can enter the lungs, being "moderately durable" once there (Speit, 2002). Although cellulose fibres, from wood pulp and textile fabric, are used without "significant concern", cellulose fibres share similar features with asbestos, including the needle-like shape and biopersistence. Moreover, the inflammatory responses of respirable cellulose fibres (wood pulp) using animal models were already reported (Cullen *et al.*, 2000). In light of these results, it seems crucial to evaluate the toxicity of the BC nanofibres. It must be remarked that, although BC cannot be enzymatically degraded in the human body, the inflammatory processes may actually degrade cellulose to some extent. Given the current focus of BC as a promising biomaterial with a variety of applications, it is relevant to evaluate not only the toxicity of BC membranes or scaffolds, but also of its degradation products, including BC nanofibres.

Indeed, although *in vivo* studies demonstrate the BC biocompatibility (Helenius *et al.*, 2006), and lack of mutagenicity (Schmitt *et al.*, 1991), no reports are available on the BC nanofibres toxicity. Although BC is not expected to be degraded *in vivo*, safety concerns makes this study mandatory. It is well accepted that *in vitro* studies using cell systems are valuable tools to clarify the cellular mechanisms involved in genototoxic effects, including DNA damage (Dusinská *et al.*, 2004 ; Speit, 2002). Therefore, the aim of this study is to evaluate the genotoxicity of cellulose nanofibres at cellular level using the single cell gel electrophoresis and the *Salmonella* reversion assays. The cell proliferation in the presence of nanofibres was also evaluated. These tests are useful as a screening tool for setting priorities because they are an inexpensive and a quick way to help single out substances that should be targeted for further testing. Furthermore, these assays were already used to demonstrate the genotoxic effect of asbestos fibres in mammalian cells *in vitro* (Dusinská *et al.*, 2004 ; Speit, 2002).

MATERIALS and METHODS

Bacterial strain, cells and culture medium

The cellulose was produced by *Gluconacetobacter xylinus* (ATCC 53582), purchased from the American Type Culture Collection, grown statically in Hestrin-Schramm medium (Hestrin and Schramm, 1954), pH 5 at 30ºC, during 5 days.
In the *Salmonella* reversion assay, four strains of *Salmonella tryphimurium* (Dr. B. N. Ames, Biochemistry Department, University of California, Berkeley, USA) were used, namely, TA97a [*his* D6610, *rfa*, Δ *uvr*^{B}, *bio*$^{-}$, pKM101 (Ap^{R})], TA98 [*his* D3052, *rfa*, Δ *uvr*^{B}, *bio*$^{-}$, pKM101 (Ap^{R})], TA100 [*his* G46, *rfa*, Δ *uvr*^{B}, *bio*$^{-}$, pKM101 (Ap^{R})], and TA102 [*his* D428, *rfa*, pKM101 (Ap^{R}), pQA1 (Tt^{R})] (Levin *et al.*, 1982; Maron and Ames, 1983).

The proliferation assays were performed using mouse embryo fibroblasts 3T3 (ATCC CCL-164), grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% newborn calf serum (Invitrogen), and Chinese Hamster Ovary (CHO), grown in DMEM media supplemented with 10% fetal bovine serum (Invitrogen), both culture medium were supplemented with penicillin/streptomycin (1 µg/ml) (Sigma-Aldrich, St. Louis, USA) and the incubation was at 37ºC, in a fully modified air containing 5% CO_2. The same conditions were used to grow CHO cells for comet assay. The cell viability was assessed using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) assay, obtained from Invitrogen.

Production of BC nanofibres

The production of bacterial cellulose (BC) was performed by growing *G. xylinus* in Hestrin-Schramm medium, pH 5. After inoculation, the culture (100 ml) was incubated, first with agitation during 8 h, and then statically at 30ºC, for 5-7 days. BC pellicles were purified in a 4% NaOH solution at 70ºC, for 90 min. BC was then neutralised by thoroughly washing with water. Finally, BC pellicles were lyophilised prior to use.

The nanofibres production, by acidic and/or ultrasonic treatment, was based on previous works (Roman and Winter, 2004; Zhao *et al.*, 2007). The acid hydrolysis was performed as follows: 20 mg of dry BC was sliced in small pieces and 2 ml of 50% H_2SO_4 (v/v) was

added. The mixture was kept at 40ºC, for 2 h with vigorous stirring. To stop the hydrolysis, 10 ml of cold water was added and the cellulose was recovered by filtration, using a membrane with a 0.45 μm pore size. Then, the cellulose was washed out with 20 ml of water and the recovered pellet was resuspended in 10 ml of water. This suspension was treated by sonication at 40 W (Branson Ultrasonic Disruptor, Sonifier II/W450) for 10 min (samples were maintained on ice during sonication). Then, the NFs suspension was centrifuged (1 h, 15000 rpm), and the pellet resuspended in water and sonicated again, in the same condition, for another 10 min. The yield of the process was evaluated by quantifying the total sugar in the samples, using the phenol-sulphuric method (Dubois *et al.*, 1956).

TEM analysis

The NFs obtained were stained with uranyl acetate and analysed by transmission electronic microscopy (TEM, Zeiss 902A Orius SC 1000; 50kV).

Evaluation of cellulose nanofibres mutagenicity by *Salmonella* reversion assay

Four *Salmonella tryphimurium* strains were used to study the potential mutagenicity effect of the cellulosic NFs. The procedure was to some extent modified from the original description by Kado *et al.* (1986). This assay was performed in miscrosuspension with or without S9 mixture (Moltox^{TM}, North Carolina, USA), using 0.1, 0.5 or 1.0 mg/ml of NFs suspension. The negative control was distilled water, and the positive controls employed were: 0.1 μg/plate 4NQO (4-Nitroquinoline 1-oxide) for the TA97a and TA98 strains; 5.0 μg/plate sodium azide for the TA100 strain; and 0.5 μg/plate mytomicyn C for the TA102 strain. Briefly, 105 μl of a mixture containing the NFs suspension and cell suspension (10^9 cells/ml) were incubated at 37ºC for 90 min. Then, 2.5 ml of molten Top agar (0.6% bacto-agar and 0.5% NaCl) was added, before plating in a Petri dish containing minimal agar (1.5% agar, Vogel-Bonner E medium). The His^+ revertant colonies were counted after 72 h of incubation at 37ºC. All experiments were repeated at least three times with three replicas. The mutagenicity of cellulose NFs was evaluated according to the following parameters: the maximum number of revertants in the presence of the NFs should be 2-fold or more relative to the negative control; a dose-dependent increase in the number of revertants should be observed (Mortelmans and Zeiger, 2000).

Proliferation assays

The proliferation assays were performed *in vitro* as follows: 1 ml of the CHO or mouse embryo fibroblast 3T3 cell suspension (10^4 cells/ml) was seeded in a 24-well polystyrene plate (TPP, Switzerland). The cells were allowed to adhere for 4 h. Before the addition of cellulose NFs, the medium with non-adherent cells was removed and the NFs containing medium (to a final concentration of: 1, 0.5 or 0.1 mg/ml) was added. A control without NFs was carried out. The cellular growth at 0, 24, 48 and 72 h of incubation was evaluated by MTT assay, a colorimetric test that gives a measure of the mitochondrial activity. The effect of NFs on the cell morphology was evaluated by microscopic observation (Nikon Eclipse TE300 Inverted Microscope).

Evaluation of cellulose nanofibres genotoxicity by single cell gel assay (comet assay)

The DNA integrity was evaluated by alkaline single cell gel assay (also kwon as comet assay) using CHO cells grown in the presence of different NFs concentration.

In this assay, 2 ml of CHO cell suspension (10^5 cells/ml) were seeded on a 6-well polystyrene plate (TPP, Switzerland). After 16 h, the medium was refreshed with medium containing the NFs (0.1, 0.5 or 1 mg/ml). Cells were incubated with NFs suspension during 48 h. Hydrogen peroxide (100 mM) and water were used as positive and negative controls, respectively. The alkaline comet assay was performed as described by Singh (Singh *et al.*, 1988). Briefly, cells were trypsinized from 6-well polystyrene plate, and resuspended in 50 μl of medium. The cell viability was determined in a Neubauer counting chamber using the trypan blue exclusion test. A volume of 10 μl of the cellular suspension were embedded in 0.5% low-melting-point agarose and plated on agarose-coated microscope slide. Then, the slides with cells were treated with lysis solution (2.5 M NaOH, 0.1 M EDTA, 0.010 M Tris, 1% Triton X-100, 10% DMSO, adjusted to pH 10) for 12 hours at 4ºC, rinsed with distilled water, and placed in the electrophoresis buffer (0.3 M NaHO, pH 13 and 0.001 M EDTA), for 20 min to allow DNA unwinding. Following electrophoresis (30 min, at 25 V and 300 mA), the slides were neutralized with 0.4 M Tris buffer (pH 7.5) and stained with ethidium bromide (20 mg/ml). The slides were analysed through fluorescence microscopy (Nikon Eclipse TE300 microscope equipped with a Nikon E600 camera, 0.488 microns/pixel). At least 300 cells per condition tested were analysed.

The DNA damages were evaluated by image analysis using the ‘‘Comet Assay IV version 4.2” image analysis system. Data collected from each cell included tail length (TL), tail migration (TMi), percent tail DNA (TI), and tail moment (TM), which correspond to the product of the comet length and the amount of DNA in the tail (Olive and Durand, 1992).

Statistic analysis

The One-way Analysis of Variance (ANOVA) was applied to statistics evaluation of the comet scores and to the proliferation assays results. The post-test Tukey-Kramer Multiple Comparisons test was used to compare the scores of the samples and positive control (PC). The analyses were performed using GraphPad Prism 3.05.

RESULTS and DISCUSSION

Production of BC nanofibres

G. xylinum synthesizes cellulose nanofibres with 40–50 nm width (the bacterial cellulose ribbons), which assemble in a static culture as a white gelatinous material (pellicle) on the surface of the culture liquid. The native cellulose consists of sets of parallel chains of β-1,4-D-glucopyranose units interlinked by intermolecular hydrogen bonds (Czaja *et al.*, 2007). Several works describe the production of nanofibres from different cellulosic sources, using acid hydrolysis (Araki *et al.*, 1999; Roman and Winter, 2004) or mechanic treatment (Zhao *et al.*, 2007). These two approaches were used in order to extract NFs from BC. The acid hydrolysis was tested using a range of acid concentrations, temperatures and treatment time. Concentrations of H_2SO_4 superior to 50% resulted in extensive hydrolysis, yielding less than 20% of the material used (data not shown). The acid concentration is in fact the critical parameter in the acid hydrolysis approach. The use 50 % H_2SO_4, for 2 h at 40ºC, yielded 50% of nanofibres. According to Zhao *et al.* (2007), sonication can also be successfully used to extract NFs from natural materials, including cellulose from wood, cotton, bamboo. This approach was also applied to BC. Using acid hydrolysis (50% H_2SO_4, 2 h, 40ºC) and sonication (20 min, 40 W), needle shaped cellulose NFs with 50-1500 nm length and 3-5 nm width, were obtained (figure 1).

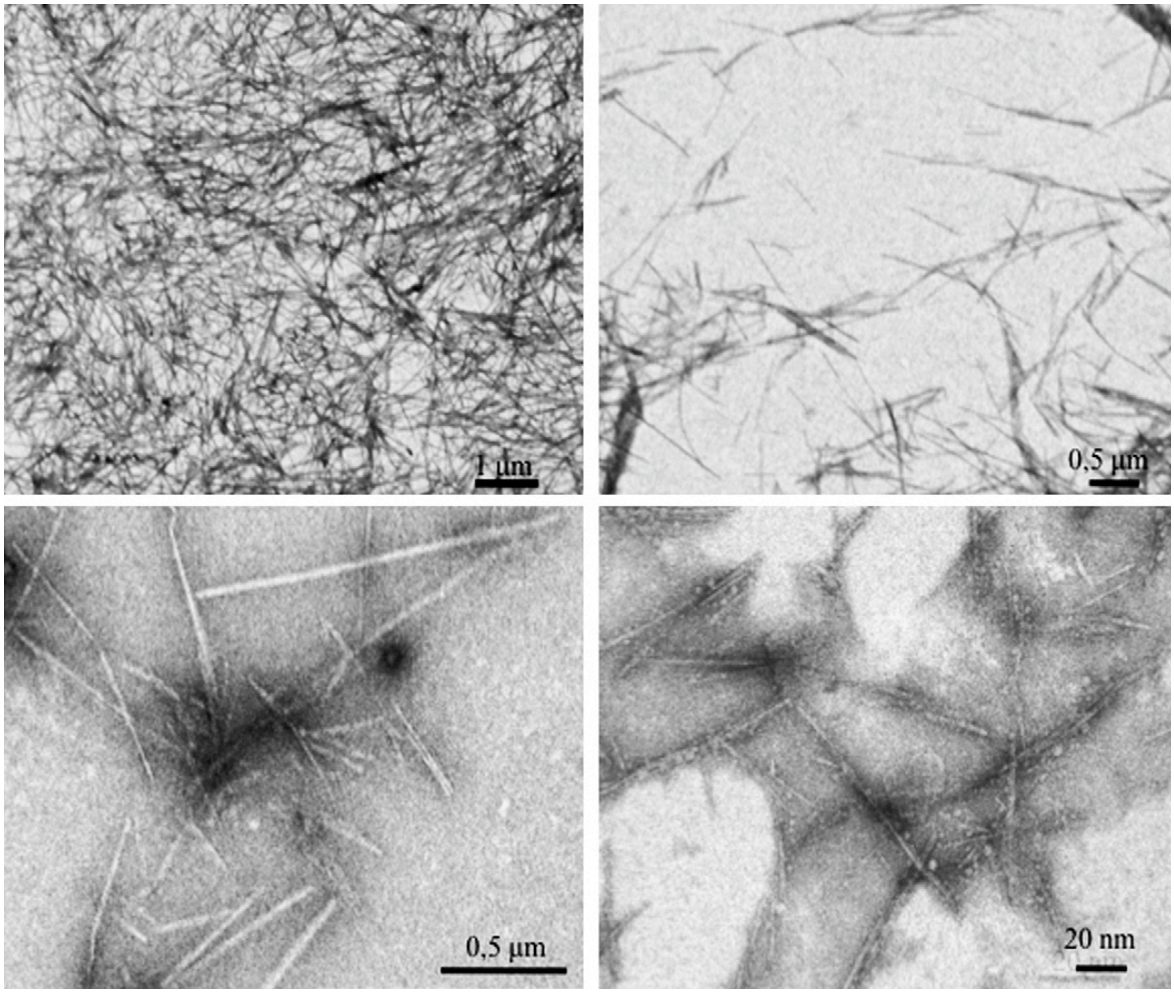

Figure 1 - TEM image of cellulose nanofibres (50kV; Zeiss 902A Orius SC 1000).

Evaluation of cellulose nanofibres mutagenicity by *Salmonella* reversion assay

The purpose of the bacterial reverse mutation assay is to evaluate the mutagenicity of the cellulose NFs, by measuring its ability to induce reverse mutations at selected *loci*, in several bacterial strains. Having into account that mutations are essential for cancer formation, the reliable characterization of mutagenicity is mandatory, while characterizing the safety of a biomaterial. The Kado test (Kado *et al.*, 1983) is a modification with improved sensitivity of the Ames test (Ames *et al.*, 1972). This is a simple, quick and inexpensive mutagenicity test, required for safety testing of a variety of compounds, including drugs, medical devices, food additives, industrial chemicals and pesticides (McCann *et al.*, 1975). Furthermore, the potential mutagenicity of BC and of some its

derivates were already accessed using the Ames assay (Schmitt *et al.*, 1991), therefore it was selected as a first approach in this work to investigate the possible mutagenicity of the cellulose NFs. The strains used were specially constructed to allow detection of mutagens acting via different mechanisms, namely frameshift mutations (TA97a and TA98 strains), base-pair substitution mutations (TA100 and TA102), detection of oxidative and alkylating mutagens and active forms of oxygen (TA102) (Hakura *et al.*, 2005). Table 1 presents the results obtained with the different strains.

Table 1 - Results obtained in *Salmonella* reversion assay.

	Revertant colonies/plate ± SD (without S9)				Revertant colonies/plate ± SD (with S9)			
Strain	TA97a	TA98	TA100	TA102	TA97a	TA98	TA100	TA102
PC	540±54	389±17	1531±183	1026±36	191±21	195±76	485±14	2356±196
NC	143±17	36±6	228±18	350±27	93±8	16±4	82±44	958±20
0.1	124±6	31±6	235±9	327±12	93±4	20±1	133±7	691±61
0.5	132±14	43±2	220±2	327±13	91±10	20±1	112±14	656±35
1.0	147±12	42±4	225±7	333±18	108±7	26±4	112±33	859±109

PC: positive control: 0.1 μg/plate of 4NQO to TA97a and TA98, 5.0 μg/plate sodium azide to TA100 and 0.5 μg/plate mytomicyn C to TA102; NC: negative control: H_2O; SD: standard deviation.

The reversion of the histidine phenotype in *Salmonella* strains is often adopted as a criteria for the classification of molecules as mutagenic. The results obtained in the presence of the cellulose NFs, without S9 mixture, correspond to the spontaneous reversion for each strain and are similar to those obtained to negative control (table 1). In the presence of S9 mixture, an increase of revertant colonies per plate, for the TA98 and TA100 strains, is detected as compared with control; however, the increases was in each case < 2-fold and does not appear to be dose-related. The results suggest that, under the conditions tested, the cellulose NFs do not present mutagenic behaviour, as described previously for BC and some fibrous BC-based materials (Schmitt *et al.*, 1991).

In vitro proliferation assay

The cellular morphology and proliferation may be affected by the presence of nanostructural patterns. Several studies analysed the proliferation of different cell lines on BC membranes, confirming its non-toxicity and applicability as scaffold for cell proliferation. However, depending on the cells used, the effect of the biomaterial on the proliferation rate and the cell morphology may be quite different (Sanchavanakit *et al.*, 2006). Several studies showed that the cytotoxicity of a nanomaterial is many times cell-specific (Cullen *et al.*, 2002). Recently, De Nicola *et al.* (2007) reported that, although carbon nanotubes do not present cytotoxic effect on human leukemic U937 cells, the proliferation rate is deeply altered. Moreover, Bottini *et al.* (2006) showed that the same nanotubes refereed above induce apoptosis in T lymphocytic cells, suggesting that cytotoxicity may be cell-specific. In addition, it has been reported that asbestos fibres inhibits the growth of CHO cells (Speit, 2002), and yet the same fibres stimulate the proliferation of different kinds of cells, *in vitro*, including fibroblasts (Bernstein *et al.*, 2005). Taking in consideration the evidence of contradictory, cell-specific effects arising from the interaction cell-biomaterial, the evaluation of the NFs effect on proliferative rate was performed both with CHO cells and fibroblasts. In both cases, the proliferation was about 15-20% lower in the presence of NFs, after 72 h of cell culture, irrespective of the concentration used (Figure 2). The lower proliferation rate may stem from the insolubility of NFs and their slow deposition on the polystyrene plate. It is known that cell proliferation is dependent on characteristics of material surface, such as its roughness. In addition, it was also described that cell proliferation on BC membrane is slower than on the cell culture plate (Backdahl *et al.*, 2006). However, the microscopic observations did not reveal differences in the cellular morphology.

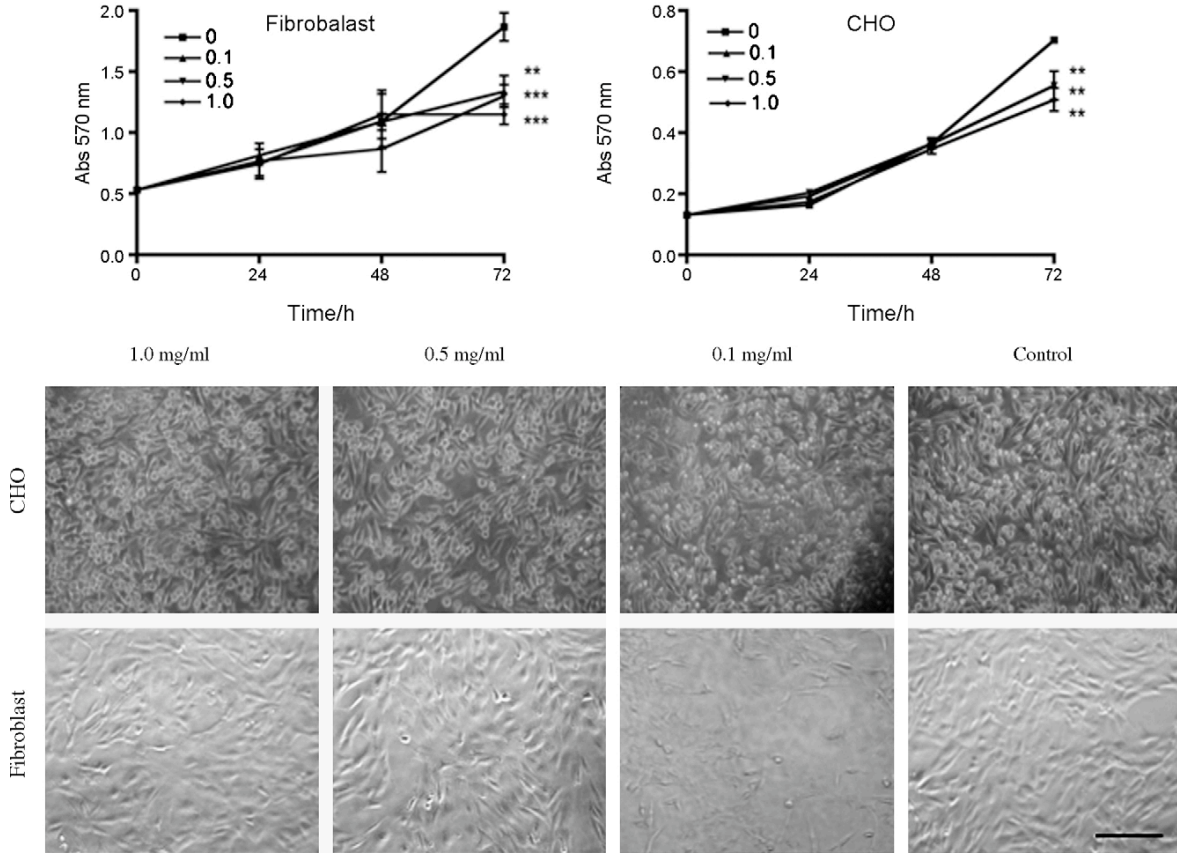

Figure 2 – MTT results from proliferation assays using mouse embryonic fibroblast 3T3 and CHO (mean ± SD; **P < 0.05; ***P < 0.005). Image obtained by optical microscopy of fibroblasts grown in the presence of cellulose NFs during 72 h. Scale bar = 20 μm.

Evaluation of cellulose nanofibres genotoxicity by comet assay

The genotoxicity of a material may be measured by analysing the damages caused on DNA. The comet assay is based on the ability of negatively charged loops/fragments of DNA to be drawn through an agarose gel, in response to an electric field. The extent of DNA migration depends directly on the DNA damage present in the cells (Collins *et al.*, 2008). The advantages of the comet assay, relative to other genotoxicity tests, include its high sensitivity for detecting low levels of both single and double stranded breaks in damaged DNA, the requirement for small numbers of cells per sample, flexibility, low cost, and ease of application (Collins *et al.*, 1997; Collins *et al.*, 2008). Moreover, the comet assay is arguably one of the most widely available tests used for genotoxicity, being already described as a reproducible assay to evaluate nanoparticles genotoxicity (Collins

et al., 1997), and suggested as a diagnostic tool for clinical management of cancer (Collins *et al.*, 2008). The nanomaterial's genotoxicity may result from a direct interaction with DNA, or from an indirect response caused by several factors, including surface stress through direct particle influences on DNA, the release of toxic ions from soluble nanoparticles, or generation of oxidative stress (Donaldson *et al.*, 2006). It has been proposed that (oxidative) DNA damage plus structural and numerical chromosome aberrations are the most sensitive genetic endpoints for detection of asbestos-induced genotoxicity detectable by *in vitro* assay. The comet assay has indeed proven to be a sensitive test to detect genotoxic effects of asbestos fibres in mammalian cell *in vitro* (Dusinská *et al.*, 2004; Speit, 2002). Therefore, cells grown in NFs – containing medium were analysed by the comet assay, in order to evaluate their genotoxicity. Cells grown on bacterial cellulose membrane were also tested as a control.

The DNA damages were evaluated by visual scoring and image analysis. Figure 3 shows a representative image obtained for each NFs concentration tested (0.1, 0.5, 1.0 mg/ml), negative and positive control (NC, PC) and the results obtained from the visual score. The results show that the DNA damages in the presence of NFs are similar to the negative control for each NFs concentration used. Around 95 % of cells present comet class 0 and 1, corresponding to no or insignificant DNA damage. The cell percentage showing comet class 2, 3 and 4 under different conditions are in the graphics of figure 3, and represent around 5 % of cell. Similar results were obtained with the cells grown on BC membranes (comet class 2: 4.7±3.72; comet class 3: 1.3±2.31, and comet class 4: 1.3±1.53).

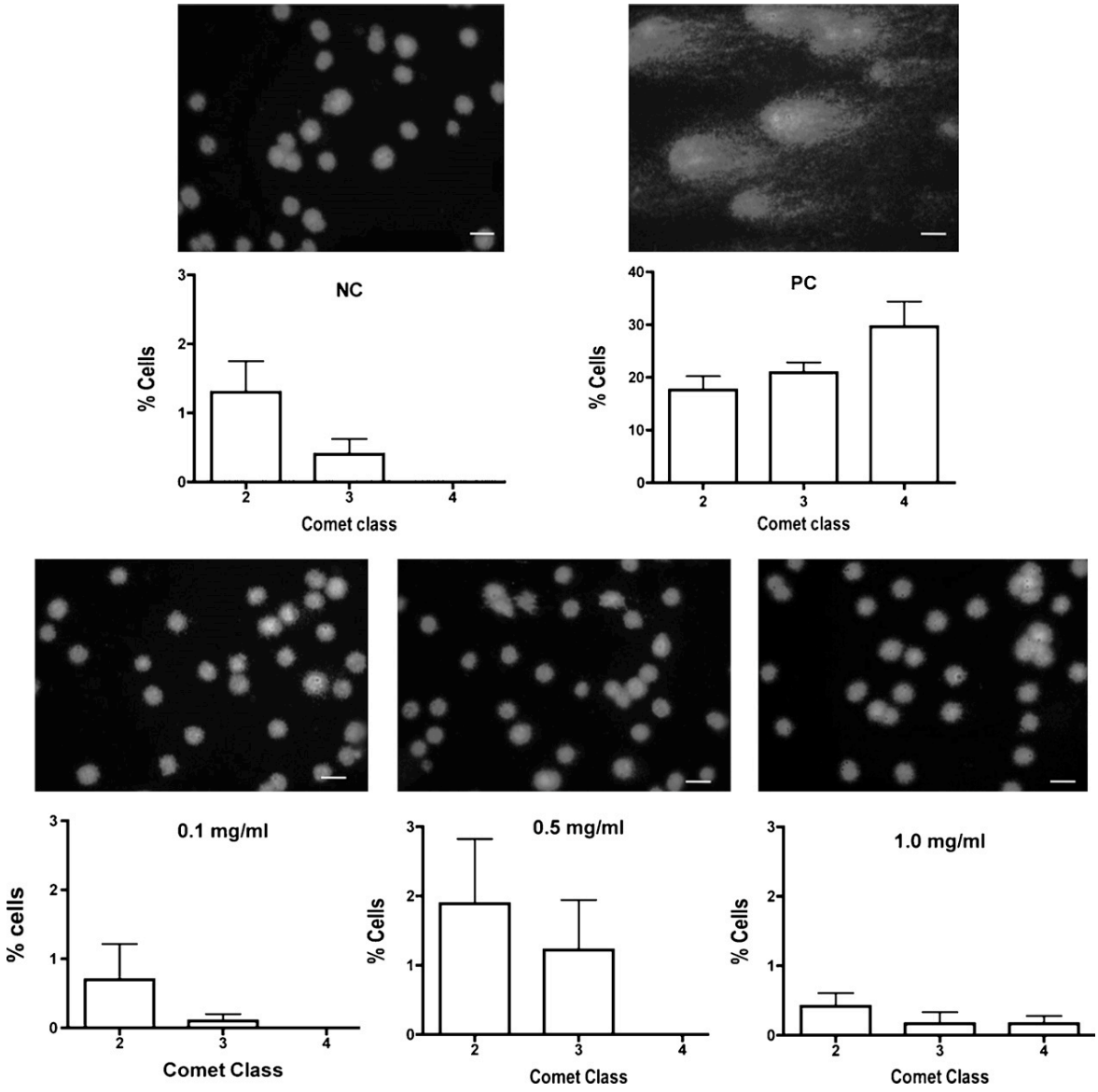

Figure 3 – Fluorescent microscopy images of ethidium bromide stained DNA and results from visual scoring in the comet assay. NC: negative control (H_2O); PC: positive control (H_2O_2); 0.1–1.0 NFs concentration in mg/ml. The images were scored and classified into five classes and given a value according to tail intensity, from 0 (no tail) to 4 (almost all DNA in the tail). Scale bar = 50 μm.

Regarding the comet parameters obtained from image analyses (table 2), tail length (TL), tail % DNA (TI), tail moment (TM) and tail migration (TMi), the NFs did not induce DNA damages under the concentrations tested, since the negative control and samples with NFs presented similar results, significantly (TL, TM, TI, TMi, $P < 0.001$) lower than the positive control. The same results were obtained for the cells grown on surface of BC

membrane (data not shown); in fact, our results confirmed the previous reports describing the non genotoxicity of BC (Schmitt *et al.*, 1991).

Table 2 – Results from images analysis using the Comet Assay IV software (mean ± SD).

Sample	Tail Length (µm)	Tail DNA (%)	Tail Moment	Tail Migration
0.1	17.78 ± 1.73	6.26 ± 1.20	0.66 ± 0.17	2.11 ± 0.82
0.5	21.25 ± 4.99	6.99 ± 3.48	1.03 ± 0.94	4.82 ± 4.25
1.0	15.88 ± 1.44	6.16 ± 1.78	0.71 ± 0.23	1.59 ± 0.55
NC	19.69 ± 3.31	6.88 ± 1.84	1.09 ± 0.67	3.94 ± 2.53
PC	101.36 ± 35.11***	49.06 ± 14.51***	25.42 ± 14.38***	83.11 ± 35.13***

PC: positive control (H_2O_2); NC: negative control (H_2O); 0.1–1.0 NFs concentration in mg/ml *** P < 0.001.

Taking together the results from visual scoring and image analysis, it may be concluded that the cellulose NFs do not present genotoxicity, under the tested conditions. Since alkaline comet assay allows for detection of DNA strand breaks, cross-links and alkali-labil sites induced by a series of physical and chemical agents it may be concluded that NFs do not induce those damages in DNA.

CONCLUSION

This work presents the first evaluation of the potential genotoxicity of nanofibres extracted from bacterial cellulose. Regarding the results of *Salmonella* reversion and comet assays, cellulose NFs did not present genotoxicity under the conditions tested, as already described for bacterial cellulose membrane. The cell culture systems have been shown to be valuable tools in fibre genotoxicity testing. Unlike *in vivo* studies, secondary inflammatory effects do not affect *in vitro* findings. Induction of DNA damages has been demonstrated for various types of asbestos fibres in several cell systems including CHO cell lines, which was not observed for BC nanofibres. Nevertheless, further studies must be performed in order to comprehensively characterize the toxicology of cellulose-based materials, since small modification in the material could result in drastic changes in cell-

material interactions. Work in progress includes the interaction of BC nanofibres with macrophages and *in vivo* assays.

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Chapter 6

Final remarks and Perspectives

A CBM from the human protein laforin was cloned and expressed in different *E. coli* expression systems (pET25b, pET29a and pGEXT4-1). The attempts to produce the soluble and functional protein were unsuccessful. The solubilization and refolding of inclusion bodies proved to be an efficient approach to produce the functional protein, with starch and glycogen affinity. This is the first report of cloning this human CBM, through IB solubilization and refolding. Nevertheless, further purification using starch affinity may be necessary to provide the separation of the functional protein. This purification step may be important to solve the 3D structure, by crystallography. In addition, future work includes *in vitro* tests to evaluate the effect of the recombinant human CBM-RGD on the interaction of cells with activated biomaterials. Regarding the results obtained with recombinant protein containing a bacterial SBM fused to a RGD, it may be concluded that CBM may be effectively used to functionalize dextrin hydrogels. Furthermore, recombinant proteins can be purified using starch, a non-expensive material. Although the enhancement of cell adhesion and spreading was observed, cell proliferation was not improved under the conditions tested. Therefore, in future work, different conditions (such RGD concentrations) may be optimized. In addition, several other small peptides could be fused to the SBM in order to obtain materials with different bioactivities.

Dextrin is a very promising biomaterial and, surprisingly, it is rather unexploited. The DEB-UM group is one of the few in the world currently developing dextrin-based biomaterials. The work presented under the scope of this thesis contributes to the characterization of dextrin hydrogels, namely the study of the *in vivo* biocompatibility and degradability of subcutaneous implants in mice. Dextrin-hydrogels presented different degradability behavior, depending on the molecule used in the cross-linking and on the DS of the polymer. Dextrin-VA is non-degradable hydrogel *in vivo*, irrespective of the DS, as described *in vitro* study. On the other hand, dextrin-HEMA hydrogel is a degradable

material and the degradability rate depends on its concentration. Despite the degradability differences, results strongly suggest that both dextrin hydrogels are fully biocompatible. The results also suggest that a hydrogel with controlled degradation rate may be obtained using different mixtures of dextrin-VA and dextrin-HEMA. This approach will be studied in a future work. These hydrogels can also be exploited as drug delivery systems through the incorporation of dextrin nanogels (already developed at DEB-UM), obtained by self-assembling. The nanogels allow the solubilization of poorly water soluble pharmaceuticals and therapeutic proteins, thus provide a smart and simple controlled delivery system.

Bacterial cellulose, like dextrin, is another very promising biomaterial. Although usually recognized as biocompatible, no information is available regarding BC nanofibres. The results from comet assay and *Salmonella* reversion assay show that BC-NFs do not present genotoxicity under the conditions tested. Moreover, the results from cell proliferation assay also confirmed their non-toxicity. *In vivo* assays, to be carried out in the future, will provide further information about their interaction with tissues, in particular whether NFs trigger acute and chronic inflammatory responses.

The following main results from this work are thus highlighted:

- The use of CBMs as a tool to coat biomaterials made of polysaccharides with bioactive peptides was demonstrated to be an effective and simple approach to functionalize the biomaterials
- The CBM from the human protein laforin was expressed and isolated in functional form for the first time, making possible structural studies for the elucidation of mechanistic aspects of this uncommon CBM
- Dextrins hydrogels, confirming previous work from our lab, exhibit excellent biocompatibility, as demonstrated by *in vivo* results.
- Biocompatibility aspects of the BC nanofibres are studied for the first time in this project. The possible toxic effects related to its needle shape (shared with asbestos nanofibres) are not confirmed, thus reinforcing the general view of BC as a highly biocompatible biomaterial.