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Development and Characterization of Dextrin Based Hydrogels
Use of Non-catalityc Domains for the Modification of Polysaccharides

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Doutoramento em Engenharia Química e Biológica

Trabalho efectuado sob a orientação do
Doutor Francisco Miguel Portela Gama
Doutora Lucília Maria Alves Ribeiro Domingues

Setembro 2008

Universidade do Minho, ___/___/_____

Assinatura: _____

Aos meus Pais, à minha Irmã e ao António

'Que a força do medo que tenho não me impeça de ver o que anseio
que a morte de tudo o que acredito não me tape os ouvidos e a boca
pois metade de mim é o que eu grito mas a outra metade é silêncio (…)
Que essa minha vontade de ir embora se transforme na calma e na paz que eu mereço
que essa tensão que me corrói por dentro seja um dia recompensada
porque metade de mim é o que penso e a outra metade um vulcão.
Que o medo da solidão se afaste, que o convívio comigo mesmo se torne ao menos suportável
que o espelho reflita em meu rosto um doce sorriso que me lembro ter dado na infância
porque metade de mim é a lembrança do que fui e a outra metade não sei
Que não seja preciso mais que uma simples alegria pra me fazer aquietar o espírito
e que o silêncio me fale cada vez mais
porque metade de mim é abrigo mas a outra metade é cansaço!
Que a arte nos aponte uma resposta mesmo que ela não saiba
e que ninguém a tente complicar pois é preciso simplicidade pra fazê-la florescer
porque metade de mim é plateia e a outra metade é a canção
E que a minha loucura seja perdoada
porque metade de mim é amor e a outra metade também.'

Oswaldo Montenegro

Agradecimentos

Sendo que se encerra esta jornada, agradeço a todos aqueles que, de uma forma ou de outra, contribuiram para a sua concretização.

Ao Prof. Miguel, antes de tudo, agradeço pelo apoio científico, mas não menos importante, pela constância e pela paciência que me dedicou, mesmo nas alturas (não raras) em que lhe esgotei todos os limites. *Obrigada Prof.*

À Prof. Lucília pelo apoio científico e pelo incentivo e motivação.

A todos os meus colegas do LTEB, de uma forma especial Catarina (*Goncinha*), Susana (*Su Margarida*) e David (*Tirando onda na's Europa*), por serem exemplos de determinação e trabalho e claro…pela indulgência para com o meu dandismo. *O dandismo não é um deleite excessivo com roupas ou elegância material.* Disse Baudelaire. *Para o dandi perfeito, essas coisas nada mais são do que o símbolo da superioridade aristocrática de sua mente*.

À Monica, porque sim. Do Benfica, às solitárias sopas de brócolos! Dos grandes amores platónicos, aos planos combinados com precisão atómica. Do Realismo do Eça, ao Romantismo decadente e demodè do Bocage. *EU SEI, TU SABES...NÓS SABEMOS!*

Ao Tiago, porque sonha os meus sonhos. De facto, nem todos os Deuses sumiram e se transformaram em prosaicas figuras humanas. Sobrou *UM*.

À Fundação para a Ciência e Tecnologia (FCT), pelo apoio financeiro através da bolsa de investigação SFRH/BD/17482/2004.

A essência da Ciência é a remissão do pecado de ser frágil a condição humana. Assim me turba de compensação a paixão que lhe tenho e à sua inerente complexidade…Oscilando em privilégio e pesar de estar ao mesmo tempo dentro e fora d'Ela. Cumpre-se O sonho, o Meu sonho, o meu Fado

Nem tudo fica por fazer JC

Abstract

Tissue engineering (TE) has emerged as a promising approach to circumvent the limitations of the existing therapies for the treatment of tissue loss or organ failure. In a parallel route, continuous advances in biotechnology led to the availability of complex natural molecules for the treatment of the *20th Century* diseases, such as AIDS, Alzheimer and cancer. These molecules, far more challenging to deliver than the classical therapeutic agents, were the driven force for the development of a new frontier research – the controlled drug delivery (CDD). TE and CDD have soon become interdisciplinary branches of science, gathering concepts from engineering, material and life sciences to develop new generations of biomedical tools which allowed overcoming clinical limitations such as donor scarcity, immunological rejection or drawbacks associated with surgery, thereby increasing patient compliance.

The development of biomedical devices has focused on the design of three-dimensional structures made from natural or synthetic materials, termed scaffolds. Hydrogels are a class of hydrophilic polymeric scaffolds, with appealing features from the perspective of biological mimicking. They have a good biocompatibility, degradability and appropriate mechanical properties, allowing for a favorable controlled interaction with living systems. Hydrogels can be used in TE, as scaffolds to support and promote tissue regeneration, and as attractive systems for the controlled release of pharmaceutically active molecules.

The goal of this thesis was to functionalize the biomaterial – Dextrin – to produce a hydrogel, as a potential alternative to the commonly used polymers for biomedical applications, namely as controlled release devices. Dextrin is a polymer composed of α-(1→4) D-glucose units, produced by partial hydrolysis of starch. The transesterification of dextrin with vinyl acrylate (VA) was carried out in anhydrous dimethylsulfoxide (DMSO), being C2 and C3 the preferred acylation positions, as revealed by solid state-NMR (nuclear magnetic resonance) analysis. Different degrees of substitution (DS) ranging from ca. 10 to 70% were obtained by controlling the molar ratio of VA to dextrin and gels were obtained by free radical polymerization of aqueous solutions of dextrin-VA. A preliminary analysis on the potential of these hydrogels for the controlled release of bioactive molecules was carried out. The protein (bovine serum albumin-BSA) diffusion coefficients on the hydrogels were calculated using the lag-time analysis. Values in range 10^{-7} cm^2/s were obtained for DS 20 and DS 40 and a smaller value of 10^{-8} cm^2/s arised upon DS increasing to 70%, revealing the dependence of the diffusivity on the crosslinking density. Further investigation has shown that the degradation is very slow under physiological conditions. However, the hydrogels could be rendered degradable through the incorporation of the enzyme amyloglucosidase, which prove to be an effective route to modulate the release profiles. Nevertheless, an alternative approach, which included the functionalization of the polymer with a methacrylate ester (HEMA), was also performed. It was possible to obtain hydrogels with distinct mechanical properties, resulting in more desirable degradation kinetics, as revealed through a rheologic analysis of the viscoelastic behavior. Finally, the biocompatibility of the hydrogels has been evaluated *in vitro*, using mouse embryo fibroblasts. The adhesion, proliferation and morphology of the cells on the hydrogel were

studied. The extracts obtained from the hydrogels, only slightly reduced the proliferation of fibroblasts (~15%). It was possible to observe that the direct seeding of the cells onto the hydrogels surfaces resulted in a reduction in the proliferation rate, as compared to tissue culture polystyrene plate. However, the results show that, although with a delay, cells are effectively able to grow, indicating that no deleterious effects are produced by dextrin hydrogels.

Cellulose is the most abundant polysaccharide on Earth. Its hydrolysis is handled by a variety of different enzymes, known as cellulases. Cellulases, hemicellulases and other polysaccharide-degrading enzymes are widely used in a variety of applications, namelly in pulp and paper industries. Despite its wide utilization, several drawbacks result from enzyme utilization. Taking the paper treatment as an example, the drawbacks include the extensive hydrolysis of polysaccharides that leads to a reduction of both fiber strength and mass. In this context, the application of carbohydrate-binding modules (CBMs) allows overcoming the limitations associated with the enzyme technology. CBMs are non-catalytic modules present in several cellulases and hemicellulases. Several studies indicated that treatment of cellulose fibers with CBMs alters the interfacial properties of the fibers.

In this work, the effect of recombinant cellulose-binding domains (CBD) on the properties of secondary paper fiber was evaluated. Two recombinant family 3 CBDs, from *Clostridium thermocellum* scaffoldin protein CipA (CBD_{CipA}) and Cellobiohydrolase A (CbhA) were used. The CbhA CBD was used either alone (CBD_{CbhA}) or fused with the internal fibronectin ($FN3_{1,2}$) module ($FN3_1$-$FN3_2$-CBD_{CbhA}). Additionally, the CBDs were chemically conjugated with an activated polyethylene glycol (PEG). The data showed that the CBD_{CipA}-PEG conjugate leads to a change on the properties of secondary fibers, as revealed by the improvement in both pulp drainage (Shopper-Riegler degree (ºSR) decreased up to 15%) and paper tensile strength. This effect is attributed to the presence of the PEG molecule, since CBDs lacking PEG were unable to modify pulp and paper properties. It is suggested that PEG mimetizes the glycosidic fraction of fungal CBDs, which is absent in the highly purified bacterial modules used here. It is concluded that the improved drainability of the pulp is attributed to the hydration and stabilization of the fibers.

Resumo

A engenharia de tecidos (TE) surgiu como uma forma promissora de contornar as limitações das terapias existentes, utilizadas no tratamento do mau funcionamento ou perda total de funções de um órgão ou tecido. Numa linha de investigação paralela, os contínuos avanços biotecnológicos, conduziram ao aparecimento de uma vasta gama de moléculas complexas para o tratamento de emergentes doenças do Século XX, tais como SIDA, Alzheimer e cancro. A forma de libertação destas novas moléculas no organismo constituiu um desafio, que acabaria por ser a força impulsionadora de uma nova fronteira de investigação – a libertação controlada de fármacos (CDD). TE e CDD cedo se tornaram ramos científicos interdisciplinares. Aplicando conceitos de engenharia e ciências da vida, uniram esforços no sentido de desenvolver novas gerações de produtos biomédicos que permitissem ultrapassar alguns dos urgentes problemas associados à prática clínica, como a escassez de dadores, a rejeição imunológica ou as desvantagens da cirurgia, melhorando os cuidados de saúde.

O desenvolvimento dos novos produtos biomédicos foi direccionado no sentido da produção de estruturas tridimensionais a partir de materiais naturais ou sintéticos, denominados scaffolds. Neste contexto surgem os hidrogéis, como uma classe de scaffolds poliméricos e hidrofilicos, com características apelativas da perspectiva do mimetismo de condições biológicas naturais, das quais se destacam a biocompatibilidade, a degradabilidade e as propriedades mecânicas, permitindo uma interacção favorável e controlada com os sistemas vivos. Os hidrogéis são utilizados em TE como suportes para promover a regeneração de tecidos, podendo também ser usados como atractivos sistemas de libertação controlada de fármacos.

Um dos principais objectivos deste trabalho consistia na funcionalização de um biomaterial – Dextrino – para a produção de um hidrogel, como alternativa aos polímeros actualmente utilizados em aplicações biomédicas, nomeadamente como sistema de libertação controlada. O dextrino é um polímero de unidades de α-(1→4) D-glucose, produzido pela hidrólise parcial do amido. A sua transesterificação com vinil acrilato (VA) foi efectuada em dimetilsulfoxido anidro (DMSO), sendo as posições C2 e C3, os locais preferenciais de acilação, revelados por ressonância magnética nuclear (NMR) de sólidos. Diferentes graus de substituição (DS) (entre 10 e 70%) foram obtidos através da alteração da razão molar VA/dextrino e os hidrogéis foram obtidos por polimerização radicalar de soluções aquosas de dextrino-VA. A avaliação preliminar do potencial destes hidrogéis como sistemas de libertação controlada, foi efectuada utilizando a proteína albumina sérica de bovino (BSA), tendo os coeficientes de difusão sido calculado por análise do *lag-time*. Valores na ordem de 10^{-7} cm^2/s foram obtidos para géis DS 20 e DS 40. Verificou-se, no entanto, uma diminuição para 10^{-8} cm^2/s, aquando do aumento do DS para 70%, assinalando a dependência da difusão na densidade de reticulação do hidrogel. Apesar de investigação subsequente ter revelado que a degradação dos hidrogéis ocorre de forma lenta em condições fisiológicas, foi possível torná-los degradáveis através da incorporação da enzima amiloglucosidase, sendo uma forma efectiva de modular os perfis de libertação. Não obstante, foi realizada uma abordagem alternativa, passando pela utilização do ester metacrilato (HEMA) na funcionalização do polímero. A

avaliação reológica do comportamento visco-elástico revelou ser possível a obtenção de hidrogéis com propriedades mecânicas distintas, resultando em cinéticas de degradação mais apropriadas. Finalmente, a biocompatibilidade dos hidrogéis foi avaliada *in vitro*, em fibroblastos embrionários de rato, através da análise da adesão, proliferação e morfologia celulares. Os resultados demonstraram que os extractos obtidos a partir dos hidrogéis induziram apenas uma ligeira redução da proliferação celular (~15%). Foi ainda possível observar que o cultivo directo das células na superfície dos hidrogéis, resulta numa redução na taxa de proliferação quando comparada com a cultura controlo. No entanto, foi demonstrado que as células são efectivamente capazes de crescer, indicando que a presença do hidrogel não produz efeitos deletérios.

A celulose é o polissacarídeo mais abundante na Terra. A sua hidrólise é levada a cabo por diferentes enzimas, conhecidas como celulases. As celulases, hemicelulases e outras enzimas responsáveis pela degradação de polissacarídeos, têm uma vasta aplicação, nomeadamente na indústria de polpa e papel. Apesar de amplamente utilizadas, vários inconvenientes resultam da acção das enzimas. No caso do tratamento de papel destaca-se a hidrólise extensiva dos polissacarídeos, que resulta numa redução de massa e resistência das fibras. Neste contexto, a aplicação de módulos de ligação a carbohidratos (CBMs), surge como uma alternativa viável, evitando as desvantagens da tecnologia enzimática. Os CBMs consistem em módulos não-catalíticos, presentes em várias celulases e hemicelulases. Vários estudos indicam que o tratamento de fibras de celulose com CBMs provoca alterações nas propriedades interfaciais das mesmas.

Neste trabalho foi avaliado o efeito de domínios de ligação a celulose (CBDs) recombinantes nas propriedades de fibras de papel secundárias. Foram utilizados dois CBDs (família 3) recombinantes de *Clostridium thermocellum*, pertencentes a dois complexos enzimáticos: *scaffoldin protein A* (CipA/CBD_{CipA}) e *Cellobiohydrolase A* (CbhA/CBD_{CbhA}). O CBD_{CipA} foi utilizado isolado ou em fusão com o módulo interno de fibronectina ($FN3_1$-$FN3_2$-CBD_{CbhA}). Procedeu-se ainda à conjugação química dos CBDs com uma molécula activada de polietileno glicol (PEG). Os resultados obtidos demonstraram que o conjugado CBD_{CipA}-PEG provoca alterações nas fibras secundárias, que resultam no melhoramento da drenabilidadedas polpas (diminuição do grau de Shopper-Riegler (ºSR) até 15%), bem como da resistência à tensão do papel. Este efeito é atribuído à presença da molécula de PEG, uma vez que na ausência deste, os CBDs isolados não são capazes de provocar alterações nas propriedades da polpa e do papel, sugerindo que a molécula de PEG mimetiza o efeito da fracção glicosídica dos CBDs fungícos, que está ausente nos módulos bacterianos puros. Conclui-se que o melhoramento na drenabilidade da polpa está relacionado com a hidratação e estabilização das fibras.

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List of Abbreviations

AMG	Amyloglucosidase
APS	Ammonium persulfate
ASC	Acid-swollen cellulose
BMP	Bone morphogenetic protein
CBD	Cellulose-binding domain
CbhA	Cellobiohydrolase A
CBM	Carbohydrate-binding module
CBS	Calf bovine serum
CDD	Controlled drug delivery
CDI	N,N'-Carbonyldiimidazole
CipA	Cellulosome integrating protein A
CP-MAS	Cross-Polarization and Magic-Angle-Spinning
2D	Two dimensional
3D	Three dimensional
DMEM	Dulbecco's modified Eagle medium
DMAP	4-(N,N-Dimethylamine)pyridine
DMSO	Dimehyl sulfoxide
DNS	Dinitrosalicylic acid
ECM	Extracellular matrix
FDA	Food and Drug Administration
FN	Fibronectin
GH9	Family 9 glycosyl hydrolase
GPS	Gel Permeation Chromatography
HA	Hyaluronic acid
HAP	Hydroxyapatite
IgG	Immunoglobulin G
IKVAV	Ile-Lys-Val-Ala-Val (signaling domain)
IPTG	Isopropyl-D-thiogalactopyranoside
MALDI-TOF	Matrix-Assisted Laser Desorption/ Ionization-Time Of Flight
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide

MWCO	Molecular weight cutoff
Ni-NTA	Ni-Nitrilotriacetic acid
NMR	Nuclear Magnetic Resonance
PBS	Phosphate buffer saline
PCL	Poly(ε-caprolactone)
PEG	Poly(ethylene glycol) and copolymers
PHEMA	Poly(hydroxyethyl mehacrylate)
PLA	Poly(lactic acid)
PNIPAM	Poly(N- isopropylacrylamide)
PVA	Poly(vinyl alcohol)
REDV	Arg-Glu-Asp-Val (signaling domain)
RGD	Arg-Gly-Asp (signaling domain)
SBM	Starch binding modules
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
SS-mPEG	Succinimidyl succinate-PEG
TCP	β-tricalcium phosphate
TCPS	Cell culture polystyrene plate
TE	Tissue engineering
TEMED	N,N,N',N'-Tetramethylenethylenediamine
THF	Tetrahydrofuran
TNBSA	2,4,6-Trinitrobenzene sulfonic acid
VA	Vinyl acrylate
YIGSR	Tyr-Ile- Gly-Ser-Arg (signaling domain)

List of Nomenclature

A	Area	(cm^2)
a_w	Water activity	(dimensionless)
C	Concentration	($g\ cm^{-3}$)
CBD_{Bound}	Molar amount of CBD adsorbed	($\mu mol\ g^{-1}$)
CBD_{Free}	CBDs in the liquid phase at the adsorption equilibrium	($\mu mol\ g^{-1}$)
$CBD_{Initial}$	Initial CBD concentration	($\mu mol\ g^{-1}$)
CBD_{Max}	Maximum molar amount of CBD adsorbed	($\mu mol\ g^{-1}$)
CPII	Cell Proliferation Inhibition Index	(%)
DR	Degree of reticulation	(%)
DS	Degree of substitution	(%)
G'	Storage modulus	(Pa)
G''	Viscous modulus	(Pa)
K_a	Adsorption equilibrium constant	($L\ \mu mol^{-1}$)
l	Membrane thickness	(cm)
m_{loss}	Mass loss during degradation	(%)
Q	Solute transferred through the membrane	(g)
ºSR	Shopper-Riegler degree	(º)
SR	Swelling Ratio	(dimensionless)
t	Time	(seconds)
t_s	Lag time	(seconds)

Greek Symbols

θ	Diffraction angle	(º)
δ	^{1}NMR peak intensities	(ppm)
Tan (α)	Tangent of phase degree	(dimensionless)

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CHAPTER 1

Introduction

Background & Aims

Boswell: But, Sir is it not somewhat singular that you should happen to have Cocker's Arithmetic aboutyou on your journey?

Dr. Johnson: Why, Sir if you are to have but one book with you upon a journey, let it be a book of science. When you read through a book of entertainment, you know it, and it can do no more for you; but a book of science is inexhaustible.

James Boswell (1740-95) | Biographer of Samuel Johnson

Two major objectives compose the aim of this thesis. The first goal was to develop dextrin hydrogels with potential applications in the biomedical field. The other one refers to the use of cellulose-binding domains in the modification of polysaccharides. The Introduction is likewise divided in two sections, presenting the main aspects and background of each of the above items. The first section describes the fundamentals of two biomedical fields of major importance – tissue engineering and drug delivery. A review on biomaterials and the design of scaffolds are included. Special attention is paid to hydrogels and a detailed description is addressed to dextrin. The second section provides an introduction to cellulose, and the enzymes involved in its degradation. The use of these enzymes in applications such as textile and paper industry is also briefly described. Finally, the biotechnological application of non-catalytic domains such as carbohydrate-binding modules (CBMs), as substitutes of the enzymatic technology, namely in the paper-making processes, is discussed.

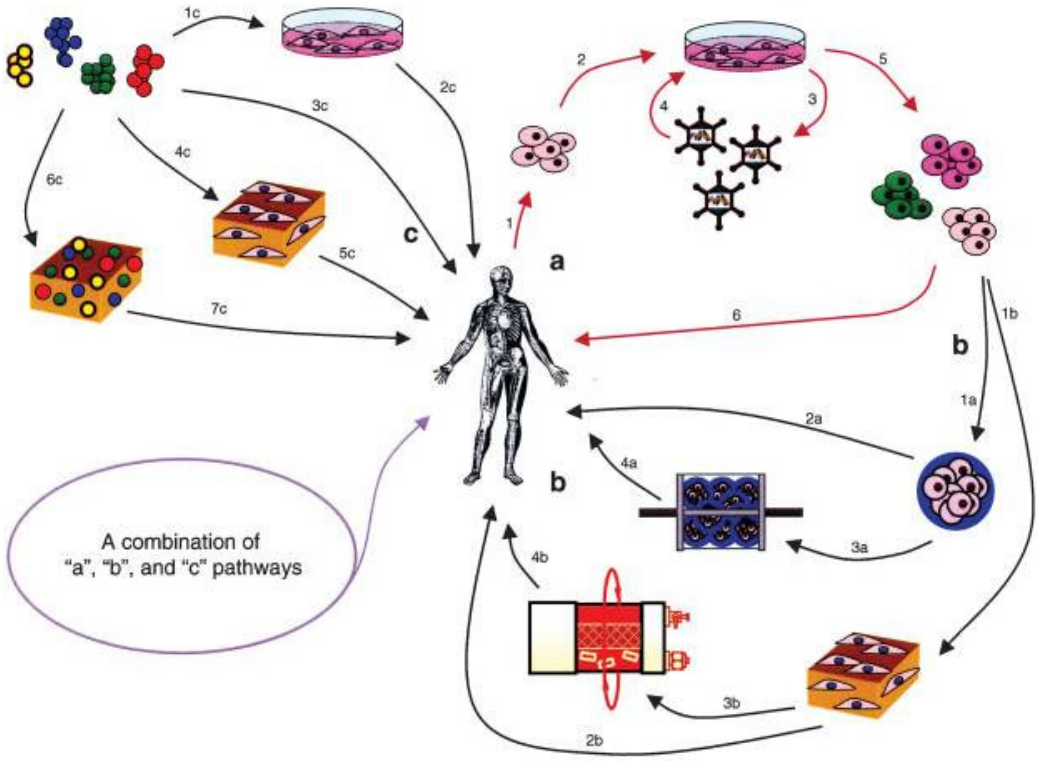
SECTION I

Development and Characterization of Dextrin Based Hydrogels

Tissue Engineering – An Overview to the Hybrid Technology

The advent of tissue engineering (TE) has been motivated by the challenge of producing tissues substitutes that might restore, maintain or improve the structural features and physiological functions of natural living tissues (Thomson et al. 1995, Freed and Vunjak-Novakovic 1998). Tissue engineering has been described in the early 90s by Langer and Vacanty (1993) as "the persuasion of the body to heal itself, through the delivery at the appropriate sites of molecular signals, cells, and supporting structures". Initial strategies for the creation of new tissues combined three basic principles: (1) The isolation and cultivation of cells, (2) the use of tissue-inducing substances, and (3) the placement of cells within suitable matrices to support their three dimensional growth (Langer and Vacanty 1993, Vacanty and Langer 1999) (Figure 1).

The direct *in vivo* implantation of isolated cells, allows overcoming the complications of surgery, as an invasive technique. Additionally, it is possible to replace only those cells that supply the needed function and also manipulate cells before infusion. In this approach there is very little of engineering. Its potential limitation include failure of infused cells in maintaining their function and it must be considered the possibility of immunological rejection (Langer and Vacanty 1993, Freed and Vunjak-Novakovic 1998) (Figure 1, pathway a).



Adapted from Baroli 2006

Figure 1. Tissue engineering strategies. To regenerate an organ, a cell explant (pathway a, 1), can be cultivated *in vitro* (a, 2) to differentiate, eventually modified genetically (a, 3–4), and expanded (a, 5) prior to be re-implanted, preferentially, in the same individual (a, 6). In a second approach, the explanted cells (pathway b) can be encapsulated (b, 1a) and implanted in the body (b, 2a) to act as an artificial organ, or encapsulated and assembled in a bioreactor (b, 3a) to serve as an external (b, 4a) artificial organ. Alternatively, cells (b, 1b) might be exposed to controlled signals and then seeded on matrices increasing the probability of a successfully integration within the host after be implanted (b, 2b), or after a period of cultivation *in vitro*, for instance, with bioreactors (b, 3b). The third approach is that of using tissue-inducing substances (pathway c) that can be added (c, 1c) prior to reinfuse exposed cells in the body. Alternatively, these molecules could be administered, delivered, and/or targeted to the exact location where regeneration is desired. They can also be used *in vitro* and on cells that are growing on a matrix(c, 4c) that will be implanted after a certain time, or that contemplate the encapsulation of such molecules in a matrix (c, 6c) to be implanted (c, 7c). Finally, it should be mentioned that regeneration may be also achieved by using approaches that could be considered combinations of pathways a, b, and c.

In the second strategy (Figure 1, pathway b), a temporary support is required to serve as an adhesive substrate for the implanted cells and a physical support to guide the formation of the new tissues. Transplanted cells adhere to the support or are encapsulated in a biomaterial, proliferate and produce the extracellular matrix (ECM), stimulating the new tissue formation. During this process, the support matrix gradually degrades and might, eventually, be eliminated (Langer and Vacanty 1993).

The third approach contemplates the use of tissue-inducing substances such as growth factors, or cytokines, before cell reinfusion (Figure 1, pathway c). One of the major disadvantages of this approach is the purification and large-scale production of the inducing substances, and also the need of a system to deliver the bioactive molecule to the desired location (Langer and Vacanty 1993, Langer 1999).

These principles and strategies are still valid and encouraging research has been carried out over the last decade, reporting on the application of tissue engineering products and strategies in clinical use (Lysaght et al. 1998, Bonassar and Vacanty 1998, Lysaght and Hazelhurst 2000). However, it must be recognized that many unforeseen problems must be overcome to achieve the ambitious goals of providing fully functional tissue replacement.

Having in mind these developments, it seems obvious that the original support materials must be tailored to provide additional functionality, besides the ability to withstand mechanical loads or to possess suitable degradation kinetics (Tessmar and Gopferich 2007). They should act as a synthetic extracellular matrix. ECM is the natural medium in which cells proliferate, differentiate and migrate, and therefore is the gold standard for tissue regeneration (Meredith et al. 1993, Bosman et al. 2003). Natural ECM is a condensed matrix mainly composed of locally secreted proteins, proteoglycans and polysaccharides, arranged as a molecular network. The homeostatic dynamic state of ECM is controlled by proteoglycans, and a number of signaling molecules, such as growth factors, which mediate cell-ECM and cell-cell interactions. All these molecules are embedded in an

amorphous, fundamental substance represented by glycosaminoglycan chains, which form the highly hydrated gel structure imbibing the matrix (Meredith et al. 1993). Thus, a suitable support for tissue engineering should mimic some of the ECM characteristics, namely providing mechanical integrity to tissues, supporting their growth, providing an environment for the host cell survival as well as the means to deliver nutrients, growth and differentiation factors for long term support of the proliferation. Additionally, they should guide cell adhesion and even recruit desirable cells, promoting the dynamic interaction with surrounding tissues (Yang et al. 2001, Drury and Mooney 2003). Furthermore, biocompatibility is an essential issue regarding their pharmaceutical and biomedical applicability, to avoid any adverse foreign host response (Hutmacher 2001).

Controlled Drug Delivery – A New Research Frontier

Despite the great attention paid to the promising developments in TE, a parallel extensive research has also been continually performed over the past two decades in another challenging field – the controlled drug delivery (CDD). CDD strategies have made a dramatic impact in medicine. Advances in biotechnology led to the availability of complex natural molecules for disease treatment (growth factors, interferons, cytokines, response modifiers, etc). In form of complex carbohydrates, proteins or DNA, these materials are, sometimes, larger in size, less stable, less soluble and much harder to deliver than the classical therapeutic agents. The development of CDD was a critical step in the treatment of diseases such as AIDS, Alzheimer and cancer, and as challenging as the new drug discovery itself (Davis and Illum 1998).

Although initially following two distinct scientific routes, TE and CDD have soon converged. The need for deliver growth and differentiation factors for long term support of the proliferating cells, turned the controllable release systems into the

fusion step in the development of new generations of biomedical tools and products based on TE principles.

Controlled drug delivery occurs when a polymer is combined with a drug or other bioactive molecule in such a way that the molecule is released from the material in a predesigned manner (Robinson and Lee 1997, Langer 1988). The release of the active agent may be constant, cyclic or triggered by an external event. The goal of controlling the delivery is to achieve more effective therapies, eliminating at the same time, the possibility for both under and overdosing. The ability to maintain the drug levels within a desired range of concentration in a targeted way, enhancing the ability to use highly toxic, poorly soluble or relatively unstable drugs, the need for fewer administrations and increasing in patient compliance are among the advantages of using controlled-delivery systems (Figure 2). Additionally, their use to minimize drug degradation and loss, to prevent harmful side-effects and to increase the drug bioavailability, has also been successfully applied (Kost and Langer 1992, Peppas 1997, Charman et al. 1999, Soppimath 2001).

The ideal drug delivery system should be inert, biocompatible, mechanically strong, comfortable for the patient, capable of high levels of drug loading, safe from accidental release, simple to administer and remove, and easy to fabricate and sterilize. Among drug carriers are polymers in different formulations (hydrogels, micro and nanoparticles), microcapsules, lipoproteins, liposomes, micelles and others. (Santini 2000, Soppimath et al. 2001, Torchilin 2001, Bae et al. 2003, Kopecek 2003, Haag 2003, Muller-Goymann 2004, Packhaeuser 2004).

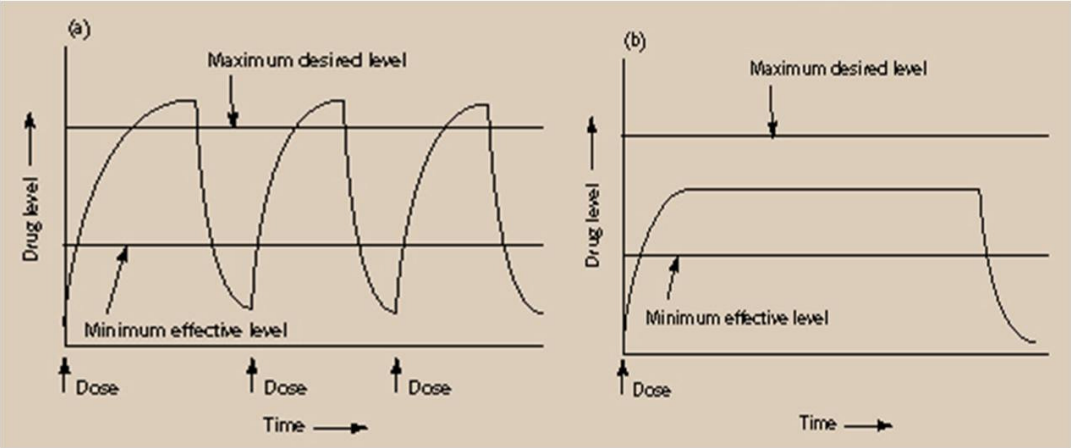


Figure 2. Drug levels in the blood with (a) traditional drug dosing and (b) controlled-delivery. With traditional tablets or injections, the drug level rises after each administration, decreasing until the next one. The blood level of the agent should remain between a maximum value (which may be toxic), and a minimum value (below which the drug is no longer effective). In controlled drug delivery the drug level in the blood remains constant, between the desired maximum and minimum, for an extended period of time.

In recent years, controlled delivery formulations became much more sophisticated, with the ability to do more than simply extend the effective release period for a bioactive agent. Current controlled release devices are capable to respond to changes in the biological environment and delivering/stop delivering the drugs based on these changes. Moreover, the delivery system can be targeted to the specific cell, tissue, or local where the drug it contains is to be delivered. The release rates are therefore determined by the design of the system, which in turn depends on the requirements of its final application (Peppas 1997, Tessmar and Göpferich 2007).

Table 1 summarizes the key characteristics of a controlled delivery system. The mode of delivery and subsequent degradation are important features in developing successful formulations. Sustained (or continuous) release of a drug involves the release at a controlled rate due to the diffusion out of the polymer or by its degradation over time. Pulsatile release is achieved by using carrier polymers that respond to specific stimuli (e.g., exposure to light, changes in pH or temperature) and is often the preferred method of delivery, as it resembles the naturally

occurring homeostatic processes. Additionally, the delivery can also be targeted (Heller 1985, Pitt 1987). In this modality, the delivery system is directed to the site of interest. Two major mechanisms can be used, passive and active targeting. An example of passive targeting is the preferential accumulation of chemotherapeutic agents in solid tumors as a result of the enhanced vascular permeability of tumor tissues compared with the healthy ones (Niculescu-Duvaz and Springer 1997, Kost and Langer 2001). The active targeting involves the surface functionalization of the delivery systems with ligands that are selectively recognized by receptors on the surface of the cells of interest (Kost et al. 1987, Robinson and Lee 1987, Saslawski et al. 1988, Chasin and Langer 1990, Manabe et al. 2004).

Table 1. Characteristics of a drug delivery system.

Type of Delivery	**Release Mechanisms**		**Administration Route**
Sustained (continuous) Pulsatile (stimuli reactive) Targeted	Diffusion	Reservoir Matrix Erosion	Peroral Parental (intravenous, intramuscular, subcutaneous) Transdermal Pulmunary Trans-tissue

Concerning the release mechanisms, there are two primary mechanisms by which bioactive agents can be released from a delivery system – diffusion and erosion. Two types of diffusion controlled systems have been developed. The first is a

reservoir device in which the bioactive agent forms a core surrounded by a polymeric barrier. These systems include membranes, capsules, microcapsules, liposomes, and hollow fibers. The second type is a polymeric matrix device in which the active agent is dispersed or dissolved. In these two systems, drug diffusion through the polymer is the rate-limiting step. Likewise, release rates depend on the polymer characteristics and its consequent effect on the diffusion and partition coefficient of the biomolecule to be released (Kost et al. 1987, Robinson and Lee 1987, Langer 1988, Chasin and Langer 1990).

In chemically controlled systems, the erosion is the mechanism underlying release control. Bioerodible (or biodegradable) systems offer the advantage of being absorbed by the body, avoiding the drawbacks of the surgical removal. In a bioerodible system the drug is ideally uniformly distributed throughout a polymer as in matrix systems (Kost 1987, Kost and Langer 1992). As the polymer surrounding the drug is eroded, the drug is release. The pendant chain system is another chemically controlled system. The drug is chemically attached to a polymer backbone chain and the release takes place by hydrolytic or enzymatic cleavage (Heller 1988, Jeong et al. 1999).

Finally, the choice of the administration route is another feature of major importance in the application of CDD technology. The delivery route is driven by patient acceptability, the properties of the drug, such as its solubility, the access to a disease location, or effectiveness in dealing with the specific disease. The most important drug delivery route is the peroral, however, parental, transdermal, pulmonary and trans-tissue routes are also very important (Davis and Illum 1998).

Biomaterials – The Foundations

Originating from their application in the biological environment, the designation of *biomaterials* is due to their ability to replace or restore biological functions and exhibit a pronounced compatibility with the biological environment (Langer et al. 1990, Hoecker 1998). Biomaterials play a major role in most tissue engineering strategies and apart from this original use, they have been increasingly applied as the pivotal substrate for cell attachment and migration and also as implantable systems for drug and cell carrying (McCulloch and Shalaby 1998, Zhou et al. 2003, Kanjickal and Lopina 2004), to activate specific cellular function in a localized region (Langer 1995, James and Khon 1996, Hutmacher et al. 2001). The development of biomaterials for medical applications has recently focused on the design of biomimetic materials that might be able to interact with surrounding tissues by biomolecular recognition, to make them capable of eliciting specific cellular responses, mediated by specific interactions (Klee and Hoecker 1999, Lucke et al. 2000). Biomaterials have been employed to conduct and accelerate otherwise naturally occurring phenomena, such as tissue regeneration in wound healing. They can also be used to induce cellular responses that might not be normally present, such as healing in a diseased subject and to block natural phenomena, such as the immune rejection of cell transplants (Langer 1995).

A wide variety of biomaterials, both synthetic and natural, is available (Table 2). Naturally derived and recombinant biomaterials that combine the beneficial aspects of both natural and many of the desirable features of synthetic materials have been designed and produced. In general, some authors claim that the latter offer some advantages, since they can be tailored to give a wide range of properties and more predictable and reproducible results that the materials derived from natural sources (Doylan and Cameron1990, Cascone et al. 1993, Hanein et al. 1995). However, natural biomaterials are more likely to induce the appropriate biological response which is fundamental in biomedical applications.

Table 2. Important biomaterials employed in medicine.

Material		**Medical applications**
Natural organic materials	Collagens Hyaluronic acid (HA) and derivates Alginate Starch based polymers Cellulose Chitosan Pullulan	Soft tissue regeneration Controlled drug delivery Cell encapsulation Cell culture surfaces
Synthetic organic materials	Poly(glycolic acid) (PGA) Poly(lactic acid) (PLA) Poly(vinyl alcohol) (PVA) Poly(ε-caprolactone) (PCL) Poly(hydroxyethyl mehacrilate) PHEMA Poly(*N*- isopropylacrylamide) (PNIPAM) Poly(ethylene glycol) PEG and copolymers	Bone and cartilage repair Opthtalmic applications Artificial skin Controlled drug delivery Cell encapsulation Cell culture surfaces
Inorganic materials	Hydroxyapatite (HAP) β-tricalcium phosphate (TCP)	Bone and other mineralized tissues regeneration

From Biomaterial to Scaffold

Depending on the final specific application, the required biomaterial and its properties must be quite different. In many cases, the biomaterial must be designed

not to interact with individual cells, but rather with multiple cells, or even whole tissues. This means that the materials must be processed into devices exceeding the dimensions of a single cell in a two (2D) or three (3D) dimensional architecture. In the late 1970s and 1980s, cells on sheets of collagen (2D), or collagen–glycosaminoglycan composites, were used in tissue regeneration in an attempt to create new skin (Bell et al. 1981, Burke et al. 1991). However, the pressure of the constant attempts to engineer virtually every human tissue (including cartilage, bone, muscle, heart valves, nerves, among others), drove the research to 3D structures that enable large numbers of cells to be housed. The term *scaffold* arose. The scaffold serves as 3D template for initial cell attachment and subsequent tissue formation both *in vitro* and *in vivo*. The scaffold provides the necessary support for cells to attach, proliferate, maintaining their differentiated state and can even define the overall shape of the tissue-engineered transplant (Langer 2000, Hutmacher 2001).

Scaffold Characteristics

The challenge for scaffold systems is to arrange an appropriate 3D configuration that shall be able to fulfill the needs of the final application, which, in general, requires balancing temporary mechanical function with mass transport to aid biological delivery and/or tissue regeneration, and do so in a way that can be carried out reproducibly, economically, and on a large scale. The requirements for a scaffold to be considered suitable in the biomedical field are complex; however, the following basic characteristics must be addressed to bring about the desired biologic response (Hutmacher 2001). (1) The scaffold should be biocompatible. Neither it nor its degradation products should induce any adverse response or toxicity. (2) The scaffold should possess the appropriate mechanical properties, to provide the correct environment, matching the intended site of implantation. (3) Additionally the scaffold should be made from material with controlled biodegradability or bioresorbability so that tissue will eventually replace the

scaffold. (4) It should have an interconnected pore network, enhancing the diffusion rates, improving oxygen and nutrient supply and waste removal, thereby facilitating the vascularization. (5) Furthermore an appropriate surface chemistry should favor cellular attachment, differentiation and proliferation. (6) Finally the scaffold should be easily processed into a variety of shapes and sizes as well as easily sterilized (Freed et al. 1994, Thomson et al. 1995, Lu and Mikos 1996, Agrawal et al. 1997, Piskin 1997, Shapiro and Cohen 1997, Simske 1997, Maquet and Jerome 1997, Kim and Mooney 1998, Middleton and Tipton 2000, Chapekar 2000, Hutmacher 2000).

Polymers in Biomedical Applications

The selection of a scaffold material is both critical and difficult. There are many biomaterials available, such as metals, ceramics and polymers. Metals and ceramics have contributed to major advances in medicine, particularly in orthopedic tissue replacements. Typical implant metals were made of cobalt and titanium, and typical ceramics included alumina, zirconia, calcium phosphate, and bioglass (Hench 1996). However, the requirement of biodegradability totally excludes the use of metals and almost excludes the use of ceramics as scaffolds (Thomson et al. 1995, Maquet and Jerome 1997). Nevertheless, inorganic compounds, such as hydroxyapatite (HAP), β-tricalcium phosphate (TCP) and combinations of both can be successfully applied in orthopedic applications for the regeneration of bones and other mineralized tissues (LeGeroz 2002, Paul and Sharma 2003). The drawbacks include the difficulty in processing complex shapes and the non-degradability. For these reasons, polymeric materials have received an increasing attention. Biodegradable polymers can be broadly classified into natural and synthetic, based on their origin.

Natural Polymers

Natural polymers, such as polysaccharides and proteins have been shown to have wide application as scaffolds. Its major advantage is that they often have an organized structure, at both molecular and macroscopic levels, thereby resulting in some favorable characteristics, such as the ability to induce tissue ingrowth and to most closely simulate the native cellular milieu. In fact, they usually contain domains that can send important signals to guide cell development at various stages. However, poor mechanical performances and large batch-to-batch variations can limit their wide applications. Also, the degradation of naturally occurring polymers almost relies on enzymatic processes, which will introduce some inevitable patient-to-patient variation in the degradation rates, affecting the predictability and reproducibility as it was already stated (Thomson et al. 1995).

Hyaluronic acid (HA) and collagen were considered as the materials of choice for scaffolds for a long time. They are two ubiquitous biopolymers found in the mammalian body and two of the principal components of ECM (McPherson et al. 1986, Laurent et al. 1996). They have been used especially for regeneration of soft tissues either alone or in combination with other agents (Lee et al. 2001, Pachence 1996). Although having, in general, poor mechanical properties, naturally derived polymers are interesting because they do not induce a host response, and may enhance the biological recognition encouraging the normal cellular functions. For the replacement of soft tissues, there are many strategies employing a combination of collagen and hyaluronic acid, since they strongly resemble the organization of ECM (Brun et al. 1999, Xin et al. 2004, Tang et al. 2007).

Alginate is a well known polysaccharide widely used due to its gelling properties in aqueous solutions. It can be extracted from marine brown algae or produced by bacteria (Kim and Mooney 1998). Due to the intrinsic properties of alginate calcium gels (biocompatibility, adhesion, porosity, and ease of manipulation) much attention has recently been focused on the delivery of proteins, cell encapsulation, and tissue regeneration. However, alginate is not biodegradable in the human body

and the gelation dependence on the calcium ions is also a drawback, since they can be lost following implantation (Kim and Mooney 1998).

Starch is a natural mixture of glucans, which can be found in the plant kingdom where it serves as a food reserve polysaccharide, utilized during plant growth. The main sources for the commercial production of starch are potatoes, wheat, corn and rice. Starch consists of amylose, a linear polymer of α-D-glucopyranosyl units linked by α-(1→4) D-glucosidic linkages, and amylopectin, a branched polymer of α -D-glucopyranosyl units containing α-(1→4) D-glucosidic linear linkages and α-(1→6) D-glucosidic linkages at the branch points (Figure 3) (Philips 1980, Fanta and Doane 1986). The ability to form a network structure, even at very low concentrations, is one of their most important functional properties, offering the mean of increasing chemical and mechanical stability. A wide range of modification mechanisms of starches is known, including self association (induced by changes of pH, ionic strength, or physical and thermal means), complexation with salts and covalent cross-linking (Shefer et al 1992, Bhattacharya et al 1995, Willet et al 1995, Van Soest and Borger 1997, Van Soest and Vliegenthart 1997, Marques et al. 2002). Starch has been extensively studied, initially due to its interest for food and paper industry, textile and pharmacology and more recently, in the biomedical field.

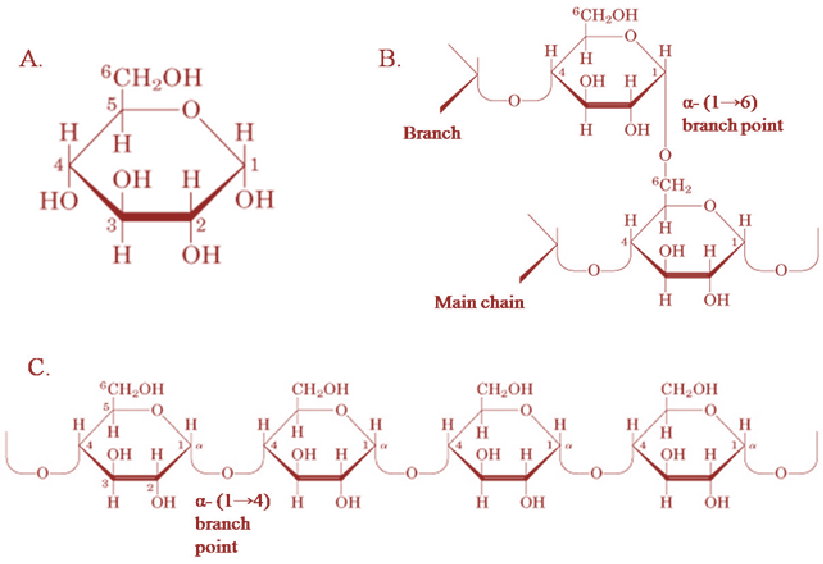


Figure 3. Structural formula of a α-D-glucopyranose residue (A), amylopectin (B) and amylose (C).

Dextran is a glucose homopolysaccharide that feature a substantial number of consecutive α-(1→6) linkages in their main chains, usually more than 50% of the total linkages. They also possess side chains stemming from α-(1→2), α-(1→3), or α-(1→4) branch linkages. Just like starch, there are many reports on the use of dextran in the pharmaceutical field, being the drug delivery a very promising one. One of the first approaches was to introduce reactive double bonds by functionalizing the polymer with glycidyl acrylate (Kamath and Park 1995). This study was the precursor for numerous others. Dextran-MA, dextran-HEMA, microparticles of functionalized dextran are being used for the delivery of imunoglobulin G (IgG), native or recombinant human bone morphogenetic protein (BMP), DNA, among others (Franssen et al. 1997, Hennink et al. 1997, Ferreira et al. 2002, Pitarresi et al. 2003, Ferreira et al. 2004, Maire et al. 2005, Ferreira et al. 2005, DeGest et al. 2006).

Cellulose is a linear polymer with repeating units consisting of cellobiose. It can undergo enzymatic degradation resulting in the formation of D-glucose units. Although cellulose is insoluble in common solvents, the hydroxyl groups are reactive and can be easily functionalized. Several derivatives of cellulose in the form of ethers, esters, and acetals, such as methyl cellulose, hydroxypropylmethyl cellulose and carboxymethyl cellulose have been investigated as candidates for various applications. All of these cellulose derivatives are soluble in a variety of solvents and can be easily processed into various forms such as membranes, sponges and fibers (Baeyer et al. 1988, Hou et al. 1991). Additionally, the good mechanical properties of cellulose make it very attractive for biomedical applications as dressings in treating surgical incisions, burns, wounds, and various dermatological disorders, and also as matrices for drug delivery applications (Swarbrick and Boyan 1991, Takahashi et al. 2001, Tate et al. 2001, Li et al. 2002, Stabenfeldt et al. 2006).

Chitosan is the deacylate derivate of chitin. Chitin is a naturally wide occurring polysaccharide that can be extracted from crustacean exoskeletons (e.g. crabs and shrimps), also existing in fungal cell walls. The biodegradation rate is determined

by the amount of residual acetyl content, a parameter which can easily be varied (Hayashi 1994, Pachence and Khon 1997). Like HA, chitosan is not antigenic and is a well-tolerated implantable material, showing biostimulating activity in the healing process of various tissues. Chemical modification of chitosan produces materials with a variety of physical and mechanical properties, very useful in applications as encapsulation, inhibition of blood coagulation, controlled drug delivery, among others (Madihally and Mathew 1999).

Pullulan is a linear bacterial homopolysaccharide originating from *Aurebasidium pullulans*. The backbone is formed by glycosidic linkages of α-(1→6) D-glucopyranose and α-(1→4) D-glucopyranose units in a 1/2 ratio (Leathers 2002). The backbone structure of pullulan resembles dextran, being also easily derivatized in order to impart new physico-chemical properties, e.g. to increase the solubility in organic solvents or to introduce reactive groups, leading to a polymeric system capable of forming hydrogels. The unique linkage pattern of pullulan endows the polymer with distinctive physical traits, including adhesive properties and the capacity to form fibers, compression moldings, and strong, oxygen-impermeable films (Leathers 2003). Several studies refer pullulan as a promising polymeric carrier for many drugs (Shingel 2004). Additionally, recent works reported that pullulan allow for smooth muscle cell adhesion, spreading, and proliferation and hold promises as scaffolds for vascular engineering (Autissier et al. 2007).

Synthetic polymers

The main advantage of synthetic polymers, over the natural ones, is that they are chemically synthesized, being therefore possible to control their molecular weight and molecular weight distribution (with variable degrees of accuracy, depending on e.g. the type of polymerization reaction), which ultimately causes profound effects on the physical characteristics of the polymer, such as strength and degradation rates (Thomson et al. 1995, Maquet and Jerome 1997). They can, thus, be

developed to meet the requirements of its final applications. Additionally, although enzymatic processes can assist the degradation, it is, in general, brought up by simple hydrolysis, which is desirable to minimize the person-to-person variations. Among synthetic materials, biodegradable polyesters approved by the Food and Drug Administration (FDA) are rapidly gaining recognition in the field of TE.

Poly (α-hydroxy acids), such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and their copolymer poly(lactic-co-glycolic acid) (PLGA) are currently the most widely investigated and most commonly used synthetic biodegradable polymers and have been widely applied in bone and cartilage repair (Athanasiou et al. 1998, Sherwood et al 2002, Uematsu et al 2005).

Poly(ε-caprolactone) (PCL), which degrades at significantly lower rates than PLA, PGA and PLGA, is less attractive for tissue engineering applications. However, PCL-based scaffolds have been studied for skin replacement and other tissue engineering applications (Ng et al. 2001, Htai et al. 2004, Li et al. 2005). Furthermore, novel degradable PCL networks, PLGA-PCL-PLGA tri-block copolymers and PCL-chitosan matrices are more hydrophilic, degrade faster and possess desirable mechanical properties as compared to PCL (Choi and Park 2002, Kweon et al. 2003 Sarasam and Madihally 2005).

Poly(N-isopropylacrylamide) (pNiPAAm) and its copolymers belong to the most intensively investigated thermoreversible materials. Recent developments on pNiPAAm-based scaffolds include their use for drug delivery (Liu et al. 2006, Yin et a. 2006, Nakayama et al. 2006, Coughlan et al 2006), cell encapsulation and delivery (Na et al. 2006) and cell culture surfaces (Hatakeyama et al. 2006).

Poly(ethylene glycol)s (PEGs) are very popular synthetic polymers frequently used in tissue engineering and drug delivery applications. Although PEG derivatives provide only end-groups for chemical modification, they are frequently used, because they are non-toxic and non-adhesive towards proteins, resulting in suitable model systems (Drury and Mooney 2003, Eiselt et al. 1999). The copolymerization of PEG with biodegradable and biocompatible polyesters yielded some interesting

solutions that may be applied as delivery systems in patches, creams, gels, injectables and implants (Jeong et al. 2000, Zitzmann and Nieschlag 2000, Chen and Singh 2005, Lee et al. 2006).

Hydrogel as a Scaffold

Among the scaffolds, hydrogels are receiving an increasing attention. They can fill irregular shaped defects and incorporate cells and other bioactive materials (Drury and Mooney 2003). Several biomedical applications require materials that possess a jelly consistency, which can set and be molded into a desired shape under physiological conditions.

The term *hydrogel*, refers to aqueous (water-containing) gels, polymer networks that are insoluble in water, where they swell to an equilibrium volume, retaining their shapes (Chen et al. 1997). The importance of hydrogels in biomedical applications was first reported in the late 1950s, with the development of PHEMA gels as a soft contact lens material (Wichterle and Lim 1960). Nowadays they are used in numerous biomedical applications (Table 3) including ophthalmic devices, biosensors, biomembranes, and as delivery systems (Andrade 1976, Peppas 1986).

Hydrogels have appealing features from the perspective of biological mimicking. They are able to retain a great quantity of water, producing a soft consistency, which resembles natural living tissues. Hydrogels have a good biocompatibility and, in general, they show low minimal mechanical and frictional irritation (Chen et al. 1997, Hoffman 2002). Furthermore they can be processed under relatively mild conditions and can be delivered in a minimally invasive manner, increasing patient compliance (Lee and Mooney 2001, Nguyen and West 200, Baroli 2006).

Table 3. Biomedical applications of hydrogels.

Type of Device	Application	References
Temporary devices	Burn and surgical dressing; degradable scaffolds; artificial skin substitutes; tissue barriers	Ramakumar et al. 2002, Mazzoli et al. 2004
Semipermanent devices	Drug delivery systems; tubular devices; artificial corneas; soft tissues substitutes and replacements; bone ingrowth sponges; soft contact lenses	Pearce et al. 1984, Allarakhia et al. 1987, Jain 1988, Chehade and Elder 1997, Nicolson and Vogt 2001, Hicks e al. 2000, Vijayasekaran et al. 2000, Gulsen and Chauhan 2005, Kwon et al. 2005, Goda and Ishihara 2006, Morrison et al. 2006, Stapleton et al. 2006, Uchino et al. 2007
Permanent devices	Hemodialysis membranes; blood oxygenators; bioartificial and biohybrid organs (eg. pancreas or liver); coatings for tubing, catheters and for cell culture devices/vessels	Kolthammer 1975, Vale et al. 1975, Widdop et al. 1975, Winchester et al. 1976, Moise et al. 1977, Klomp et al. 1983, Ronel et al. 1983, Yanagi et al. 1989, Honiger et al. 1997, Brahim et al. 2002, Risbud et al. 2003, Nakabayashi and Iwasaki 2004, Ferramosca et al. 2005, Garg et al. 2005

Hydrogels are 3D hydrophilic polymeric networks, composed of homopolymers or copolymers, containing chemical or physical crosslinks. The hydrophilicity of the network is due to the presence of chemical residues as hydroxylic (–OH), carboxylic (–COOH), amidic (–CONH–), primary amidic ($–CONH_2$), sulfonic ($–SO_3H$), and others that can be found in the polymer backbone or as lateral chains (Flory and Rehner 1943, Flory 1950, Flory 1953, Peppas and Mikos 1986, Brannon-Peppas 1990). The insolubility of the gel in water is due to the presence of a three-dimensional network, where an equilibrium between dispersing and cohesive forces exist (Peppas et al. 2000, Hoffman 2002[1]). The cohesive forces are responsible for preventing further penetration of water and can be generated by

covalent bonds between the chains of the polymer network (chemical crosslink) or by cooperative and associative forces such as hydrogen bonds or Van der Waals interactions (Peppas and Mikos 1986). Hydrogels can be either neutral or ionic, depending on the ionization of the side groups. In addition to the ability of imbibing large amounts of water, certain hydrogels are sensitive to the physiological or biological environment in which they are inserted. These responsive hydrogels can exhibit swelling changes due to the external pH, temperature, ionic strength or electromagnetic radiation (Qiu and Park 2001, Jeong et al. 2002).

Hydrogel networks can be classified as natural or synthetic (based on the origin of the starting material), degradable or nondegradable, and according to the preparation methods, they can also be divided in physical or chemical. Physical hydrogels can be prepared in various manners including ionic or hydrogen bond interaction, crystallization, protein interaction, self-assembly in a supramolecular structure or micellar packing. Chemical hydrogels are produced by crosslinking materials through chemical or polymerization reaction (Peppas et al. 2000). This thesis will focus on the chemical hydrogels, specially produced by polymerization reaction. In this case, the synthetic or natural starting materials (monomer, polymers) should have, or should be modified to have at least a vinyl residue (e.g. acrylic acid, methacrylic acid, hydroxyethylmethacrylate, cinnamic acid, among others). The functionalized monomers are then polymerized in the presence of small quantities of crosslinking agents, bearing multi-vinyl residues, in a so-call chain-reaction polymerization, commonly known as radical polymerization. However, starting materials possessing different functional groups such as –OH, –COOH, –CONH or $–CONH_2$, can also be crosslinked through covalent linkages between the polymer chains and complementary reactivity, such as amine-carboxylic acid (–NH–/–COOH, isocyanate-hydroxyl (–NCO/–OH) or by Schiff base formation (Hennink and van Nostrum 2002).

Physical, Chemical and Toxicological Properties of Hydrogels

Selection or synthesis of the appropriate hydrogel is governed by the physical properties, the mass transport properties and the biological interaction requirements that are best suited for a given application.

Mechanical Properties

Mechanical properties of hydrogels are very important for pharmaceutical applications. For example, the integrity of a drug delivery device during the lifetime of the application is crucial. The system must protect a sensitive therapeutic agent, such as protein, maintaining its integrity until it is released out of the system. Changes in the crosslinking density of the hydrogels have been routinely applied to achieve the desired mechanical properties. Increasing the crosslinking will result in a stronger gel with a more brittle structure, which sometimes is not desirable, since it shall interfere with the release profiles. Hence, there is a commitment between a relatively strong but yet elastic structure (Anseth et al. 1996).

Degradation Behavior

Another important feature of the hydrogel is the degradation behavior. The desired kinetics for hydrogel degradation depends on the final application. Degradation is essential in many small and large molecule release applications and in functional tissue regeneration. Ideally, the rate of scaffold degradation should be adequate for the controlled release of bioactive molecules or reflect the rate of new tissue formation. Degradation of hydrogel may occur according to different pathways. There are three basic degradation mechanisms: hydrolysis, enzymatic cleavage and dissolution. Backbone degradation will generate low molecular weight, and generally soluble units. This type of degradation is typical of hydrophilic polymer

networks. Most of the synthetic hydrogels are degraded through hydrolysis of ester linkages. As hydrolysis occurs at a constant rate, the degradation can be manipulated by the composition of the material (Metters et al. 2000, Saito et al. 2001). In addition, it is common that both backbone and crosslinking degradations occur simultaneously. The crosslinking density is once again of major importance in determining the final properties of the material. In the case of enzymatic cleavage the degradation will depend on both the cleavage sites in the polymer and the amount of available enzyme in the hydrogel environment (West and Hubble 1999, Mann et al. 2001).

Swelling

As it was mentioned earlier, hydrogels have the ability to increase their volume while maintaining their shape. The initial and relaxed state of a hydrogel is called *dry*. In contact with water or an aqueous solution, hydrogels swell - the water molecules are drawn into the network. Since these molecules are going to occupy some space, meshes of the network will start expanding, allowing the uptake of other water molecules. The stretching caused by the swelling process is counterbalanced by the covalent or physical crosslinking of the network, preventing its destruction and creating an equilibrium swelling state. In highly crosslinked hydrogels, the mobility of the polymer chains is hindered, lowering the ability to swell, which can ultimately affect both the diffusion and the release profiles (Peppas et al. 2000).

Diffusion Characteristics and Release Profiles

In a hydrogel, the rate of diffusion depends on both the material and molecule characteristics and interactions. Gel properties such as polymer fraction, polymer size and crosslinking density, determine the structure and porosity of the gel. As a consequence, diffusion rates will be affected by the water content, the molecular

weight and size of the diffusion species, the swelling and the degradability of the material. The actual mechanism of drug release depends on whether diffusion or erosion is the rate-controlling step. For erodible systems, polymer dissolution after crosslink cleavage plays a critical role in determining overall release profiles. Degradation of crosslinks increases the mesh size of the gel, allowing for diffusion to be facilitated and therefore, the release is erosion-controlled. On contrary, the erosion of the matrix can be much slower than the diffusion of the molecule through the polymer. The release is then considered diffusion-controlled (Dijk-Wolthuis et al. 1997, Amsden 1999). While either diffusion or erosion can be the predominant factor for a specific type of polymer, in most cases, the release kinetics is a result of a combination of these two mechanisms (Sujja-areevath et al., 1998; Lee, 1981; Tahara et al., 1995; Efentakis and Buckton, 2002).

Biocompatibility

Apart from favorable physico-chemical and mechanical properties, the most important requirement for a hydrogel to be used in medical applications is its biocompatibility in a specific environment, together with the non-cytoxicity of its degradation products. Most of the toxicity problems associated with hydrogels are the unreacted monomers, oligomers and initiators, that leach out during application. These leachables can exhibit varying levels of reactivity and consequently toxicity. In addition, it is also necessary to consider the potential toxicity of the degradation products. Therefore, the knowledge of the degradation processes and the effects that the by-products might have is crucial for long-term success of the hydrogel application (Kirkpatrick 1992, Del Guerra et al. 1995, Wallin 1995, Del Guerra et al. 1996, Kohn and Langer 1996, Ratner 1997).

Improving Hydrogel Properties

Recent advances in the development of hydrogels include the incorporation of cell recognition molecules on the surface or the bulk of the materials. Early studies reported on the use of long chains of ECM proteins such as fibronectin (FN), vitronectin and laminin for surface modification, with successful promotion of cell adhesion and proliferation (Humphries et al. 1986). More recent trends leave behind the long chain proteins, using instead, short peptide fragments as signaling domains, which are more stable during the modification process and can be massively synthesized. The most commonly used peptide for modification is Arg-Gly-Asp (RGD) (Figure 4), the signaling domain derived from fibronectin and laminin, Tyr-Ile- Gly-Ser-Arg (YIGSR), Arg-Glu-Asp-Val (REDV) and Ile-Lys-Val-Ala-Val (IKVAV) (Hubbell et al. 1991, Ranieri et al. 1995).

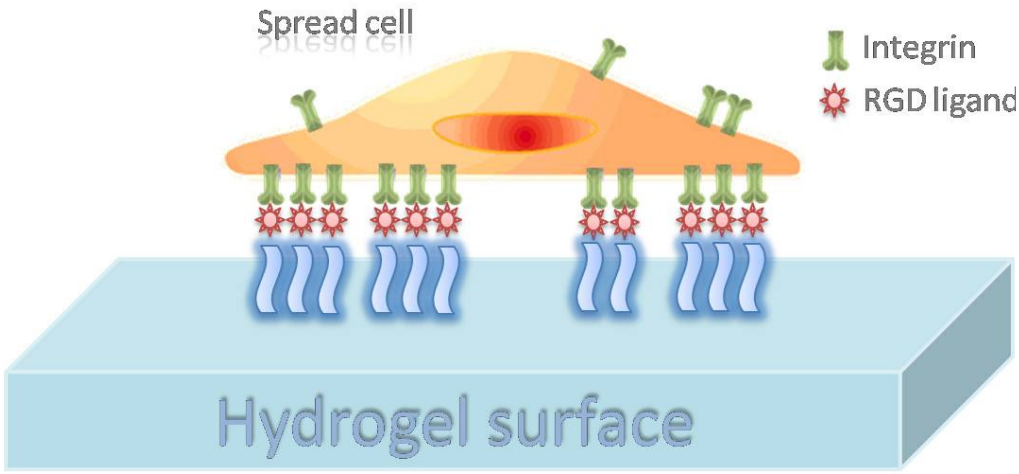


Figure 4. Schematic representation of a functionalized hydrogel surface. The RGD ligands mediate cell adhesion through the interaction with the integrins of the cells.

The surface coating with these bioactive molecules, serving the purpose of mimicking ECM, allows for the modulation of cellular functions such as cell attachment, proliferation, and differentiation. Additionally, the cell-signaling peptides are incorporated into the hydrogel backbone and not limited to the surface. Another feature includes the incorporation of enzymatically degradable sequences, allowing the degradation to be modulated by the presence of enzymes that

specifically recognize cleavage sites within the hydrogel. This is a very fine approach in hydrogels designed for controlled release, since the cleavage of the sequence releases the bioactive agent to which is linked.

Dextrin

As referred previously, the selection of the material for a scaffold, in this case a hydrogel, remains a key factor to achieve the ambitious goals of the constantly evolving biomedical field. In spite of the wide range of biodegradable polymers available, the increasing demand continues to feed the interest not only in the development of new materials, but also in improving the performance of existing ones. Having this in mind, we attempted to produce hydrogels from a naturally derived starch based polymer – dextrin.

Dextrin is a glucose containing polymer primarily linked by α-(1→4) D-glucose units, which also possesses α-(1→6) linkages (Figure 5). Dextrin has the same general formula as starch, but smaller and less complex. It is produced by partial hydrolysis of starch, by the use of acid, enzymes, or a combination of both.

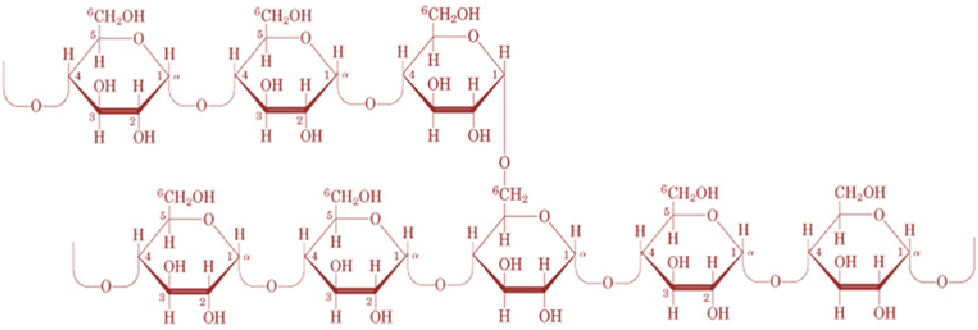


Figure 5. Schematic representation of a dextrin molecule highlighting the predominant α-(1→4) linkages, also evidencing the presence of α-(1→6) branch point.

Dextrin is a widely used material with a variety of applications, from adhesives to food industry and textiles. Moreover, several studies refer its application in a large number of areas such as medicine, pharmacy and biology (Wong and Mooney 1997, Lederer and Leipzig-Paganini 2000). Recent work reported on the ability of

dextrin conjugates to exhibit anti-endotoxin activity as well as important role in the regulation of inflammatory response (Avetisyan et al. 2006, Davtyan et al. 2006, Davtyan et al. 2007).

Dextrins can have different oligosaccharide profile, properties and molecular compositions depending on the starch and on how it is digested (Marshal et al. 1999). Relevant properties include hygroscopicity, fermentability, sweetness, stability, gelation, solubility, and bioavailability. Starch contains both linear α-(1→4) amylose and branched α-(1→6) amylopectin, the relative amounts depending on the type of starch (Figure 3). Natural starches contain 10-20% amylose and 80-90% amylopectin. Enzyme hydrolysis of starch with α-amylase efficiently hydrolyzes the α-(1→4) linkages, but not the α-(1→6) ones, leaving behind a small amount of high molecular mass residues. On the other hand, hydrolysis with a α-(1→6) specific enzyme (e.g. pullulanase) will render a higher proportion of linear α-(1→4) oligosaccharides, which are more susceptible to retrogradation and gelling (Chronakis 1998). Generally, acid hydrolyzates will contain larger amounts of residual high molar mass oligomers than their enzyme hydrolysis counterparts (Lloyd and Nelson 1984). In summary, all starch hydrolyzates will typically contain an assortment of branched and linear oligosaccharides (Marshal et al. 1999).

Chemical Modification of Dextrin

Despite the wide investigation and application of dextrin, this is, to our knowledge, the first attempt to use it as a starting material for hydrogel production. However, various approaches have been already explored for the production of starch-based hydrogels. These approaches include one (Castellano et al. 1994, Pascual et al. 1996, Zhang and Zhuo 2000, Elvira et al 2002 , Bajpai and Saxena 2004) or two-step free radical graft polymerization of hydrophilic vinyl monomers in the presence of a cross-linker (Heller et al. 1990, Crescenzi et al. 2002, Ferreira et al. 2002, Masci et al. 2002, Bhuniya et al. 2003), cross-linking by chemical reactions

with complementary groups (Lenaerts et al. 1991, Shiftan et al. 2000, Seidel et al. 2001, Seidel et al. 2004), radiation-induced polymerization and cross-linking (Rosiak and Ulanski 1999, Ceresh et al. 2002, Zhai et al. 2002, Yoshii et al. 2003) and physical self-assembly processes (Akiyoshi et al. 1993, Akiyoshi et al. 1996, Akiyoshi et al. 1997, Janes et al. 2002, Simon et al. 2003, Duval-Terrié et al. 2003).

Dextrin is not capable of gelling. As it was described, to produce chemical hydrogels, the starting raw materials should contain a functional group (Radley 1976, Wurzburg 1986, Riedel and Nickel 1999).

Chemical derivatization of polysaccharides has profound effects on macroscopic behavior (e.g. solubility, stability and viscosity characteristics). Among the many possibilities of modification, the creation of reactive double bonds through a transesterification reaction is the principal process by which it is possible to functionalize sugars, producing sugar esters (Figure 6a). This process has been studied extensively with regard to the chemical phenomena involved (Park et al. 2003, Vargha and Truter 2005, Jeong et al. 2006). The final product of this reaction has side chains (vinylic groups) attached to the polysaccharide backbone and polymerizes through well-established free radical methods (Mark and Mehltretter 1972, De Graaf et al. 1998).

The goal of this work was to use the ester, vinyl acrylate (VA), as the acyl donor to the esterification of the hydroxyl groups of dextrin (acyl acceptor) (Figure 6b). The double bonds allow further chemical reactions such as secondary crosslinking of the final product. The degree of substitution (DS) refers to the average number of substituted polysaccharide hydroxyl group and is of major importance in determining overall properties of the resulting hydrogel, such as the degradation behavior or the release profiles.

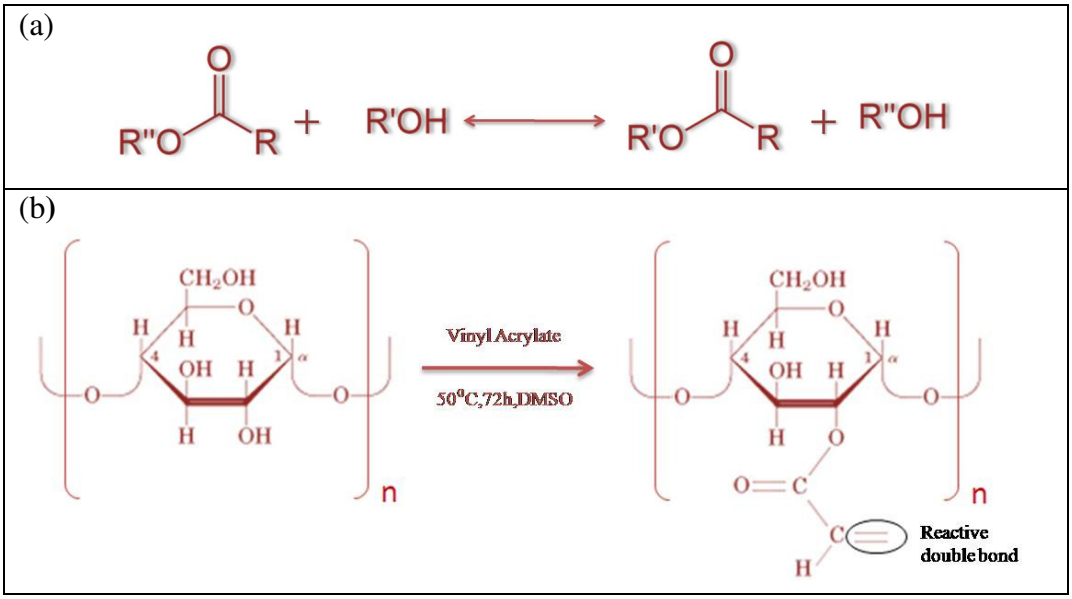


Figure 6. Generic representation of a transesterification reaction (a). Synthesis of dextrin-vinyl acrylate (VA), evidencing the reactive double bond on the final product.

Aims

The main goal of this work was to functionalize the biomaterial – Dextrin – to produce a hydrogel, and further characterize the obtained networks. As it was described in detail, due to their unique properties, hydrogels are forefront candidates for biomedical applications, namely as controlled release devices. In light of this final application, the requirements for a material to be considered suitable for biomedical use must be considered. Likewise, the following key characteristics were studied:

- Biocompatibility
- Mechanical Properties and morphology
- Diffusivity and degradation rates

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SECTION II

Use of Non-Catalytic Domains for the Modification of Polysaccharides

Polysaccharides

Polysaccharides are biological materials which occur naturally in a wide variety of plants, microorganisms and animals. They offer an extraordinary source of carbon and energy, thus representing a central component of the carbon cycle. The functionality of polysaccharides is closely related to their molecular structure and configuration (Bayer et al. 2004).

Cellulose

Cellulose is the most abundant polysaccharide on Earth. It consists of extended and long chains of poly-β-(1→4) glucose (Figure 1). The fibers are constituted by linear chains in which glucose residues are twisted by 180º. The repetitive unit is the dimer cellobiose instead of glucose, as in starch. Cellulose is highly stable and insoluble in water. Most of the cellulose in nature is found in the cell walls of plants, where it is usually the major component. The cell wall itself is a very complex structure that also contains hemicellulose, xyloglucans, lignin, pectin and proteins, which confer resistance to degradation (Figure 1) (Gibeaut and Carpita 1993). Cellulose is a material with convenient properties for quite different applications and industries (Belgacem et al. 1995). The excellent mechanical properties, wide availability associated with low cost production (both from vegetable and microbial sources), varied morphology, geometry and surface

properties, recycling possibility, make cellulose a privileged target for modification, leading to products with different properties.

In a typical cellulose-degrading ecosystem, a variety of cellulolytic bacteria and fungi work in association with related microorganisms to convert insoluble sugars which are then assimilated by the cell. This hydrolytic process is handled by a variety of different enzymes, known as cellulases (Lamed et al. 1983, Richmond 1991, Vian and Reis 1991, Delmer and Amor 1995, Warren 1996).

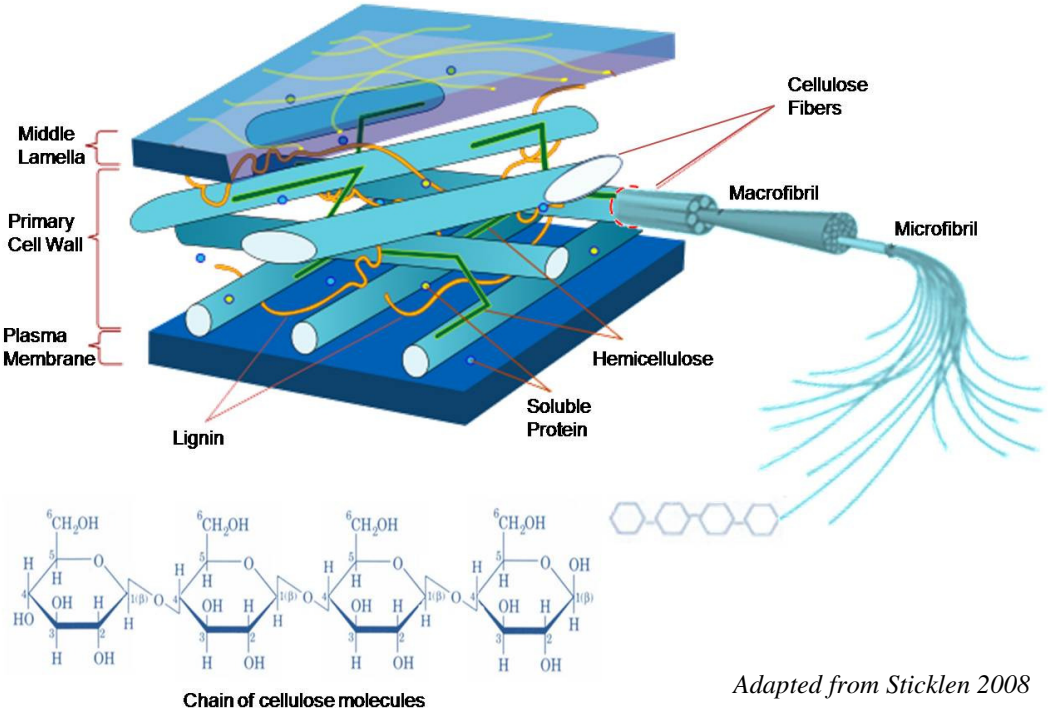

Figure 1. Cell wall structure evidencing cellulose fibers. Schematic representation of a cellulose molecule highlighting the β-(1→4) linkages.

Cellulases belong to the glycosyl-hydrolase family of enzymes, being responsible for the hydrolysis of the β-(1→4) linkages in cellulose. Microorganisms adopt fundamentally two strategies to breakdown cellulose, both aerobic and anaerobic (Henrissat and Davies 1997). Cellulolytic fungi and aerobic bacteria secrete relevant quantities of monomeric extra-cellular enzymes, each of them having specific action and acting in synergy to elicit effective hydrolysis. On contrary, the

anaerobic bacteria produce enzymatic complexes called cellulosomes, multienzymatic systems present in the surface of the cells, which adsorbs and degrade cellulose (Coughlan and Ljungdahl 1988, Shoham et al. 1999, Bayer et al. 2004). Up to 11 different enzymes are aligned, thus ensuring a high local concentration, together with the correct ratio and order of the components. For these reasons, it is believed that the anaerobic system may be metabolically advantageous over the aerobic one (individual enzymes). Besides the higher absorption efficacy of the degradation products, due to the positioning of cellulosomes on the cell surface, the close proximity between the cells and enzymes allow for a better control of the metabolic activity (Boisset et al. 1999, Schwarz WH 2001).

Cellulosome of *Clostridium thermocellum*

Clostridium thermocellum (*C. thermocellum*) is an anaerobic thermophilic bacterium, considered to have the highest rate of cellulose utilization (Johnson et al. 1982, Arai et al. 2001, Lynd et al. 2002, Demain et al. 2005). The hydrolysis is mediated by a large multi-enzyme complex termed *cellulosome* (Figure 2).

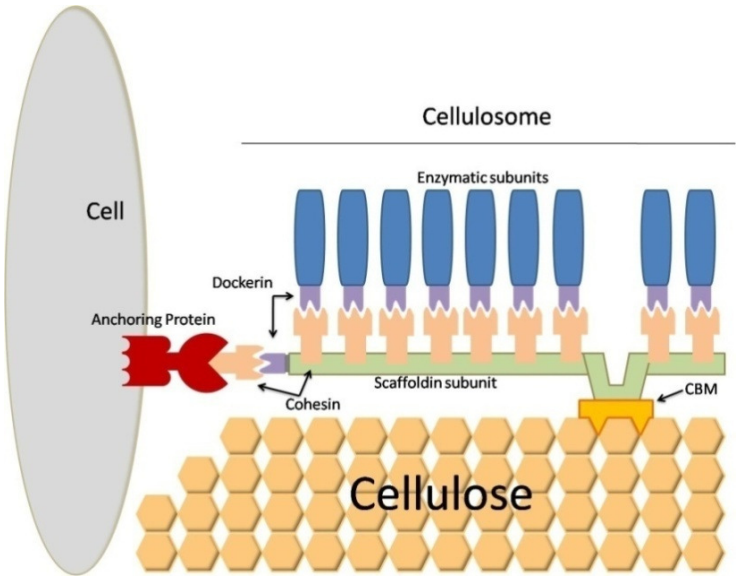

Figure 2. Simplified schematic view of the interaction between *C. thermocellum* cellulosome and its substrate. CBM – Cellulose-binding Module.

The principal component of the cellulosome is a scaffoldin subunit, a large enzyme-integrating protein (CipA – Cellulosome integrating protein) that contains cohesin modules, normally in multiple copies, for incorporation of the catalytic domains, including β-(1→4)-endoglucanases, cellobiohydrolases, hemicellulases and other cellulosomal components (Figure 2) (Bayer et al. 1994, Béguin and Lemaire 1996). The enzymes contain a complementary module, the dockerin domain that binds to the cohesin modules, mediating the integration of the enzymes into the complex of the scaffoldin subunit. The scaffoldin also frequently includes a carbohydrate-binding module (i.e., a cellulose-binding domain), commonly referred to as CBM, through which the complex usually recognizes and binds to the substrate. The cellulosome is anchored to the plant cell wall primarily through the action of the internal family 3 CBM located in CipA. Cellobiohydrolase A (CbhA) is one of the most important catalytic components of the cellulosome. It is composed of an N-terminal family 4 CBD, an immunoglobulin-like domain, a family 9 glycosyl hydrolase catalytic domain (GH9), two fibronectin like repeats ($FN3_{1,2}$), a family 3 CBD and a C-terminal dockerin domain (Felix and Ljungdahl 1993, Bayer et al. 1998, Béguin and Alzari 1998, Zverlov et al. 1998, Shoham et al. 1999, Blum et al. 2000, Kurokawa et al. 2001, Kataeva et al. 2002, Kataeva and Ljungdahl 2003, Kataeva et al. 2003).

Carbohydrate-binding Modules (CBM)

Not only cellulases, but also other enzymes involved in the hydrolysis of insoluble polysaccharides, are typically modular enzymes with a distinct carbohydrate-binding domain joined to a catalytic domain through a linker – highly glycosilated peptide chain. Due to their high specificity and substrate recognition mechanisms, these modules have a fundamental role in the enzymatic degradation of plants and in polysaccharide storage.

A carbohydrate-binding module (CBM) is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme, having carbohydrate-binding activity. CBMs were previously classified as cellulose-binding domains (CBDs) based on the initial discovery of several modules that bound cellulose as their primary ligand (Gilkes et al. 1988, Tomme et al. 1988). However, new modules meeting the CBM criteria, which are capable to bind carbohydrates other than cellulose, are continually being found.

CBMs are organized in 52 families (CAZY server, http://www.cazy.org/fam/acc_CBM.html). This classification of carbohydrate-binding domains is based on amino acid sequence similarity and is expected to aid in the identification of both CBMs and its functional residues, to predict binding specificity and polypeptide folding and even reveal evolutionary relationships. Family 1 to 13 corresponds to CBMs which have a least one cellulose-binding domain (Tomme et al. 1995, Boraston et al. 2004).

Modification of Polysaccharides

The adoption of industrial environmentally friendly processes is finally turning mandatory, bringing biotechnological processes to the forefront of research. Under its broad definition, biotechnology encompasses activities such as fermentation, cell immobilization, and enzyme technology. Extensive basic and applied research on cellulases, hemicellulases and other polysaccharide-degrading enzymes has not only generated significant scientific knowledge but has also revealed their potential in biotechnology. At present, they are used in food, brewery and wine production, animal feed, textile and laundry, pulp and paper industries, as well as in agriculture, in wastewater treatment, systems to remove oxygen-demanding substances and suspended solids and for research purposes. The demand for these enzymes is

growing rapidly and has become the driving force for intensifying its research (Bhat and Bhat 1997, Bhat 2000).

Enzymes are among the most promising examples of new technologies designed to help the textile industry meeting the challenges of strict environmental regulations, global competition and new-market driven demands for specific fabric properties. Cellulases are used in the treatment of cotton fibers, namely in the stone wash process, substituting the use of stones to produce used-like aspect of the garments (Miettinen-Oinonen 2004). They are also used in the de-pilling process, which consists in remove the small fibers – pills – present in the surface of cotton (Heikinheimo 2002).

Enzymes have also several uses in the paper industry and have long been applied in paper production, for the modification of both fiber and paper properties (Bajpai 1999). These enzymes include xylanases, endoglucanases, cellulases and hemicellulases (Suurnäki et al. 1994, Senior and Hamilton 1993, Gen and Li 2003, Seo et al. 2000, Jackson at al. 1993, Pala et al. 2001). Currently, the most important application of enzymes includes the use of xylanases in biobleaching pre-treatment of kraft pulp (Vikarii et al. 1994). This process allows for the reduction, or even elimination of the hypochloride used in the bleaching process for the lignin oxidation. Endoglucanases have found their function in two important processes, de-watering and de-inking. One of the most important stages in paper production is the de-watering step, in which water is removed from the formed sheet using a combination of vacuum and heat. Cellulases can assist in this step by solubilizing small particles that are strong water holders (Pommier et al. 1989, Pommier et al. 1990, Jackson et al. 1993, Woodward et al. 1994, Bhardwaj et al. 1996). The same mechanism is responsible for lowering the energy consumption in mechanical pulp production by improving the beatability, drainage and solubility of the pulps. Additionally, the use of cellulases for glue removal has proved to be efficient, even more than the conventional alkaline chemicals (Sykes and Klungness 1997).

Use of Non-catalytic Domains – An Emerging Field

The attractiveness of biotechnology lies in its potential to increase specificity on the reactions to save energy, lowering the associated cost in reactions, keeping in mind the development of environmentally friendly processes. Despite its wide utilization, well established in the above discussion, several drawbacks result from enzyme utilization. Taking the paper treatment as an example, the drawbacks include the extensive hydrolysis of polysaccharides that leads to a reduction of both fiber strength and mass. In this context, the application of CBMs allows overcoming the limitations of the enzyme technology.

Nowadays, CBMs are widely used. They can be used on the purification of recombinant proteins (Jiang and Radford 2000). Textile applications include fabric targeting of recombinant enzymes that do not possess a native affinity for the cellulosic fibers, such as amylases, proteases and lipases (von der Osten 2000). Other substances can also be targeted to cellulosic fabrics. Fragrance-bearing particles conjugated to CBMs can be added to laundry powder, hence reducing the amount of fragrance needed in the product (Berry 2001). Another application consists in the expression of CBMs at the cells surface, such that they may be immobilized in cellulosic materials (Nam et al. 2002, Lethio et al. 2003). CBMs are also successfully applied as analytical tool in research. An example is the development of a system based on CBMs that allows for a rapid detection of pathogenic microbes in food samples (Shoseyov et al. 1999).

Due to its ability to adsorb to cellulose without degrading it, CBMs have great biotechnology potential. The ability to fuse CBDs with other molecules of different sizes, charges, hydrophobicities, catalytic activities or bioactivities, allow these molecules to be potentially interesting and useful on the activation and modification of cellulose fibers. Inspiring research into CBM-cellulose fiber interactions is continuously being carried out (Kitaoka and Tanaka 2001, Levy and Shoseyov 2002). Several studies have indicated that treatment of cellulose fibers with CBMs alters the interfacial properties of the fibers. This phenomenon was first

observed by Cavaco-Paulo et al. (1999), who demonstrated that treatment of cotton fibers with a CBM alters their affinity to dye. Later on, Suürnakki et al. (2000) showed that treatment of bleached chemical pulp with endoglucanases, cellobiohydrolases, and the catalytic domains from *Trichoderma reesei* could also change the interfacial properties, having a beneficial effect on pulp properties such as viscosity and strength after refinement. Another study demonstrated that treatment of fibers recycled from old paperboard containers with CBMs improve both the tensile and burst indexes, as well as increase the pulp drainage rate (Pala et al. 2001, Pala et al. 2002). Following studies described an increase in the surface area of cellulosic fibers as a consequence of cellulosic aggregate disruption (Din et al. 1991, Kitaoka and Tanaka 2001, Pinto et al. 2004). Kataeva et al. (2002) have also demonstrated the non hydrolytic disruption of cellulose fibers by the family 3 CBM of CbhA of *C. thermocellum.* This non-hydrolytic disruptive activity has also been ascribed to the $Fn3_{1,2}$ module. Additionally, it was suggested that $Fn3_{1,2}$ affects hydrolysis more significantly when it is attached to its appended catalytic domain, in what seems to be a synergistic process. This thesis will focus on the potential of two recombinant family 3 CBDs (belonging to CipA and CbhA of the *C. thermocellum*) and a fusion protein with the $FN3_{1,2}$ module (also from CbhA) to modify secondary paper fibers.

As described earlier, CBMs are produced by a large number of fungi and bacteria and have the ability to potentiate the catalytic efficiency of the associated catalytic domains. They may be obtained through the proteolysis of cellulases, providing large amounts of peptides in a rather rapid procedure (Pinto et al. 2004, Pinto et al. 2006). In addition they can be produced using the recombinant DNA technology (Creagh et al. 1996, Bothwell et al. 1997, Linder et al. 1999, Bayer et al. 2000). In fact, recombinant forms of these proteins containing his-tags, provide a flexible system which allows overcoming the purity limitations of the proteolysis approach. However, CBMs obtained through these methods have not the highly glycosilated peptide sequence (Pinto et al. 2004, Pinto et al. 2006). Thus, in current work,

recombinant CBM were chemically modified with PEG, expected to mimetize the properties of the native CBM glycoconjugates.

Pegylation

First reports of the use of PEG to modify the interfacial/biological properties of different molecules, remount to the late 70s and the studies on the alteration of immunological properties of BSA modified with PEG (Abuchowski et al. 1977). The coupling of PEG to other molecules can be used to attain various ends. It can be used to alter solubility characteristics both in aqueous or organic solvents (Inada et al. 1986), to increase the stability of proteins in solution (Berger and Pizzo 1988), for modulation of the immune response (Delgado et al. 1992), to extend the half life of the modified substances, among others. The PEG molecule consists of repeating units of ethylene oxide that terminate in hydroxyl groups on either end of a linear chain. In solution PEG is a highly mobile molecule that creates a large exclusion volume for its molecular weight, far larger than proteins of comparable size. It can be conjugated to other molecules through two principle approaches; (1) direct reaction with the hydroxyl groups, (2) reaction of the polymer with a functional molecule – activation. The most common activation methods, the one that has been used in this work, is the creation of amine-reactive derivates that can be linked with proteins and other amine-containing molecules through a carboxilate end, producing a stable conjugate (Figure 3) (Zalipsky et al. 1991, Miron and Wilchek 1993).

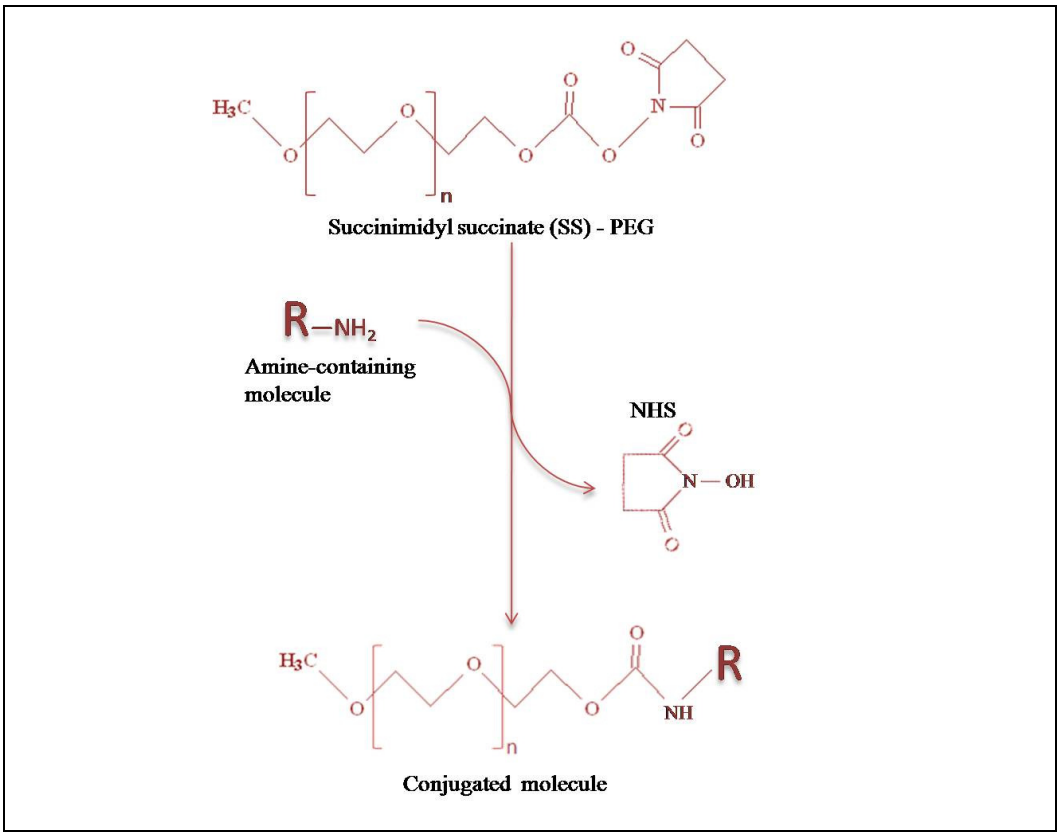

Figure 3. Structure of SS-mPEG, showing the activating succinimidyl succinate terminal group, highly reactive toward nucleophile amine-containing molecules.

Aims

The main objective of the present work was to produce and purify two recombinant family 3 CBM (belonging to CipA and CbhA of the *C. thermocellum*) as well as a fusion protein with the $FN3_{1,2}$ module ($FN3_1$-$FN3_2$-CBD_{CbhA}). Having in mind the challenging applications of the CBM as "environment-friendly" technology, the potential of these proteins to modify secondary paper fibers was also analyzed. Finally, the CBMs were chemically conjugated with an activated PEG, as this molecule mimetize the glycosidic fraction that is lacking in the highly purified bacterial CBMs. The technical properties of the paper fibers and paper sheets resulting from the CBM treatment were analyzed.

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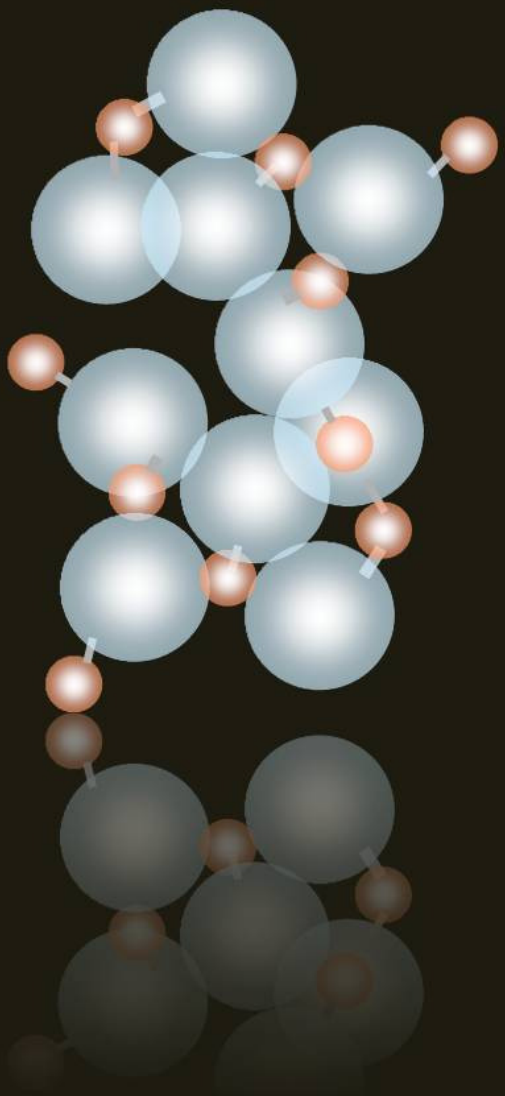
CHAPTER 2

Production and characterization of a new dextrin based hydrogel

Summary

Dextrin is a polymer composed of α-(1⟶4) D-glucose units produced by partial hydrolysis of starch. In this work, the transesterification of the soluble polysaccharide with vinyl acrylate (VA) was carried out in anhydrous dimethylsulfoxide (DMSO). The effect of the water activity and of the enzyme Proleather, on the reaction rates, was analyzed. Different degrees of substitution (DS) ranging from ca. 10% to 70% were obtained by controlling the molar ratio of VA to dextrin. Gels were obtained by free radical polymerization of dextrin-VA, with different degrees of substitution and monomer concentration, in water. A comprehensive solid state-NMR analysis of the hydrogels was performed. These hydrogels are being developed as scaffold materials for bioactive molecule and cell delivery, tissue engineering and a variety of other biomedical applications.



Carvalho JM | Gonçalves C | Gil AM | Gama FM
Eur Pol J | 2007 | Published

Introduction

Hydrogels are a class of three-dimensional, highly hydrated polymeric networks (Peppas et al. 2000). They are composed of hydrophilic polymer chains, which can be either synthetic or natural. The structural integrity of hydrogels depends on crosslinks established between the polymer chains, by chemical bonds and physical interactions. Hydrogels are providing new opportunities for the development of a variety of medical applications (Peppas et al. 1996), including drug delivery systems (McCulloch and Shalaby 1998), scaffold materials to organize cells into a three-dimensional architecture, tissue replacements (Kane et al. 1996) wound dressings (Ishihara et al. 2002, Chen et al. 2003) and immobilization of proteins and cells, among others. Since the clinical use of hydrogels is increasing, considerable efforts have been made in order to develop new hydrogels from a variety of synthetic and natural materials (Drury and Mooney 2003). In this work, we report a procedure to prepare dextrin hydrogels. Dextrin is a glucose-containing saccharide polymer linked by α-(1→4) D-glucose units, having the same general formula as starch, but smaller and less complex. This polysaccharide is produced by partial hydrolysis of starch, which can be accomplished by the use of acid, enzymes, or a combination of both. Dextrin is a widely used material with a variety of applications, from adhesives to food industry and textiles. Moreover, because of the biocompatibility and degradability, starch-based materials are presently being used to prepare biodegradable hydrogels with technological applications in a large number of areas such as medicine, pharmacy and biology (Wong and Mooney 1997, Marques et al. 2002). Various approaches have been already explored for the production of starch hydrogels. These approaches include one or two-step free radical graft polymerization of hydrophilic vinyl monomers in the presence of a cross-linker, crosslinking by chemical reactions with complementary groups, radiation-induced polymerization and crosslinking, and novel self-assembly processes (Zhang et al. 2005) However, compared with our approach, some of these chemical strategies show disadvantages, such as the use of catalysts (e.g. 4-

dimethylaminopyridine (4-DMAP) or pyridoxine) (Ferreira et al. 2002) which are difficult to remove from the reaction mixture and could dramatically increase the toxicity of the material. To prepare the hydrogels, dextrin was modified with vinyl acrylate (VA) using two different approaches, chemical or enzymatic. Chemical synthesis is broadly used, however presenting several drawbacks, such as the need for protection and deprotection of functional groups to prevent unwanted racemization, as well as the lack of stereoselectivity. The use of enzymes under mild conditions has allowed, in some cases, overcoming these limitations, also enabling modification of molecules that could not otherwise be altered (Cooney and Heuter 1974, Zacks and Klibanov 1985, Klibanov 1997, Klibanov 2001). Apolar solvents are generally considered to better preserve the enzymatic activity that polar ones (Sergeeva et al. 1997, Akkara et al. 1999). However, Ferreira et al. (2002) have recently reported the used of the *Bacillus subtilis* protease Proleather FG-F to catalyze the transesterification of dextran with VA, in anhydrous dimethylsulfoxide (DMSO). The enzyme-catalyzed system developed by Ferreira and colleagues, using dextran and inulin, was in this work used for dextrin modification. The starch polysaccharide functionalized with reactive double bonds was cross-linked by free radical polymerization in aqueous solution. The structure and morphology of starch networks in the hydrogel were characterized by cross-polarization magic-angle-spinning 13C NMR spectroscopy (CP-MAS 13C NMR).

Materials and Methods

Materials

Proleather FG-F, a protease from *Bacillus subtilis*, was obtained from Amano Enzyme Co. Dextrin - Koldex 60 (Mw= 45700 Da, as determined by gel permeation chromatography (GPC) analysis) was a generous gift from Tate and Lyle. Vinyl acrylate (VA) was from Aldrich, N,N,N',N'-tetramethylenethylenediamine (TEMED) and ammonium persulfate (APS) were purchased from BioRad, dimethylsulfoxide (DMSO) and acetone were from

AppliChem and polyethyleneglicol (PEG 200 g/mol) was obtained from Riedel-d Haën. DMSO was dried with 0.4 nm molecular sieves, at least overnight, before use. Regenerated cellulose tubular membranes, with 3500 MWCO, were obtained from Membrane Filtration Products.

Pretreatment of Proleather and determination of proteolytic activity in DMSO

Proleather FG-F was "pH adjusted" as described by Ferreira et al. (2002). The enzyme was resuspended in 20 mM phosphate buffer at pH 8. After freezing, the sample was lyophilized on a CHRIST ALPHA2-4 lyophization unit (B. Braun Biotech International) for 48 h. The percentage of active enzyme in commercial powder was 8.9 % (w/w) as obtained by BCA Protein Assay (Pierce) using BSA as protein standard. The proteolytic activity of Proleather FG-F in either 0.2 M phosphate buffer pH 8 or DMSO was determined with casein as substrate. Briefly, in the presence of a protease the casein is cleaved, exposing primary amines. The compound TNBSA (2,4,6-Trinitrobenzene sulfonic acid) reacts with these exposed amines thereby producing an orange-yellow colour that can be quantified as 450 nm absorbance (Abs_{450}). Control blanks for each sample were included in the assay to correct the effect of primary amines on proteins in the protease sample. The absorbance values were then converted to equivalent subtilisin (a *Bacillus subtilis* protease) concentration using a calibration curve. One unit of protease activity (U) is defined as the production of 1 mM of free amine per minute per milligram of enzyme.

Enzymatic and chemical synthesis of dextrin-VA

The enzyme-catalyzed modification of dextrin was performed as described by Ferreira et al. (2002) with few modifications. Briefly, dextrin (4 g) and an amount of VA ranging from 382 to 2600 µL were dissolved in anhydrous DMSO (60 mL). The reaction was started by adding 600 mg of "pH adjusted" Proleather FG-F. The reaction mixtures were then incubated at 50 ºC, with magnetic stirring, for 72 h.

Figure 1 shows the chemical reaction of dextrin modification. After this time, the mixtures were dialyzed for 5 days against HCl aqueous solution, pH 3, at 4 ºC. The aqueous solutions of dextrin-VA were precipitated in a 4-fold excess of acetone and further centrifuged at 9000 rpm for 15 min. Finally, the precipitates were lyophilized for 48 h. The chemical modification was carried out in the same conditions, but in the absence of enzyme.

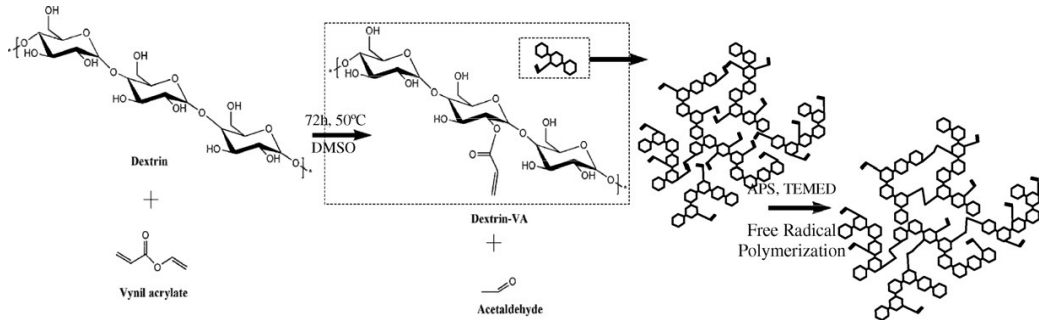


Figure 1. Dextrin-VA synthesis and formation of the hydrogel networks.

The degree of substitution (DS, defined as the amount of acrylate groups per 100 glucopyranose residues) was determined by titration according to Vervoort et al. (1997) Dextrin-VA samples (100 mg) were dissolved in 2.00 mL of 0.1 M NaOH (standardised) and stirred for 72 h at room temperature, to carry out the alkaline hydrolysis of the ester. Molar consumption of NaOH was determined by back titration with 0.1 M (standardised) HCl, using phenolphthalein as indicator. Additionally, a liquid RMN analysis of the DS was also performed and the values compared.

Determination of Water Amount and Activity (a_w)

Water content was determined by the Karl Fisher titration method. Water activity of DMSO solutions with different amounts of water was established using a microclimate method. A small amount of sample is disposed in a plate as a thin layer. The plate is then placed in a gas-tight container, where relative humidity is fixed with saturated salt solutions (LiCl, $MgCl_2$, KI and $(NH_4)_2SO_4$). Whenever the sample and the saturated salt solutions do not have the same water activity, a water

transfer among the samples occurs, quantified by weighting. The equilibrium is reached when constant mass is observed, by sorption or desorption. The water activity of the sample is determined according to the procedure described by Multon and Bizot (1978) and shown in figure 2.

Preparation of dextrin-VA hydrogels

Dextrin-VA hydrogels were obtained by free radical polymerization of aqueous solution of dextrin-VA, with different degrees of substitution and monomer concentration (Figure 1). Two different amounts of dextrin-VA (300 and 400 mg) were dissolved in 900 µL of 0.2 M phosphate buffer, pH 8.0, and bubbled with nitrogen for 2 min. The gelation reactions were initiated by adding 90 µL APS (80 mg/ml in 0.2 M phosphate buffer, pH 8.0) and 90 µL TEMED (13.6 % (v/v) in water, pH adjusted to 8.0 with HCl) and allowed to occur for 30 min at room temperature. All the recipients in which gelation took place were coated with a polyethyleneglicol solution (PEG 200 g/mol) to simplify the removal of the hydrogel following polymerization.

Solid state NMR Spectroscopy

The CP-MAS ^{13}C NMR technique is a convenient solid-state analytical method for the characterization of hydrogels due to the insolubility of their networks. The ^{13}C NMR spectra were recorded on a 400 MHz Bruker FT NMR (Fourier transform nuclear magnetic resonance) spectrometer equipped with CP-MAS (cross-polarization and magic-angle-spinning) accessories. The dry samples were placed in a 7 mm rotor and spun at a rate of 4.5 kHz. Spectra were acquired with a contact time of 5 ms, spectral width 300 ppm, 2 K time domain points and the number of transients indicated in the figure captions. All spectra were processed with a linebroadening (LB) factor of 30 Hz in order to improve signal to noise ratio.

X-ray powder diffraction measurements

Monochromatic Cu-Kα radiation (wavelength = 1.542 Å) was produced by a Philips PW1710 X-ray powder diffractometer. The samples were exposed to the x-ray beam with the x-ray generator running at 40 KV and 30 mA. The scanning regions of the diffraction angle 2θ were 3º - 65º, step interval 0.02 and scan rate 2º C/min.

Results and Discussion

A. Production of dextrin-VA

In previous studies, Ferreira and colleagues (2002) demonstrated the successful enzymatic acryloilation of the polysaccharide dextran, solubilized in anhydrous DMSO. In this work, a similar dextrin modification was attempted using the same enzyme-catalyzed method. The proteolytic activity of the enzyme Proleather FG-F in either 0.2 M phosphate buffer pH 8 or DMSO was assessed (Figure 2). As can be seen, although with a smaller activity, the enzyme remains catalytically active when solubilized in a DMSO solution. Additionally, a time-course reaction, carried out for six days, showed that the enzyme is very stable in DMSO, conserving nearly 80 % of the initial activity.

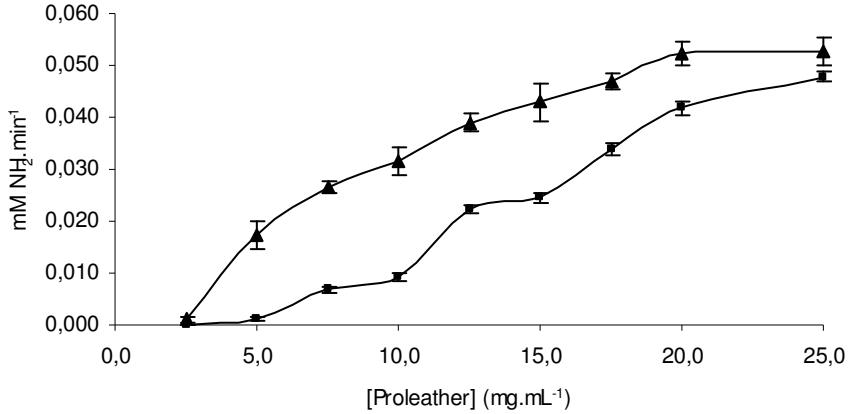


Figure 2. Proteolytic activity in 0.2 M phosphate buffer pH 8 (▲) or DMSO (■) using casein as substrate. The proteolytic activity is expressed as the rate of NH2 formation.

The synthesis of dextrin-VA was performed both in the presence or absence of 10 mg/mL Proleather FG-F, for 72 h at 50 ºC in DMSO. The results in Table 1 show that dextrin-VA with different DS (ranging from ca.10 to 70 %) can be obtained, both in the presence or absence of the enzyme, by varying the concentration of the acyl donor. The modified polymer was successfully recovered by precipitation with an excess of acetone, after dialysis. The obtained reaction yields, in the range 41-62%, were superior to values referred in other similar works (Ferreira et al. 2002).

Table 1. Degree of substitution (DS) obtained with different VA concentrations obtained enzymatic and chemically, with dextrin and dextran as substrates.

Dextrin				**Dextran**			
With Proleather		Non-catalysed reaction (Without Proleather)		With Proleather		Non-catalysed reaction (Without Proleather)	
DS (%)[a]	Efficiency[b]	DS (%)[a]	Efficiency[b]	DS (%)[a]	Efficiency[b]	DS (%)[a]	Efficiency[b]
11	73	10	73	10	67	0	0
14	70	13	70	13	65	0	0
19	76	20	76	16	64	0	0
67	67	59	59	44	44	5	5

[a] Determined by titration of the molar consumption of NaOH (alkaline hydrolysis of the ester)

[b] Ratio of the experimental to the theoretical DS (Calculated as molar ratio of VA to dextrin glucopyranose residues in the reaction mixtures)

A comparative study, using dextrin and dextran, was carried out in the presence and absence of the enzyme. Dextrans are soluble polysaccharides characterized by a predominance (higher than 95%) of α-(1→6) backbone linkages and varying proportions of α-(1→2), α-(1→3) and α-(1→4) linkages typically at branch points. Dextrins are partially hydrolyzed glucose homopolymers composed exclusively of α-(1→4) backbone linkages. As already stated, Ferreira and colleagues showed in

previous work that the presence of the Proleather enzyme is mandatory for a high DS to be obtained, using dextran as substrate, as confirmed in this work (Table 1). Interestingly, the spontaneous non-catalysed reaction is very effective in the case of dextrin (reaction efficiency always higher than 59%). As a matter of fact, the presence of enzyme, for this substrate, does affect neither the reaction yield nor kinetics. It thus appears that the non-catalysed reaction occurs faster and to a higher extent with dextrin as substrate rather than with dextran. Proleather is an efficient catalyst for dextran transesterification, but not of dextrin. Indeed, the reaction reaches similar conversion degrees for different DS and reaction times (data not shown) irrespective of the presence of enzyme. These results clearly show that, contrarily to previous results obtained with dextran and inulin, dextrin acylation is not catalysed by Proleather.

Characterization of dextrin-VA obtained with and without Proleather

The ester positions on the glucopyranosyl residues were determined based on additional signals present in the 13C NMR Spectrum of dextrin-VA produced both with and without Proleather (Ferreira et al. 2002). The two positional isomers in the main dextrin backbone are at the glucopyranosyl positions 2 and 3. The respective ^{13}C NMR assignments are presented in Table 2.

Table 2. ^{13}C and ^{1}H NMR assignments of the glucopyranosyl ring (δ, ppm) on dextrin-VA with DS 20%.

Carbon	**Dextrin-VA**					
	unsubstituted		2-substituted		3-substituted	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	5.42	102.4	5.65	99.06	5.47	102.30
2	3.65	74.26	4.86	75.53	*	*
3	3.86	73.89	4.25	73.67	5.44	78.06
4	3.69	79.45	3.69	79.45	3.69	79.45

5	3.99	76.05	3.99	76.05	3.99	76.05
6	3.88	63.18	3.88	63.18	3.88	63.18

*Signals not identified.

Bi-dimensional (HSQC) NMR experiments were carried out in order to evaluate the isomer ratio in dextrin-VA. The area of the three peaks, C1, C1-S2 and C1-S3, was determined using a Lorentzian adjust, and the isomer ratios calculated. For dextrin-VA with DS 20% obtained by chemical reaction, an isomer ratio of 70:30, at positions 2 and 3, was found. For the enzymatic reaction a similar substitution pattern was observed (the ratio of isomers at positions 2 and 3 was 76:24). Additionally, the regioisomer at position 2 was more highly favored (two times). Again, it appears that the enzyme does not influence the reaction, since the isomers obtained are the same, irrespective of the presence of the enzyme. Thus, this work demonstrates that it is possible to regioselectively modified dextrin without using enzymes.

Effect of water amount and activity on the transesterification reaction

The transesterification reaction was carried out using mixtures of DMSO with different amounts of water, corresponding to different values of a_w (Figure 3). The values of a_w and the corresponding dextrin DS obtained are show in and 2 and table 3. Table 3 shows that the reaction is switched towards synthesis for a_w <0.3. Low amounts of water (in the range 0 to 20%) correspond to similar a_w, and lead to similar DS. Higher values of a_w (≥0.4) result in a dramatic reduction of dextrin modification. These results are clearly different from the effect of a_w described by Ferreira and colleagues for the catalysed reaction, with dextran. In this case, a very low amount of water is mandatory for the enzymatic reaction to be carried out, while in our experiment (with dextrin) as much as 20% of water (a_w≈0.3) may be present without loss of synthesis reaction.

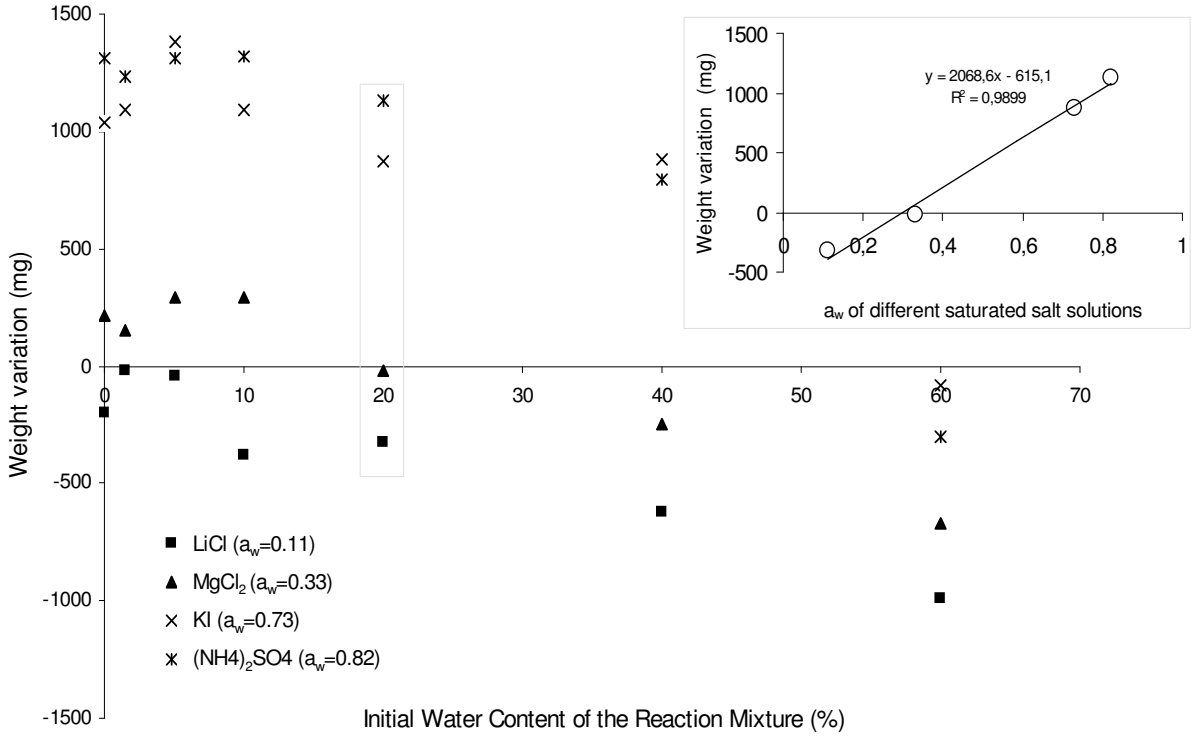


Figure 3. Weight variation of the samples containing DMSO, VA and enzyme, with different initial water content, after equilibrium with saturated salt solutions. Insertion shows the a_w calculation (a_w=0.3, for y=0) for an initial water content of 20%.

Table 3. Water activity of reaction mixtures with different amounts of water and DS (the VA to dextrin ratio correspond to a theoretical DS of 20%) obtained after transesterification reaction.

Water Content (%)	**Water activity***	**DS (%)[c]**
0.015	0.22 (0.11-0.33)	17
0.15	0.17 (0.11-0.33)	17
5	0.15 (0.11-0.33)	18
10	0.25 (0.11-0.33)	14
20	0.30 (0.11-0.33)	14
40	0.41 (0.33-0.73)	0

60	0.95 (0.82-1)	0
80	~1	0
100	1	0

*a_w estimated as shown in figure 2. The actual a_w value is in the range between brackets.

B. Hydrogel production and NMR characterization

The acrylate groups in dextrin-VA were polymerized using APS and TEMED as free radical initiators. Aqueous solutions of dextrin-VA with different DS (ranging from ca. 10 to 70%) and with two concentrations (33% and 44% (w/v)), were polymerized at room temperature, and gelation occurred within less than 5 min. The obtained hydrogel slabs were almost transparent, the opacity increasing with decreasing in DS values, which is likely related to the better solubility of the more highly substituted material. The hydrogels were characterized using the solid state NMR technique of ^{13}C CP-MAS. This analysis, as described ahead, allowed the detection of VA adsorbed to dextrin, covalently linked to dextrin, and finally of the VA reticulating the hydrogel.

In the ^{13}C CP/MAS spectra of dextrin or similar samples, the peak chemical shifts are sufficiently similar to those observed in the solution state to allow peak assignment to be made. In order to identify the environments of non-linked dextrin and VA moieties, a sample of dextrin wetted with vinyl acrylate was prepared and analysed. The spectrum obtained (Figure 4) clearly shows the glucopyranosyl carbons in the 58-110 ppm and the acrylate carbons in the 120-175 ppm region. Signals at 90-105 ppm and 58-65 ppm are attributed to C1 and C6 carbons in hexapyranoses, respectively. The overlapping signal at about 68-78 ppm is associated with C2, C3 and C5. The resonance that appears at 82 ppm is thought to arise from C4 carbons in noncrystalline material (Langer et al. 1992). Regarding VA carbons, the peak resonating at 128 ppm arises from the carbons engaged in the double bond whereas the peak at 163 ppm corresponds to the carbonyl carbons (arrow in Figure 4). Both these positions should thus be characteristic of the VA environments when simply adsorbed to the dextrin. And 5 shows the downfield

expansions of the 13C CP-MAS NMR spectra of the same non derivatized dextrin sample, together with those of samples of dextrin-VA obtained with different degrees of substitution.

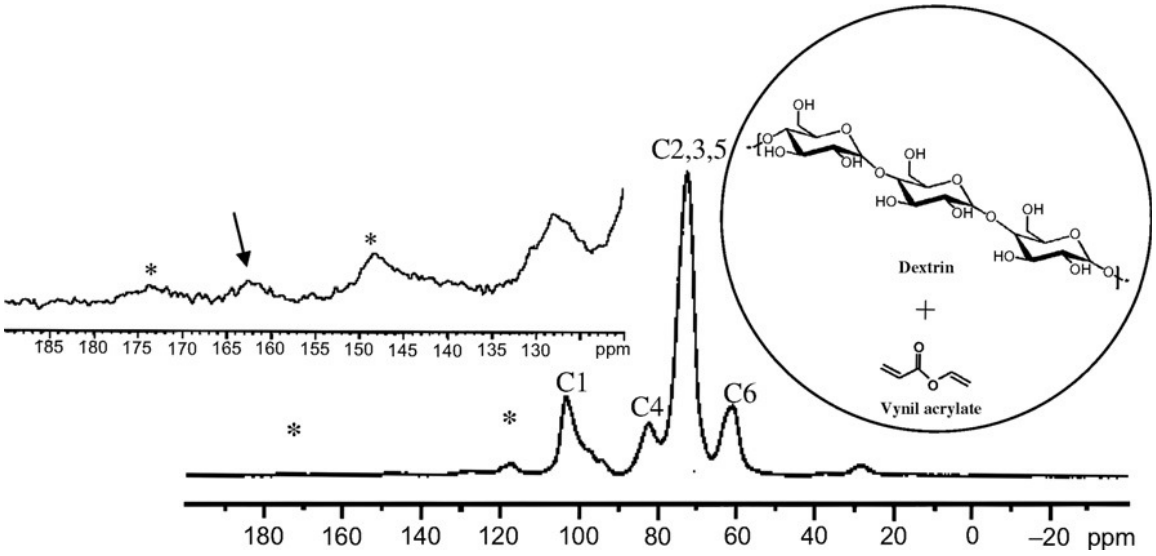


Figure 4. ^{13}C CP-MAS NMR spectra of dextrin mixed with VA. Bands indicated with * are spinning side bands. Number of scans (NS) =16729.

It is clear that the transesterification reaction leads to a shift of the VA carbonyl resonance from 163 ppm to 167 ppm. Indeed, and in spite of the relatively poor signal to noise ratio of the spectra (Figure 5), the area of this signal was found to increase steadily with the degree of substitution. It is possible, therefore, to distinguish the grafted (167 ppm) and non grafted (163 ppm) acrylate groups, with basis on the CP-MAS spectra of the samples. In the course of dextrin-VA preparation, extensive dialysis is crucial for the effective removal of unreacted vinyl acrylate. The effectiveness of the dialysis could, thus, be evaluated by NMR of the consistent presence of the 163 ppm environment (unreacted VA monomers), under conditions of incomplete dialysis.

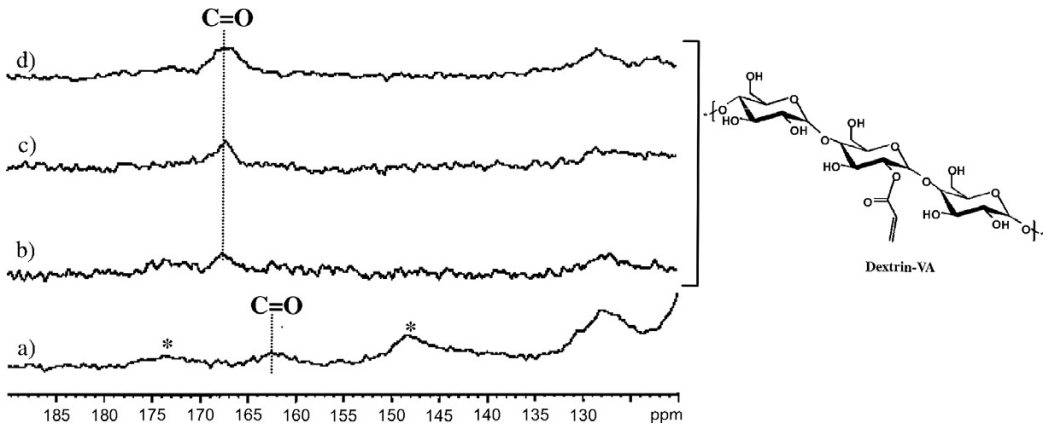


Figure 5. Downfield expansions of the ^{13}C CP-MAS NMR spectra of (a) dextrin mixed with vinyl acrylate and dextrin-VA with different degrees of substitution (DS) (b) 6.6%, NS=6228 (c) 8.7%, NS=6706 (d) 15.3%, NS=18827; the degrees of substitution were determined by liquid state NMR of the solubilised dextrin-VA sample. Peaks indicated with * are spinning side bands.

The spectrum of dextrin-VA hydrogel (containing cross-linked acrylate groups) is shown in Figure 6b. It is interesting to note that the carbonyl resonance is now broadened and shifted from 167 ppm in dextrin-VA, to 175 ppm in the hydrogel. The broadening reflects the heterogeneity of cross-linked VA environments and the lowfield shift results from the chemical changes taking place around the carbonyl groups upon the cross-linking process. The signal detected at 40 ppm after polymerization is attributed to the CH_2 (methylene) carbons of the cross-linked VA groups whereas the peak at 30 ppm simply corresponds to residual DMSO (arrows in Figure 6).

The peak at 167 ppm completely disappears in the hydrogel spectrum (Figure 6b), meaning that all grafted acrylate groups are involved in reticulation. When using a hydrogel prepared from a dextrin-VA with a higher degree of substitution (DS 67%), it is possible to detect grafted acrylate groups not contributing to reticulation, since after polymerization, a peak at 167 ppm is still present (Figure 7). The possibility of using the NMR peak areas to quantify the degree of reticulation (DR, percentage of grafted acrylate groups that are crosslinked) is currently being explored and will be reported in a subsequent paper.

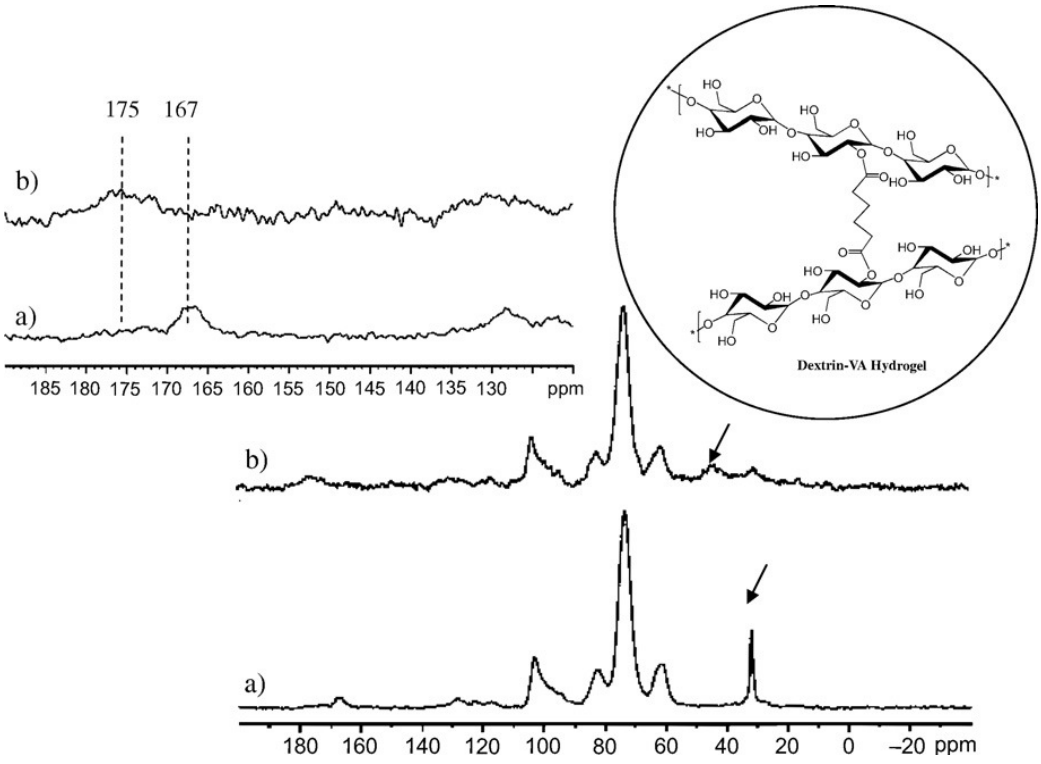


Figure 6. Full ^{13}C CP-MAS NMR spectra and lowfield expansions for (a) dextrin-VA DS 15%, NS=18827 (b) dextrin-VA DS 15% hydrogel, NS=62916.

Possible structural differences in the polysaccharide involved in the two samples, dextrin-VA and the corresponding hydrogel, may be more easily detected using the hydrogel prepared with higher DS. The differences observed in the C1 and C4 dextrin resonances in the 13C CP-MAS NMR spectra shown in figure 7 suggest that substantial conformational changes occur in the dextrin polymers in both materials. Indeed, the C1 and C4 resonances are broadened and displaced to higher field after polymerization (arrows in Figure 7). These are the two carbon sites involved in glycosidic bonds and would therefore be expected to be the most sensitive to changes in the polysaccharide conformation). Furthermore, the broadening and shift to higher field of the C4 resonance have been interpreted

before as indicative of an increase in polysaccharide crystallinity (Bociek and Gidley 1985). This could indeed be confirmed by the X-ray diffraction patterns of both samples (Figure 8) which suggest an increase in polymer crystallinity as a result of reticulation.

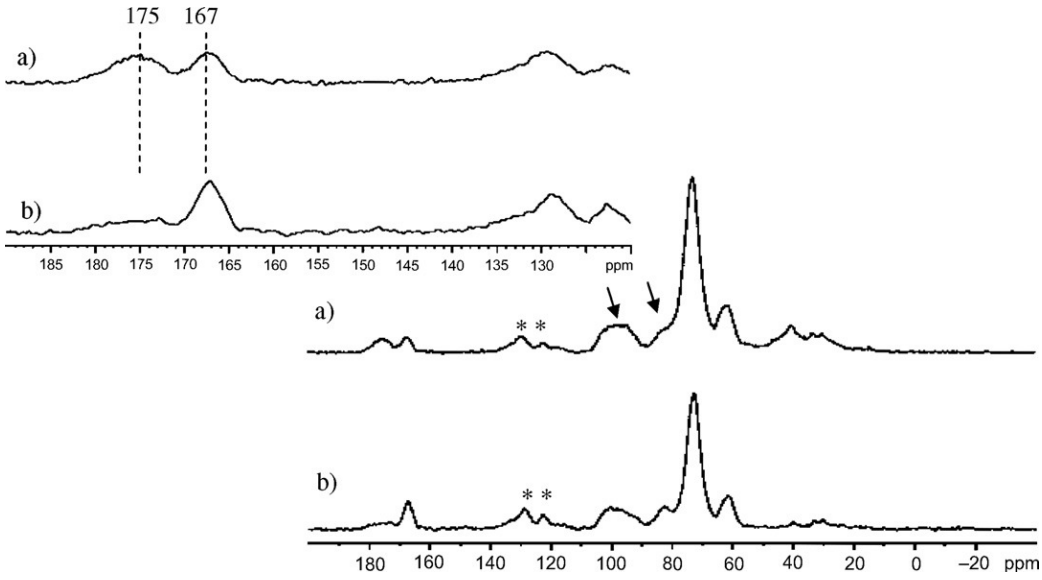


Figure 7. Full ^{13}C CP-MAS NMR spectra and lowfield expansions for (a) dextrin-VA DS=67%, NS=20821 (b) dextrin-VA DS=67% hydrogel, NS=20898. The signals indicated with * correspond to spinning sidebands**.**

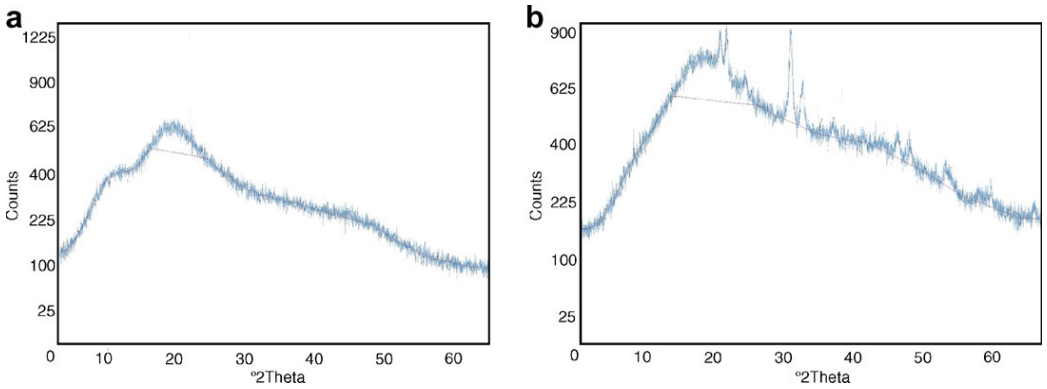


Figure 8. X-ray powder diffraction patterns obtained from (a) dextrin-VA DS 67% (b) dextrin-VA DS 67% hydrogel.

Conclusions

A method previously developed by Ferreira and colleagues was in this work applied in the production of dextrin hydrogels. Surprisingly, it was verified that, although Proleather effectively catalyses the transesterification of acrylate molecules to dextran and inulin, dextrin is not a substrate for this enzyme. Nevertheless, it has been possible to produce dextrin with varying amounts of grafted acrylate groups, by varying the VA concentration. Also surprising was the finding that the regioselectivity of the non-catalysed reaction is comparable to the one obtained with the enzyme-catalysed reaction, C2 and C3 being the preferred acylation positions. Derivatized dextrin can be polymerized in aqueous solution by radical polymerization with APS and TEMED.

Combining the ability to tailor the hydrogel properties to fit a wide range of characteristics, with the biocompatibility of the starch-based materials, it is possible to obtain dextrin crosslinked networks which might be very attractive for biomedical applications. Although starch hydrogels have been reported by other authors, the approach used in this work is different, and the basis for a comprehensive NMR structural analysis has also been developed. Current work is in progress with the purpose of quantifying, through solid NMR analysis, the several kinds of VA present in the hydrogels. Rheological and biocompatibility studies have been performed and will be described elsewhere.

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CHAPTER 3

New dextrin-vinylacrylate hydrogel: studies on protein diffusion and release

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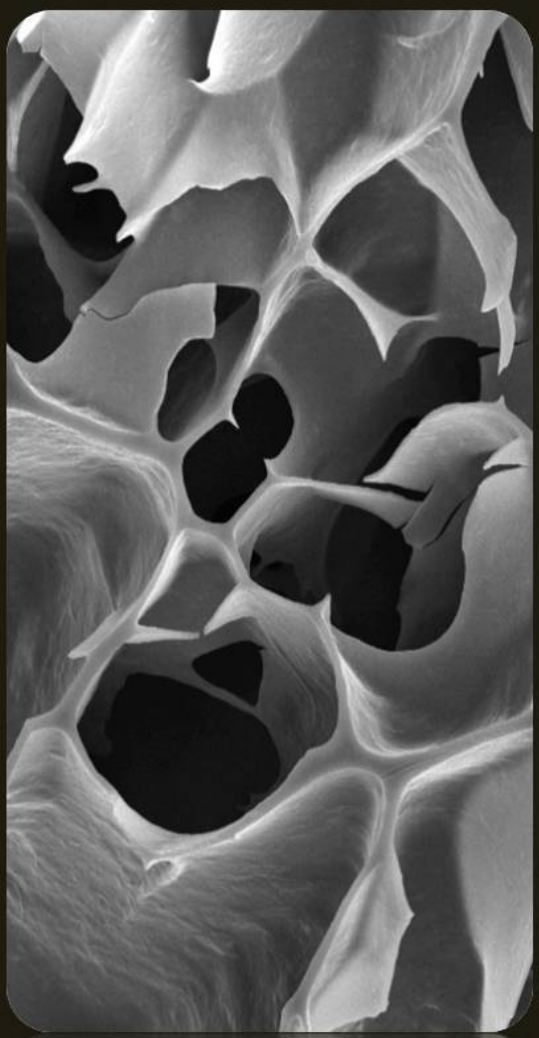
Carbohydr Pol | 2008 | Published



Summary

New dextrin hydrogels with degrees of substitution (DS) from ca. 10 (DS10) to 70% (DS70) were prepared by radical polymerization of aqueous solutions of vinylacrylate(VA)-derivatized dextrin. A preliminary analysis on the potential of these hydrogels for the controlled release of bioactive molecules was carried out. The protein (bovine serum albumin-BSA) diffusion coefficients on the hydrogels were calculated using the lag-time analysis. Values in range 10^{-7} cm^2/s were obtained for DS 20 and DS 40 and a smaller value of 10^{-8} cm^2/s arised upon DS increasing to 70%, revealing the dependence of the diffusivity on the crosslinking density. The release of BSA from dextrin-VA hydrogels, in the presence of amyloglucosidase was shown to be mainly dependent on the diffusion and, to a smaller extent, on the degradation kinetics. The protein release can be tailored from days to months by varying the DS.

Introduction

Biodegradable hydrogels are useful for a variety of medical applications (Dordick et al. 1994, Park and Park 1996, Han et al. 2000). In general, hydrogels have a good biocompatibility and the soft rubbery consistence resemble natural tissues, allowing for a favorable controlled interaction with living systems (Vyavahare and Kohn, 1994). Hydrogels may be used in the field of tissue engineering, as scaffolds to support and promote tissue regeneration and also as attractive systems for the controlled release of pharmaceutically active molecules (Peppas 1996, Kane et al. 1996, McCulloch and Shalaby 1998, Peppas et al. 2000). A drug delivery vehicle positioned in the proximity of the site of disease or injury can release the drug in the desired location, this way reducing the side effects that usually result from systemic administration. Additionally, depending on the crosslinking density, the hydrogels structure can restrict the diffusion of macromolecules, being able to deliver the therapeutic agent over extended periods of time. Indeed, improving in the patient compliance and extension of product life are major advantages of the drug delivery systems (Hubbell 1995, Ratner 2002). Ideal systems for drug delivery are degradable, permeable, porous and capable of maintaining a desired shape. In previous work, we have described a new dextrin based hydrogel, which could possibly match these properties (Carvalho et al. 2007). Dextrin is a glucose containing polymer linked by α-(1→4) D-glucose units, having the same general formula as starch, but smaller and less complex.

In this study, we aim at evaluating the diffusion of two different molecules in the hydrogel matrixes, glucose and a model protein, bovine serum albumin (BSA). This will allow achieving a second goal, to evaluate the potential of the hydrogels as controlled release systems. For these purposes, the enzyme amyloglucosidase was used to modulate the release of proteins entrapped in the hydrogel. Thus, the degradation rate of the network may be controlled through the crosslink density of the hydrogels and the amount of enzyme present.

Materials and Methods

Materials

Dextrin - Koldex 60 starch was a generous gift from Tate & Lyle (Decatur, IL, USA). Vinyl acrylate (VA) was from Aldrich, N,N,N',N'-tetramethylenethylenediamine (TEMED), ammonium persulfate (APS) and Protein Assay were purchased from BioRad, dimethylsulfoxide (DMSO) and acetone were from AppliChem and polyethyleneglicol (PEG 200 g/mol) was obtained from Riedel-d Haën. DMSO was dried with 0.4 nm molecular sieves at least overnight before use. BSA was from Sigma (Mw= 67 kDa). Regenerated cellulose tubular membranes with 3500 molecular weight cutoff (MWCO) were obtained from Membrane Filtration Products (Seguin, USA)

Carbohydrate analysis

Dextrin sugars were released by 1 M sulfuric acid hydrolysis at 100 ºC for 2.5 h and analysed as their alditol acetates by gas chromatography using a Carlo Erba 6000 with a split injector (split ratio 1:60) and a flame ionisation detector as described by Mafra et al. (2001). For determination of the linkage glycosidic composition, the dextrin sample was activated with powdered NaOH in dry dimethylsuphoxide and methylated with CH3I (Ciucanu and Kerek 1984). The methylated material was dissolved in dichloromethane:water (1:1, v/v), and the organic phase was recovered, washed several times with water, evaporated, and freeze dried. The methylated polysaccharides were hydrolyzed with 2 M TFA at 121 ºC for 1 h, reduced by $NaBD_4$ and acetylated in the presence of acetic anhydride and 1-methylimidazole. The partially methylated alditol acetates were analysed by GC-MS using an OV-1 capillary column as described by Cardoso et al. (2007). Triplicates were made of all analyses.

Preparation of dextrin-VA hydrogels

Dextrin-VA monomers were synthesized from dextrin in DMSO in the presence of different amounts of vinyl acrylate. The transesterification reaction was carried out at 50ºC for 72 h. The degree of acrylate substitution (DS) was determined by proton nuclear resonance spectroscopy (^{1}H-NMR) in D_2O as previously described (Carvalho et al. 2007). Briefly, ^{1}H-NMR spectra were recorded in D_2O (10 mg in 1 mL) and DS was calculated using equation 1:

$$\text{DS } \% = \frac{x}{y} \times \frac{7}{3} \times 100$$

where, in the NMR spectra, x is the sum of the peak intensities corresponding to the protons from vinyl group (δ in the range of 6.00-7.00 ppm) and y is the sum of the peak intensities of all dextrin protons (δ in the range of 0.00-6.00 ppm), excluding the water peak (δ around 4.80 ppm) (Figure 1). The hydrogel slabs with different DS (from ca. 10 to 70%) were prepared by radical polymerization of aqueous solution of Dextrin-VA. These solutions were prepared by dissolving dextrin-VA (300 or 400 mg) in buffer (900 µL) (phosphate buffer 0.2 M, pH 8.0 with 0.02% sodium azide) and bubbling with nitrogen for 2 min. For the release experiments, before gelation, a BSA solution (in phosphate buffer) was added yielding a final concentration of 2 g/L. The gelation reactions were initiated by adding 90 µL APS (80 mg/ml in phosphate buffer) and 90 µL TEMED (13.6 % (v/v) in water, pH adjusted to 8.0 with HCl) and allowed to occur for 30 min at room temperature. For the preparation of dextrin-VA/amyloglucosidase (AMG) hydrogels, different amounts of enzyme solution (diluted to 10 U/mL in phosphate buffer) was added to the dextrin-VA solution before the addition of the gelation reagents, as described above.

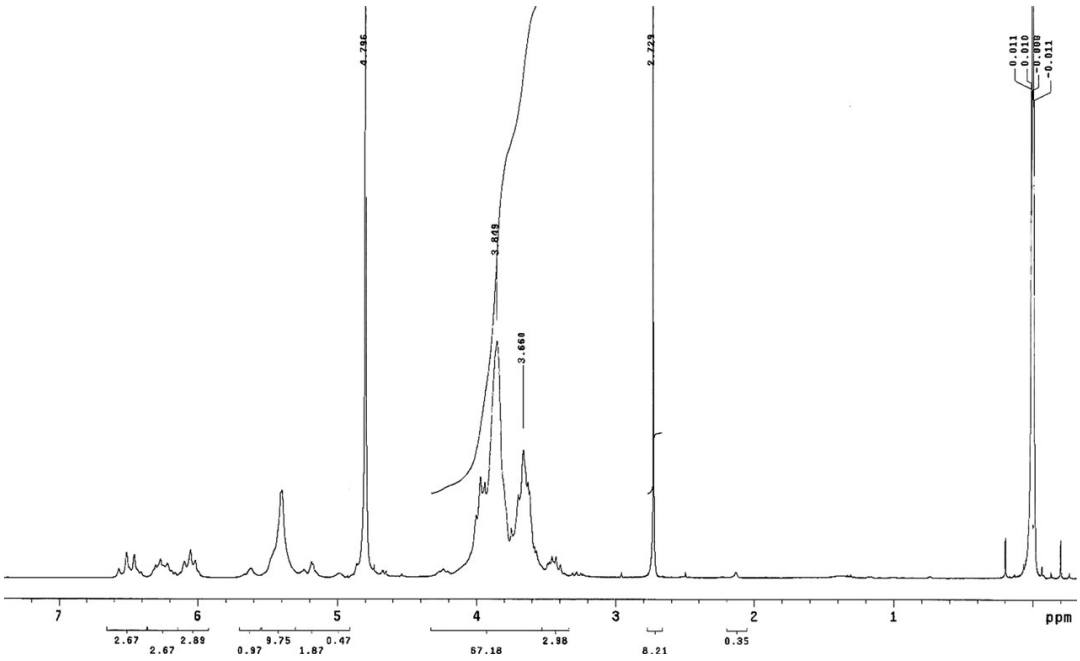


Figure 1. ^{1}H-NMR spectra of dextrin-VA in D_2O. DS= 20%.

SEM analysis

The internal and external structure of the hydrogels was analysed by SEM. Samples were either flash-freeze with liquid nitrogen or frozen at -80 ºC. Freeze-dried samples were gold sputter coated (SC 502 Golden Sputter, Fison instruments). All micrographs were taken using an S 360 Scanning Electron Microscope (Leica, Cambridge).

Release experiments

After polymerization, dextrin-VA hydrogels (with DS ranging from 20 to 70%), with or without AMG, were transferred to individual containers. To maximize the area for free diffusion, a perforated polypropylene support was placed at 5 mm from the bottom of the containers. The hydrogels were placed on the supports, submerged with 30 mL of PBS, stored at 37 ºC and gently shaken at 100 strokes per minute. Samples (100 µL) were taken at regular intervals and replaced by fresh

buffer. The protein concentration in the samples was measured by using the BioRad protein assay (microassay procedure).

Diffusivity experiments

The diffusion cell is a modification of Teixeira et al. (1994) (Figure 2). It is made of Perspex and consists of two chambers of 60 mL, divided by a Perspex plate, in which the hydrogel (Ø 20 mm; thickness of 0.5 mm) is inserted. The whole structure is held together with screws. The Perspex plate is supported by a squared mesh and sealed with O-rings. Agitation was obtained using magnetically driven bars in both chambers.

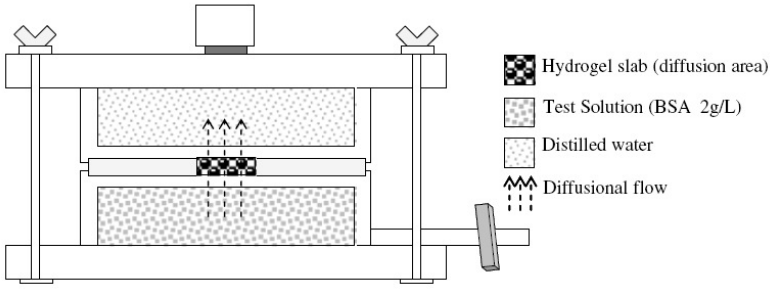


Figure 2. Diagram of diffusion chamber (Lateral view).

For the diffusivity experiments, hydrogels with different DS (20, 40 and 70) were evaluated and the diffusion coefficients of BSA and glucose were calculated. Samples were collected at regular time intervals and replaced with distilled water. For the BSA assay, protein concentration was measured by using the BioRad protein assay (microassay procedure) and in the glucose assay, the soluble sugars were determined using the dinitrosalicylic acid (DNS) method. Concentration of glucose or BSA in the lower chamber was 100 g/L or 20 g/L, respectively. The diffusion coefficients were calculated using lag-time analysis (Teixeira et al. 1994),

$$Q_{ts} = \frac{ADC_1}{l}\left(t_s - \frac{l^2}{6D}\right)$$

where Q is the total amount of solute transferred through the membrane (g), A the area (3.14 cm^2), D the diffusion coefficient (cm^2/s), C the concentration (g/cm^3), l the membrane thickness (0.5 cm) and t the time (seconds). The intercept of the linear part of the curve obtained by plotting Q vs. time is the so called "lag time", t_s, Solving the equation for $t=t_s$ (the corresponding $Qt_s=0$), allows the calculation of D. At least three independent assays were performed for each experiment.

Results and Discussion

Dextrin characterization

The dextrin used to produce the hydrogel was only composed by glucose. Methylation analysis showed the presence of liner (85.3%), non-reducing terminal (11.2%) and branched (3.5%) residues (Table 1). The proportion of terminal residues was 3 times higher than the proportion of branched residues. In a large molecular weight amylopectin molecule, the amount of terminal residues tends to be similar to the amount of branched residues. However, the shorter the molecule the higher the proportion of terminal residues in relation to the branched ones. In this sample, by the proportion of 3:1 it can be inferred the presence of linear structures (1 terminal and no branching residues) and structures with one branching point (2 terminals to 1 branching residue) in similar amounts (Table 1 and Figure 3). By the difference between the abundance of the estimated terminal residues and those corresponding to the abundance of the branched residues, the amount of terminal residues in the main backbone can be estimated as 7.7%. Taking this into account, the average degree of polymerization of the dextrins can be estimated by the sum of the abundance of all residues (100%) divided by the abundance of terminal residues in the main backbone (7.7%), that is, the dextrin had an average degree of polymerization of 13 glucose residues where one in each two molecules is a branched structure (Figure 3).

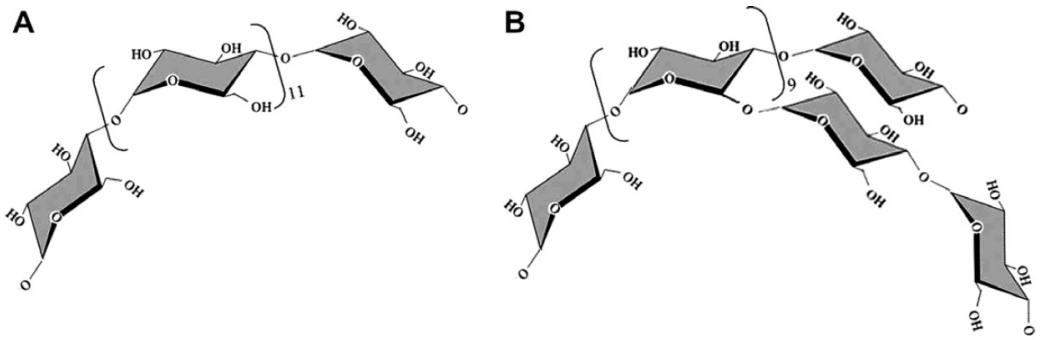


Figure 3. Structure representative of the dextrin molecules used to form dextrin-VA hydrogels. A. Linear structure. B. Branched structure.

Table 1. Glycosidic-linkage analysis of the dextrin used to prepare the hydrogel.

Linkage	**Relative abundance**
Terminal-Glc*p*	11.2
(1→4)-Glc*p*	85.3
(1→4,6)-Glc*p*	3.5
Average degree of polymerization	13
% Branching points	3.5

Diffusivity experiments

The release of an active agent from a polymeric network consists on the diffusion of the agent through the bulk of the polymer (Amsden 1998, Amsden 1999). Depending on the hydrogel network reticulation, the effective release of entrapped material may be controlled by the erosion/degradation of the polymer or by the mass transfer properties (Amsden 1998, Kanjickal and Lopina. 2004). To optimize the release characteristics of dextrin-VA hydrogels, a fundamental understanding of the diffusivity mechanism of biomacromolecules through the matrix is required. The diffusion coefficient is affected by the molecular size of the solute and the characteristics of the polymer network, such as the crosslinking density, capable of affecting diffusivity to a large extent.

Figure 4 shows the different diffusion behavior of BSA (67 kDa) and glucose. After a lag-phase, the total amount of solute transferred through the hydrogel is proportional to the time. The diffusivity coefficients were determined using hydrogels with different DS, ranging from 20 to 70% (Table 2). The diffusion coefficient of BSA on the DS 20 hydrogel was $4.2 \pm 1.6 \times 10^{-7}$ cm^2/s. This value is only slightly lower than the one obtained by Lévesque et al. (2005), for BSA diffusivity in water (5.9×10^{-7} cm^2/s) and in the order of magnitude of the value obtained with Dex-MA hydrogels ($3.1 \pm 0.2 \times 10^{-7}$ cm^2/s). The diffusivity in water and in the hydrogel is thus in the same range, suggesting that these matrices probably have continuous water and polymer phases with interconnected pores. In fact, SEM analysis of three-dimensional hydrogel slabs revealed a highly porous structure (Figure 5).

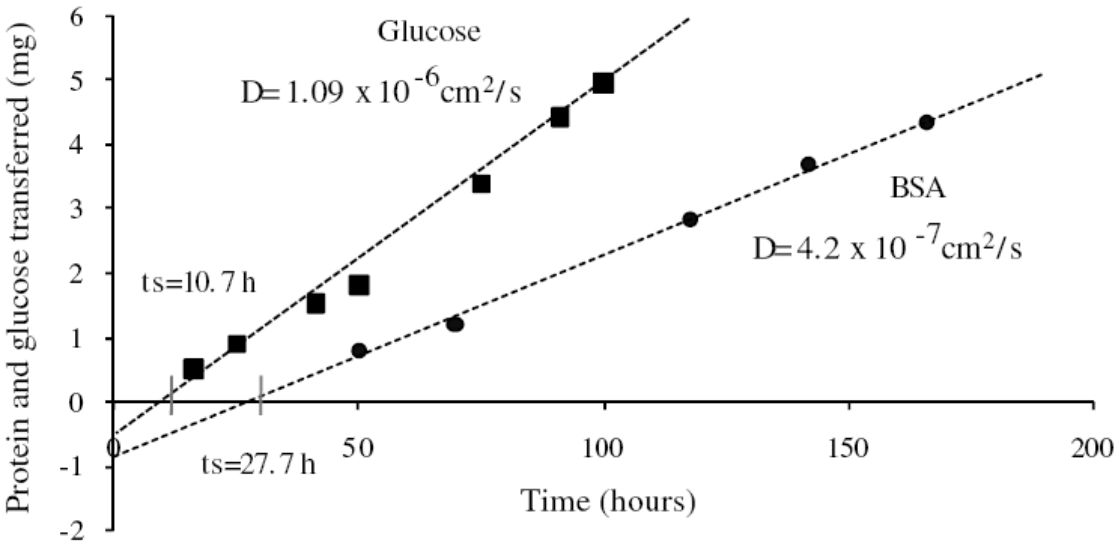


Figure 4. Protein and glucose transferred through the membrane (DS=20%), *vs* time, in the diffusivity experiments.

Table 2. Diffusivity coefficients of BSA and glucose for different hydrogel DS and hydrogel mass loss values after 20 days.

Hydrogel DS	Diffusivity Coefficient (cm^2/s)		Hydrogel Mass Loss (after 20 days (%))
	BSA	Glucose	
20	$4.2 \pm 1.6 \times 10^{-7}$	$1.1 \pm 0.7 \times 10^{-6}$	18.3
40	$1.5 \pm 1.2 \times 10^{-7}$	$8.7 \pm 1.1 \times 10^{-7}$	15.8
70	*	ND	12.1

ND- Non determined

*- No diffusion of BSA was detected after 80 days, implying a reduction of the diffusion coefficient by at least one order of magnitude.

Hydrogels with lower DS (10 and 20%) exhibit irregular pores, in the size range 20-70 μm. However, we were able to alter the pore morphology by raising the DS up to ca. 70%, thereby obtaining a material with much lower porosity. The diffusion coefficient obtained with a DS 40 hydrogel was $1.5 \pm 1.2 \times 10^{-7}$ cm^2/s. As expected, increasing the DS reduces the mobility of the biomolecules through the matrix. For a DS 70, a smooth surface is observed by SEM (Figure 5) and large pores, and consequently interconnectivity, are no longer observed. As a matter of fact, a diffusion assay with a gel with DS 70, revealed no diffusion of BSA through the gel after approximately 80 days, implying a reduction of the diffusion coefficient by at least one order of magnitude. The ability to change the DS, thereby altering the crosslinking density and the whole structure of the network, allows modulating the diffusivity across the networks.

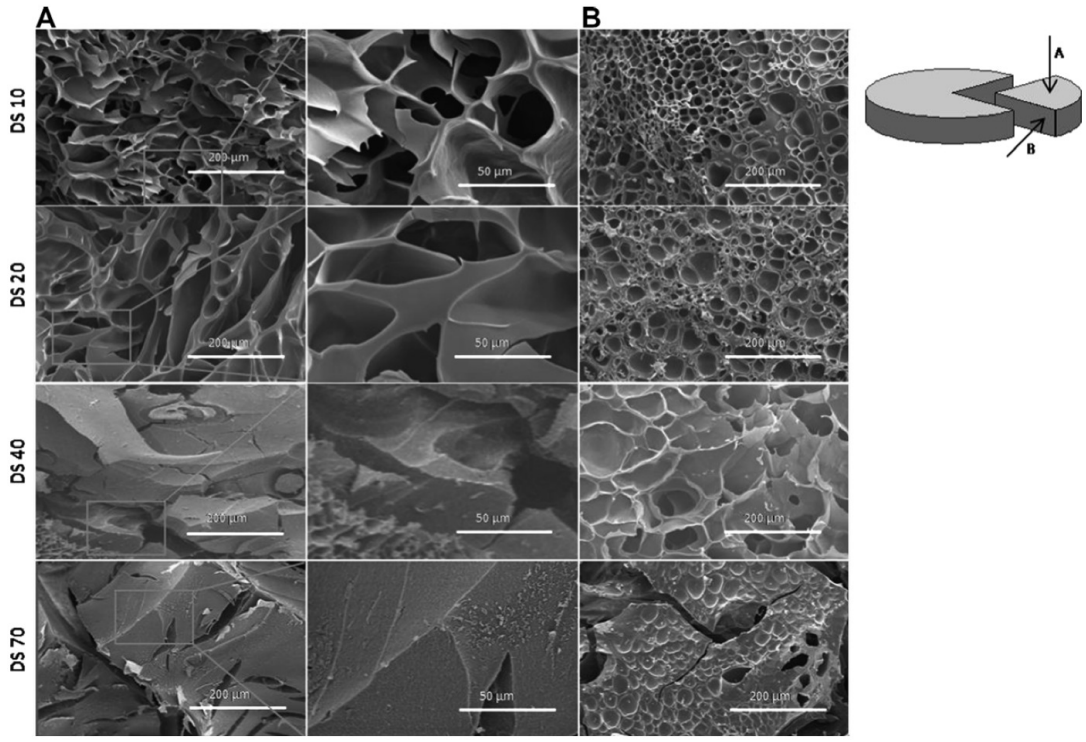


Figure 5. SEM micrographs of hydrogel inner (A) and outer (B) surfaces with different DS, ranging from 10 to70%.

Finally, diffusion coefficients of $1.09 \pm 0.7 \times 10^{-6}$ cm^2/s and $8.7 \pm 1.1 \times 10^{-7}$ cm^2/s were obtained for glucose, using hydrogels with DS of 20 and 40 %, respectively. These values are comparable to the one obtained by Ramos et al. (2006) for the diffusion of glucose in chitosan-dextrin-VA hydrogels and, as expected, higher than those obtained for BSA.

Release experiments

For most biomedical applications, namely in drug delivery, hydrogels that degrade in clinically relevant time-scales are favored over non-degradable ones. In addition to spontaneously degradable hydrogels, enzymatic degradation is also an interesting solution to obtain optimal degradation profiles, which result in proper rates of drug release (Mason et al. 2001, Okano and Kikchi 2002, Rice et al. 2006).

The release of BSA from dextrin-VA hydrogels was studied. The enzyme AMG was added to the dextrin-VA solutions before gelation, to analyze the effect of the enzymatic hydrolysis on the model protein release. Figure 6 illustrates the cumulative protein release in the different assays.

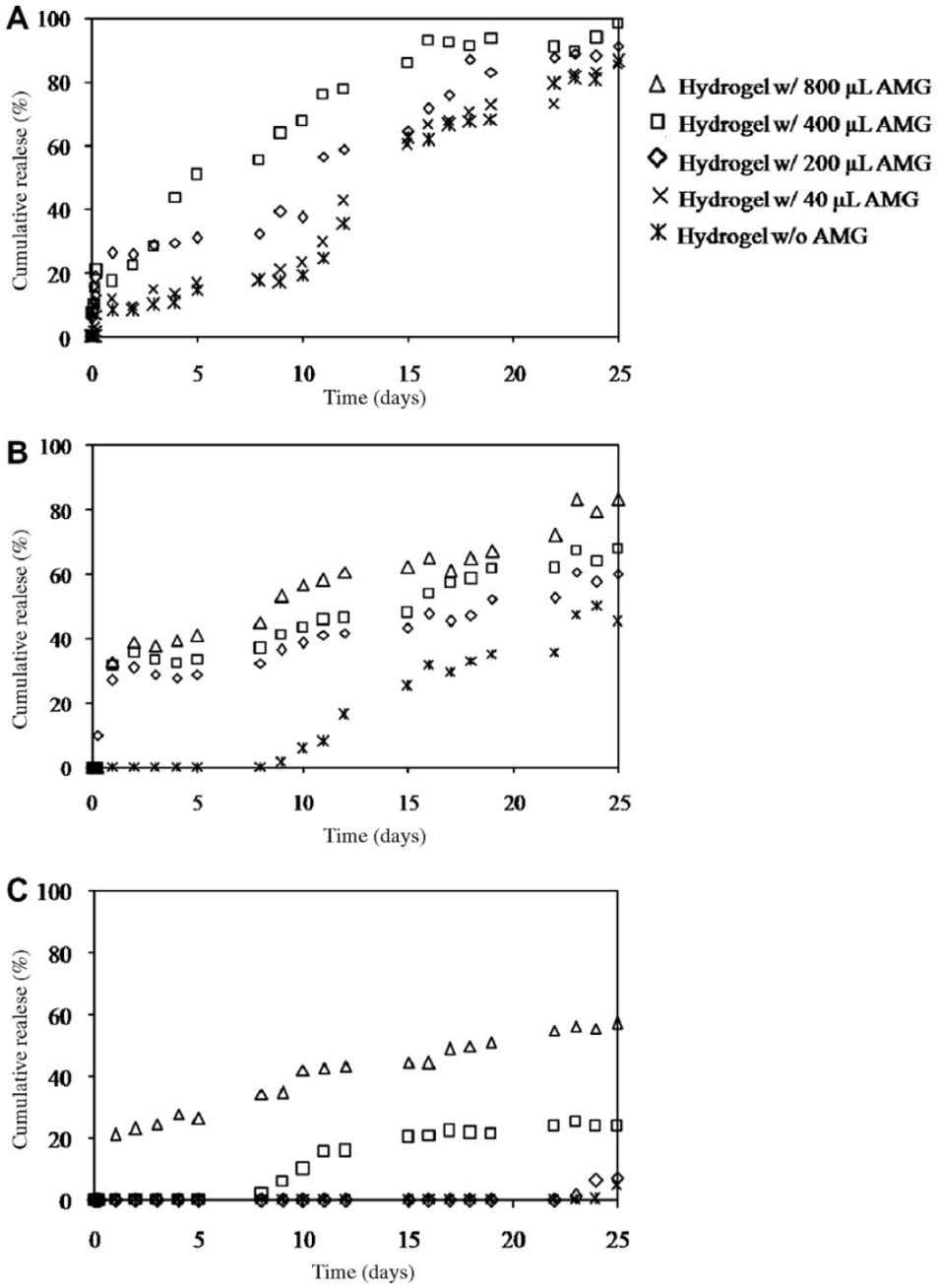


Figure 6. Cumulative release of BSA (2 g/L) from dextrin-VA hydrogels, DS 20 (A), 40 (B) and 70 (C). The data points are averages of at least three repeats that deviated less than 5% of the total amount of protein in the hydrogels.

For the DS 20 hydrogel (Figure 6A), a two stage release of protein is observed in all hydrogel samples, the rate slowing down through the time course of the experiment. In the initial incubation period (from 0 to 6 h), only a small amount of BSA was released from all samples, corresponding to between 10 and 25% of the total amount of protein in the hydrogels. After approximately 20 days, most of the protein was effectively released from the gel, in all assays.

As it is well known, diffusion might not be the only factor determining the delivery rate, degradation playing also a critical role in determining overall release profiles. Degradation of crosslinks increases the mesh size of the gel, allowing for diffusion to be facilitated. Thus, the release of a molecule entrapped in a hydrogel network is controlled by (1) the diffusion through the matrix or (2) by hydrolysis of the network crosslinks, followed by an erosion process with mass loss (Dijk-Wolthuis et al. 1997, Amsden 1999). While either diffusion or dissolution can be the predominant factor for a specific type of polymer, in most cases, the release kinetics is a result of a combination of these two mechanisms (Sujja-areevath et al. 1998, Lee 1981, Tahara et al. 1995, Efentakis and Buckton 2002).

As can be seen in Table 2, after 20 days of incubation, for DS 20, only approximately 20% of the hydrogel had undergone solubilization, according to dry weight measurements. Such mass loss reflects the release of non-reticulated material, since no significant degradation take place in the absence of the enzyme. As a matter of fact, the incubation of the hydrogel in water for up to 3 months did not lead to further mass release. Diffusion, rather than the enzymatic degradation, is the governing factor for the release in these hydrogels (DS 20). Indeed, all of the protein is released even in the case where no AMG is used, which again shows that the protein is able to freely diffuse in the hydrogel network. The presence of increasing amounts of enzyme leads to higher BSA release rate, an effect that may be classified as moderate. As a matter of fact, as shown in the previous section, the protein diffusion in this hydrogel compares with the diffusion in water, hence the moderate effect observed in the presence of AMG.

As can also be seen in figure 6, for higher DS values the total amount released from degrading hydrogels (i.e., with AMG) was always much larger compared with the control (without enzyme), a process that seems to occur in an enzyme-concentration dependent manner. These results reveal that the presence of the enzyme, by hastening the degradation of the hydrogels, significantly affect the release rate (Figure 6, B and C). As can be seen, namely for the DS 70 hydrogel sample, in the absence of the enzyme, almost no release is detected, showing that, contrarily to the DS 20 gel, in which the release is mostly diffusional, for hydrogels with a rather high DS, the release behavior is driven by the enzyme concentration. In these cases, the higher crosslinking density prevents the free diffusion of the molecules. The network mesh is tighter, with subsequent reduced mobility. Likewise, the enzymatic cleavage of polymer chains raises the degradation process, reducing the time scale of the slow natural bulk erosion, being thereby responsible for the control of the release rate.

These results show that the control of DS and of enzyme concentration allow for the establishment of release systems, controlled over different ranges of time, from days (low DS, higher enzyme) to months (higher DS, no enzyme). As reported by Meyvis et al. (2001) with dextran-MA hydrogels, more recently by Vlugt-Wensink et al. (2006) and confirmed with our diffusivity results, an increase in DS of the hydrogel, thereby increasing the crosslinking density is an expeditious method to lengthen the duration of protein delivery. Taken together, all these variables can provide a versatile method of fine tuning the protein release rate.

Conclusions

The BSA release from the dextrin-VA hydrogel with DS 20 is mainly controlled by the diffusion rate. For hydrogels with higher DS, the enzymatic hydrolysis/erosion governs the release rate. By using hydrogels with different DS and enzyme

concentration, it is possible to control the release rate from days to weeks, or even months.

Acknowledgements

This research was supported by Fundação para a Ciência e a Tecnologia under grant SFRH / BD / 17482 / 2004 and FUNCARB POCTI/BIO/45356/2002.

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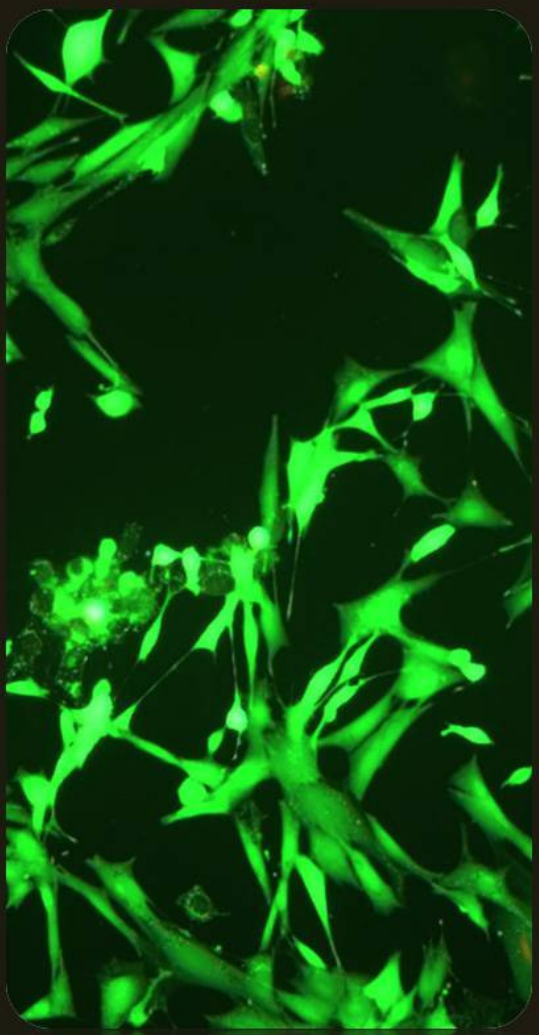
CHAPTER 4

In vitro biocompatibility assessment of dextrin-based hydrogels

Summary

The biocompatibility of dextrin-based hydrogels, obtained from dextrin-VA (vinyl acrylate) by radical polymerization, has been evaluated *in vitro*, using mouse embryo fibroblasts. *In vitro* tests were performed in accordance with the guidelines from ISO10993: 1) The toxicity of the extractable materials was evaluated; 2) Direct contact assays allowed an estimation of hydrogel biocompatibility; 3) The adhesion, proliferation and morphology of the cells on the hydrogel were also studied. The extracts obtained from the dextrin hydrogel, only slightly reduced the proliferation of fibroblasts (~15%). Direct seeding of the cells onto the hydrogels surfaces resulted in a reduction in the proliferation rate, as compared to tissue culture polystyrene plate. However, the results show that, although with a delay, cells are effectively able to grow, indicating that no deleterious effects are produced by dextrin hydrogels. The cells preferably do not spread on the gel surface, growing in large aggregates and exhibiting a round morphology. The biocompatibility of the hydrogels made of different polysaccharides have been described – dextran, starch and inulin, among others – but this is to our knowledge the first study on the biocompatibility of a dextrin hydrogel, with promising applications in the biomedical field.



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J Biomed Mat Res A | 2008 | Submitted

Introduction

Hydrogels are three-dimensional networks composed of hydrophilic polymers, capable of absorbing substantial amounts of water, thus resembling natural living tissues. Their specific chemical and physical properties make hydrogels potentially suitable for a wide variety of biomedical purposes, including contact lenses, membranes, biosensors and bioactive scaffolds for tissue engineering (e.g. linings for artificial hearts or material for artificial skin) (Sgouras and Duncan 1990, Peppas 1996, Kane et al. 1996, McCulloch and Shalaby 1998, Peppas et al. 2000, Ishihara et al. 2002, Chen et al. 2003, Drury and Mooney 2003). Hydrogels can also be applied as implantable delivery systems and as protein- and cell-resistant coatings (Levy and Andry 1991, Schacht et al. 1993, Di Silvio et al. 1994, Draye et al. 1998).

The biocompatibility of the hydrogels is an essential issue regarding their pharmaceutical and biomedical applicability. A material is considered biocompatible when it does not damage adjacent cells, does not lead to significant tissue scarring, nor originates a side response that decreases its function (Hansen et al. 1989, Behl et al. 1994, Berridge et al. 1996). In general, hydrogels have a good biocompatibility. Since their hydrophilic surface has a high interfacial free energy, proteins and cells from body fluids have a low tendency to adhere (Peppas 1996, Hern and Hubble 1998, Hoffman 2002). Moreover, the soft and rubbery consistence of hydrogel matrixes minimizes irritation of the surrounding tissues.

Recently, we have reported the production of a new dextrin hydrogel (Carvalho et al. 2007). Dextrin polymers with a varying number of functional groups (VA) attached to the macromolecular structure (degree of acrylate substitution, DS), may be produced. These polymers reticulate by radical polymerization, hence allowing the control of network properties like the swelling ratio, erosion and mass loss profiles. Herein, we report the results of in vitro cytotoxicity tests performed with dextrin-VA hydrogels and its extracts, through direct and indirect contact assays, following the guidelines from ISO10993. Additionally, observations using both

fluorescence and phase-contrast inverted light microscopy were also carried out as indicators for cell viability and morphology.

Materials and Methods

Materials

Dextrin was a generous gift from Tate & Lyle. Dimethylsulfoxide, *N*,*N*,*N*',*N*'-tetramethylenediamine (TEMED), and ammonium persulfate (APS) were purchased from Aldrich (Milwauke, WI). Dextrin acrylates (dextrin-VA) with different DS were synthesized as described previously (Carvalho et al. 2007). Dulbecco's modified Eagle medium (DMEM) with 4500 mg glucose/L, L-glutamine, 110 mg sodium piruvate/L and $NaHCO_3$ (Sigma) was supplemented with 10% calf bovine serum (CBS) (Gibco, UK) and 1% of a Penicillin/Streptomycin solution (Sigma). This medium is further referred to as DMEM complete medium. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was obtained from Sigma and MTS CellTiter 96 was from Promega. Transwell plates (96 and 24-wells) were purchased from Orange Scientific (Belgium). For the fluorescence microscopy assays, the LIVE/DEAD ® Viability/Cytotoxicity Kit for mammalian cells, from Molecular Probes (USA), was used. Positive and negative controls, latex rubber and high-density polyethylene were from U.S. Pharmacopeia. All other chemicals and solvents used in this work were of the highest purity commercially available.

Cell culture

Mouse embryo fibroblasts 3T3 (ATCC CCL-164) were grown in DMEM complete medium, at 37°C, in a fully humidified air containing 5% CO_2 (IR auto Flow). The cells were fed every 2–3 days. When cells reached confluence, the culture medium was discarded and the cells were detached with 2 mL of 0.25% (w/v) trypsin-EDTA [1:250, from porcine pancreas (Sigma)] solution for 15 min at 37°C, and 6

mL of DMEM complete medium was added to inactivate the trypsin after cell detachment. The cells were then centrifuged (10 min, 1000 rpm) and resuspended in culture medium before use.

Solutions of dextrin, dextrin-VA, TEMED, and APS

All solutions were prepared on the day of application. Solutions of 30-40% of dextrin or dextrin-VA (DS 10, 15, 20, 40 or 70%) per millilitre of DMEM complete medium and solutions of 2.5, 10, 50, and 100 μL of APS or TEMED per millilitre of DMEM complete medium were sterilized through a 0.22-μm filter (Orange Scientific, Belgium), with the exception of 400 mg/mL dextrin-VA solutions for which a 0.45-μm filter (Orange Scientific) was used because of the higher viscosity. Before cell seeding, hydrogels obtained after polymerization were rinsed with PBS (3x) and then washed twice with DMEM complete medium.

Mitochondrial metabolic activity assay

MTT solution (0.45 mg/mL in DMEM complete medium without phenol red) in an amount equal to 10% of the culture volume was added to each well. After 3 h of incubation at 37°C, the MTT solution was removed and the insoluble formazan crystals formed in the bottom of the wells were dissolved in a dimethylsulfoxide volume equal to the original culture volume (Mosmann 1983). The absorbance was measured at 570 nm using a plate reader (SynergyHT, BioTek). MTS test was additionally carried out. In this method, the absorbance of the formazan at 490 nm can be measured directly from the assay plates without additional processing.

Direct contact assay

A fibroblast suspension (3 mL) containing $3x10^4$ cells/mL was plated into each well of a six-well plate, to yield a final density of 10^4 cells/cm^2. After reaching a state of sub-confluence (after 24 hours), hydrogel discs (Ø 4 mm, 2 mm thickness)

were placed on the wells, in direct contact with cells. After 24 h of incubation, the cell morphology and viability was assessed with the Live/Dead fluorescence labelling. Positive and negative controls (discs of latex and agar gel) were also used.

Indirect contact/ Extraction assay

To evaluate the cellular proliferation inhibition index (CPII) for solutions of dextrin, dextrin-VA, TEMED, and APS, 100 µL of a fibroblast suspension containing 3 x 10^4 cells/mL was plated into each well of a 96-well plate, together with the test solutions. Because each well has a surface area of 0.31cm^2 the final seeding density was about 10^4 cells/cm^2. The medium was refreshed every day with the tests solution. The metabolic activity of the cells was measured with the MTT test, 72 hours after the addition of test solutions. The CPII was calculated using Equation (1):

$$CPII = 100 - \left(\frac{DO_{540} \text{ of test culture}}{DO_{540} \text{ of control culture}} \times 100 \right) \quad (1)$$

To assess whether leachable compounds can affect cell viability, mouse embryo fibroblasts were exposed to extracts obtained from hydrogels prepared with different DS (10 to 70%) values and initial water contents (70 and 75%). The CPII of the culture in contact to extracts (indirect contact) was also assessed. In this case, after polymerization, the hydrogels were immersed in DMEM supplemented with 1% Penicillin – Streptomycin solution (at room temperature for 6 days) and then autoclaved. Afterwards, 10% CBS solution was added to the extracts that were then filtered and incubated for 72 hours with the cultures.

Adhesion assay

The cell adhesion in dextrin-VA hydrogels was evaluated. Filtered (0.45 µm) dextrin-VA solutions (50 µL) were placed into each well of a 96-well plate.

Polymerization started by the addition of filtered APS (5 μL) and TEMED (5 μL) solutions, under sterile conditions. After 2 h of polymerization, the hydrogels formed at the bottom of the wells were washed, first with PBS and secondly with DMEM complete medium (3x200 μL); then, 100 μL of a fibroblast suspension containing $3x10^4$ cells/mL was plated into each well, to yield a final density of 10^4 cells/cm^2. The culture medium was refreshed (100 μL) every two days. For the control assays, cells were grown directly in the bottom of the wells. After 72 hours of incubation, the hydrogels were washed with PBS (3x100 μL) to remove floating cells, and the cell layer was trypsinized and counted using a cell counting chamber (Neubauer, Germany). Additionally, the cell proliferation was estimated using the mitochondrial metabolic activity of the cells grown onto the hydrogel surface, in a parallel experiment. Two methods were used, MTT and MTS, (applied directly on the cell layers grown on top of the hydrogel). The discrepancy between the results obtained, led us to addopt a slightly changed protocol: the cells were detached from the hydrogel surface, before conducting both the colorimetric and counting assays, an approach that resulted in more accurate and reliable results.

Microscopy assays

At regular time intervals, cell growth and morphology were evaluated by observation with a phase-contrast inverted light microscope. Cytotoxic effects were ranked in cellular death, cell adhesion ability and induction of morphological changes. Additionally, cells were labelled with LIVE/DEAD Viability/Cytotoxicity Kit and observed by fluorescence microscopy.

Results and Discussion

In order to evaluate the cytotoxicity of dextrin, acrylate derivatized dextrin, dextrin hydrogels and their leachable products, we have studied the effects of these

materials in a mouse embryo fibroblast culture, namely in the cell proliferation and adhesion on the hydrogel, morphological features and cell death.

Extraction Assay

As previously described, upon exposure to aqueous solutions, hydrogels can release leachable products and, therefore, the cytotoxicity of hydrogel extracts must be evaluated. For that purpose, fibroblast cultures were exposed to extracts of hydrogels, prepared using dextrin-VA with different DS and initial water contents. The cell viability was quantified using the MTS assay. The results are shown in Table 1. The extracts induced a reduction inferior to 17% in the mitochondrial metabolic activity of fibroblasts, as compared with the control. No significant differences were found in hydrogels with different DS. The initial water content, 70 and 75%, (corresponding to initial monomer concentrations of 44 and 33%, respectively) of the hydrogel network also does not produce major differences in the CPII.

Table 1. Cell proliferation inhibition index of mouse embryo fibroblasts, cultured in the presence of extracts obtained from dextrin-VA hydrogels with different DS and initial water content.

DS (%)[a]	**CPII (%)[c]** **Initial Water Content (%)[b]**	
	70	**75**
10	12.4 ± 2.3	14.8 ± 3.1
15	14.2 ± 4.5	11.9 ± 1.7
20	15.4 ± 1.6	13.1 ± 1.9
40	14.8 ± 2.1	15.5 ± 1.2
70	17.3 ± 2.7	14.9 ± 4.2

[a] Degree of substitution defined as the amount of acrylate groups per 100 glucopyranose residues, determined by liquid NMR Spectroscopy

[b] Water content of hydrogels with different initial monomer concentration

[c] Cell proliferation inhibition index assessed by MTT measurements ± Standard Deviation

Additionally, the cytotoxicity of dextrin-VA hydrogels was also assessed using a direct contact method. For that purpose, we used a hydrogel disc and a positive and negative control, latex rubber and agar, respectively. Each sample was placed on top of a sub-confluent cellular layer on a six-well plate, as described in the material and methods section. Figure 1 shows the results obtained with the fluorescence labelling of the cell layers.

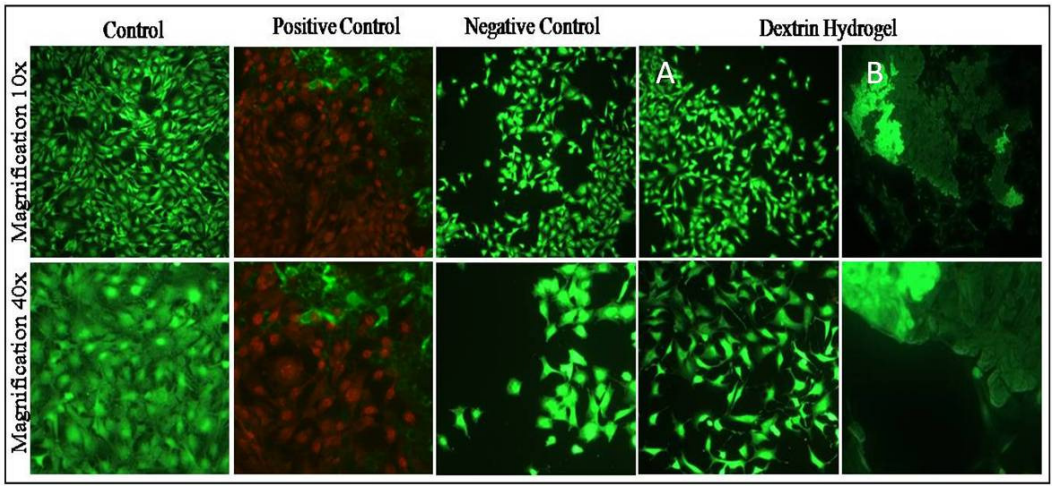

Figure 1. Fluorescence labelling of mouse embryo fibroblasts. Micrographs show the control culture, the cell layers under the positive (latex rubber) and negative (agar) controls, and the cell layers under (B) and around (A) the DS 20 hydrogel, after 24 hours of incubation (magnification 10 and 40x).

As can be seen, the latex rubber is, as expected, highly cytotoxic. Some cells were floating in the medium and the ones that remained attached are dead as well (red labbeled). Differences in the morphology were found in the cells underneath the hydrogels. These cells appeared clustered, when compared to the control cultures,

but all of them remained alive, as shown by the green fluorescence. The cell proliferation under the dextrin hydrogel was lower than the one observed in the control plate. Massia and Stark (2001), and more recently Ferreira and colleagues (2004) using human skin fibroblasts, have already reported this effect which is related to mechanical compression of cells or poor oxygenation and nutrient diffusion, due to the physical presence of the network. The cells in contact with the negative control (agar) and the dextrin hydrogel exhibit similar proliferation and morphology, thus, the hydrogels can be considered non-toxic.

Since the toxicity of the crosslinked hydrogel extracts can be caused by the leakage of any of hydrogel components, we determined the toxicity induced by individual hydrogel components. For this purpose, solutions of dextrin, acrylate derivatized dextrin (dextrin-VA) monomers with different DS values (10, 15 and 20%), APS, and TEMED were incubated with fibroblast cultures for 72 h, and the cellular viability was assessed by the MTT assay (Figure 2). As can be seen, the initiators APS and TEMED affected the metabolic activity in a concentration dependent manner, when added to the cells in the concentration range 2.5–100 µg/mL. Cytotoxicity of these compounds increase with the concentrations. The lower concentration of both APS and TEMED (2.5 µg/ml) did hardly exert any cytotoxicity (Figure 2). In contrast, at 100 µg/ml, the CPII for APS and TEMED was greater than 95%, indicating, as expected, highly cytotoxic effects, which is likely due to the reaction of the free radicals with the biological molecules (Figure 3A). However, the toxicity of these molecules is considered irrelevant, since they are consumed in the polymerization reaction, and on the other hand, due to their low molecular weight, are easily extracted from the hydrogel.

Under the same conditions, dextrin monomers revealed only moderate cytotoxic effects (Figure 2). The cytotoxicity of dextrin was tested in two relevant concentrations, 300 and 400 mg/ml, which were the ones used to produce hydrogels with different initial water contents (75 and 70%, respectively). In both cases, cellular metabolic activity did not significantly change in comparison with control cells (CPII < 20%). CPII values ranging from 18 to 35% (depending on the concentration) were previously reported by Ferreira et al. (2004) for dextran with a

molecular weight of 70 kDa using human skin fibroblasts and a similar cell culture assay.

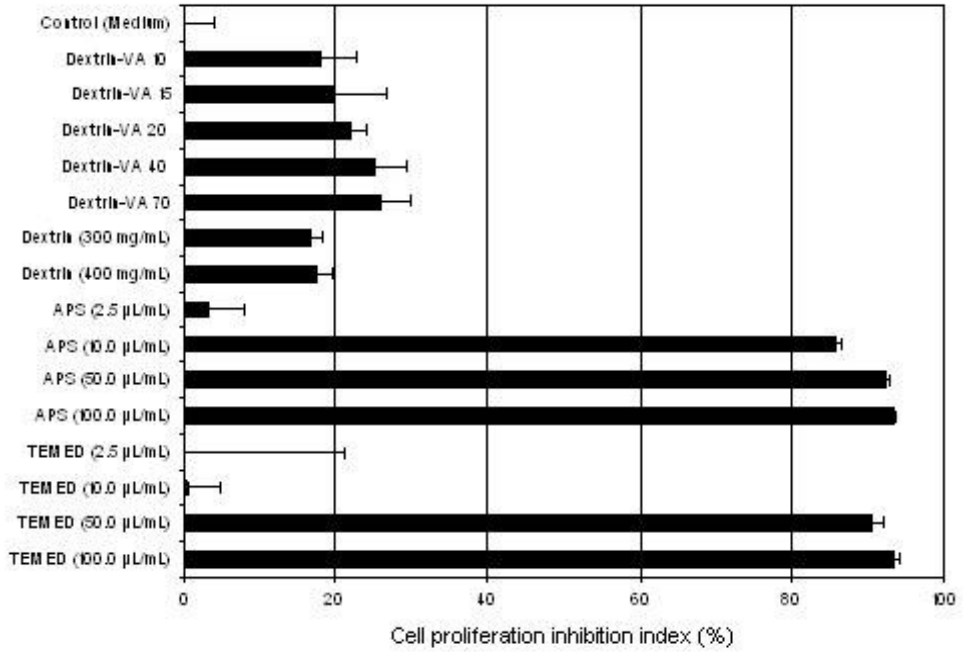

Figure 2. Cell proliferation inhibition index (mean% ± SD) after 72 h for the polymers dextrin, acrylated dextrin (dextrin-VA), and for the initiators used in the polymerization reaction APS and TEMED. Data are from a representative experiment, performed in triplicate.

Furthermore, incubated with the acrylate derivatized dextrin, the fibroblasts showed a loss in viability of about 18–23%, depending on the DS of the material. A more substituted dextrin-VA sample (higher DS value) produced higher inhibition in the cell proliferation. Compared with the results obtained with non-derivatized dextrin, a slightly higher CPII is found, probably related to the presence of vinyl groups. At the end of the culturing period, relevant morphological deviancies were observed. When compared to control cultures, the treated cells shown spider-like structures and intracellular granula were also visible (Figure 3B and C).

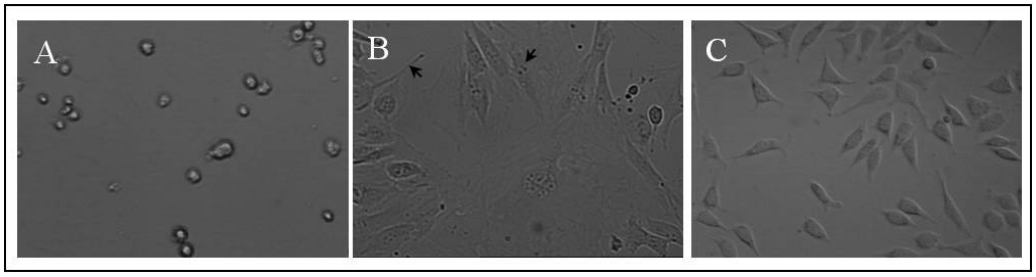

Figure 3. Mouse embryo fibroblasts, cultured for 24 h in the presence of 100 µg/mL of APS (A), 300 mg/mL DS 20 (also representative for the other DS values) (B) and control culture (C). Highly cytotoxic effects of APS are shown in micrograph A, the cells are dead and floating in the medium. When compared to control cultures, dextrin-VA-treated cells contained intracellular granula (see arrows) and have spider-like structures (magnification 10x).

Cell Adhesion and Proliferation on Dextrin Hydrogels

The cell adhesion on the hydrogel surfaces was evaluated using samples with different DS (20 and 70%). The results were expressed as a percentage of the adhesion on cell culture polystyrene plates (TCPP), estimated by cell counting. Additionally, the mitochondrial activity of the adherent cells was also assessed in a parallel experiment. This study showed that the hydrogels are, as expected, less adhesive than TCPP. The resistance of the hydrogel surfaces to cell adhesion is, as described for other polysaccharides such as dextran, presumably due to poor protein adsorption onto the hydrophilic and nonionic polymer (Osterberg et al. 1995, Frazier et al. 2000, De Groot et al. 2001). Indeed, cellular adhesion on hydrophilic materials such as hyaluronic acid and PEG-based hydrogels was reported to be extremely sparse (Mann et al. 2001, Ramamurthi and Vesely 2002). In other cases, as recently described in pullulan hydrogels using smooth muscle cells cell adhesion and moderate growth is observed (Autissier et al. 2007). Viability tests indicated that fibroblasts adhered to some extent (50 to 60%) as compared to the adhesion on TCPP and are capable to grow on dextrin hydrogels. In the first stage of the culture, the cells appear round and then attach to the gel surface. As can be seen in Table 2, after one hour, less than 30% of the cells were attached to the hydrogel surface, irrespective of the DS tested; after 4 hours, more than 50% of the initial cells remained associated to the gels.

Table 2. Mouse embryo fibroblasts adhesion onto dextrin-VA hydrogels with different DS values. (% estimated by cell counting after trypsinization).

DS (%)	**Adhesion (%)**	
	After 1 hour	**After 4 hours**
10	26.6 ± 4.8	57.6 ± 3.7
15	24.9 ± 1.3	55.4 ± 2.9
20	23.4 ± 2.0	56.5 ± 4.4
40	22.8 ± 3.8	59.1 ± 2.7
70	21.3 ± 4.6	57.4 ± 3.7

Since the gel reticulated in the bottom of the wells is slightly opaque, we used fluorescence labelling to facilitate the evaluation of the cell morphology. After 24 hours, small aggregates of round cells are visible (Figure 5). Most of the hydrogels surface is cell-free. After 3 days, larger cell aggregates are visible, and the cells start spreading out from these aggregates. The Live/Dead assay demonstrates the non-toxic character of the material, since no dead cells are observed. We then determined the cell proliferation using two metabolic assays (MTT and MTS).

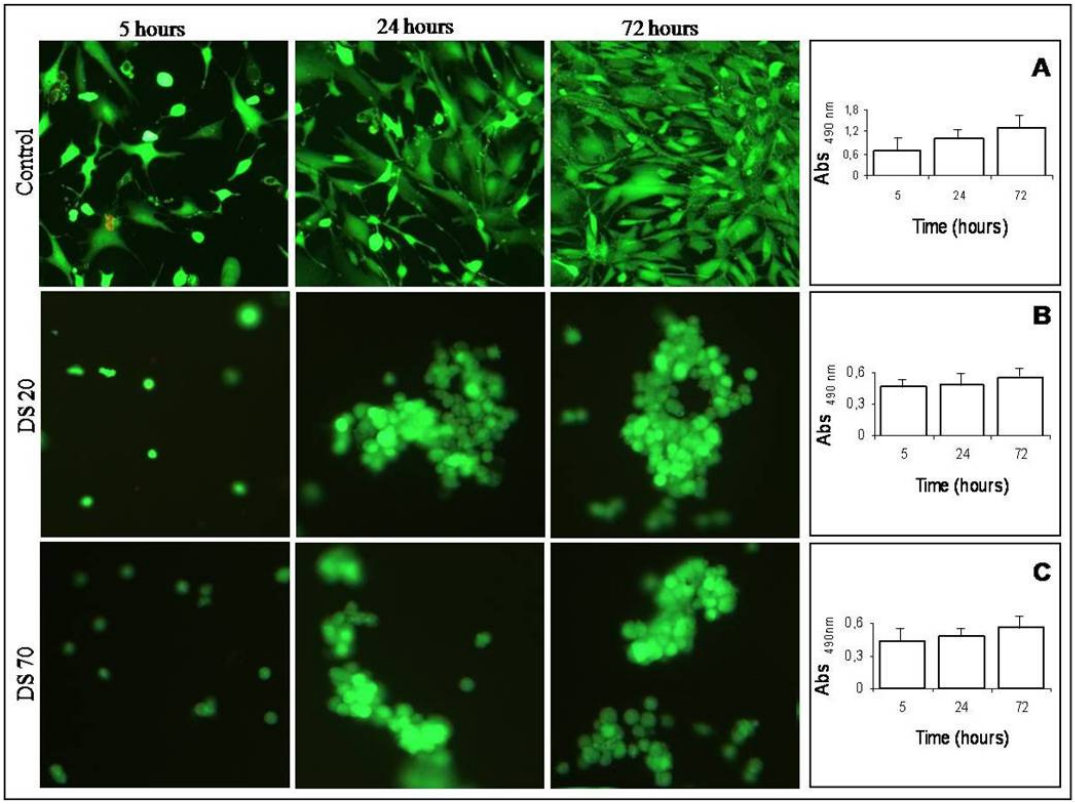

Figure 4. Fluorescence labelling of mouse embryo fibroblasts. Micrographs show control culture and cells seeded on the surface of a DS 20 and DS 70 hydrogels, after 5, 24 and 72 hours of incubation (magnification 10x). A, B and C graphics correspond to the MTS absorbance values at 490 nm through time course of the assay.

Cell viability assays, such as MTT or MTS, are commonly used to measure cell proliferation in biocompatibility studies. Both tests measure the metabolic activity of viable cells. They are based on the reduction of the tetrazolium salt MTT by the enzyme succinate dehydrogenase, which is localised in the mitochondria of viable cells. The reaction produces a water-insoluble formazan salt (in the case of MTT, but not of MTS) which must be solubilized. Discrepancy between the results obtained wih different methods, led us to perform a comparative study. The results are presented in Figure 5. Differences in the CPII value are found using the two methods, MTT and MTS. As previously referred, in these methods, the reagent is added directly to the well. Retention of formazan crystals on the hydrogel was detected. As a matter of fact, at the end of the experiment, even after crystal

dissolution, the gel remains purple-stained. The MTS method seems to be more effective on solubilizing the formazan crystals, leading to lower CPII values. Since, even with MTS, the gels still exhibited a slightly purple stain, a different approach was used. The cells were detached from the hydrogel, collected, and only then the MTS assay was performed. A more accurate and reliable estimation of CPII could thus be obtained. As a matter of fact, several reports in the literature ignore this interference that may be quite significant in the case of hydrogels, as shown in this work. CPII obtained this way is also shown in Figure 5. As can be seen, 72 hours after the cell seeding on the hydrogel surfaces, cell proliferation inhibition reaches about 50% when compared to control cultures. The direct seeding of the cells onto the hydrogels surfaces resulted in a substantial reduction in the proliferation rate. However, as can be seen by the non-normalized absorbance values (Figure 4A, B and C), the results show that although with a delay, cells are effectively able to grow, indicating that no deleterious effects are produced by dextrin hydrogels.

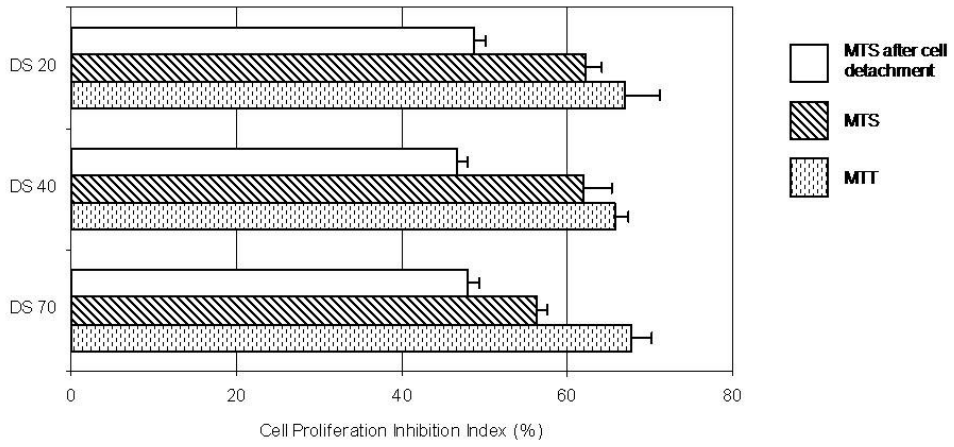

Figure 5. Cell proliferation inhibition index (CPII) (mean% ± SD) after 72 h for different dextrin hydrogel discs. CPII obtained using MTT, MTS and MTS after cell detachment. Data are from two representative experiments, each one performed in triplicate.

Conclusions

Dextrin-based hydrogels constitute a promising biomaterial that may be used for a variety of applications. The results suggest that the dextrin hydrogel have negligible cell toxicity, allowing cell adhesion and proliferation. Additionally, the modification of the surface of the hydrogels with RGD peptides, using recombinant starch binding modules (SBM) fused to the bioactive peptides, was proved to significantly improve the cell adhesion and surface spreading (ongoing work). This is a significant advantage on using a starch derived hydrogel (in this case, dextrin), since the availability of SBMs allows a wide variety of bioactive peptides to be driven to the hydrogels surfaces. Furthermore, the interaction of this hydrogel with different cell lines and living tissues – subcutaneous implants – is currently being analysed.

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CHAPTER 5

Degradation kinetics and rheological properties of new class of degradable dextrin-based hydrogels

Summary

A new class of degradable dextrin-based hydrogels (dextrin-HEMA) was obtained, by using a method based on the activation of the hydroxyl groups of HEMA with CDI, followed by their coupling with the polymer, yielding a derivatized material that can be polymerized in aqueous solution to form hydrogels. Non-degradable dextrin-VA hydrogels have been produced in previous work, having desirable biocompatibility characteristics, but failing in the degradability requirement of the controlled release. A comparative study of the stability of the dextrin-HEMA hydrogels and dextrin-VA ones revealed that the firsts are effectively hydrolyzed under physiological conditions. A severe mass loss process occurred in dextrin-HEMA gels over the time, until a complete dissolution is accomplished. Moreover, the rheological analysis revealed that dextrin-HEMA hydrogels have lower storage module (G') values, compared with the dextrin-VA ones, indicating that physical structuring is less pronounced when dextrin is modified with methacrylate side groups. The results evidenced that the dextrin functionalization with HEMA, may be an effective route to modulate the hydrogel characteristics, namely the degradation kinetics, tailoring the release profiles to fit the purpose of the controlled delivery.

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2008

Introduction

Hydrogels are a class of three-dimensional, hydrophilic polymeric networks that can absorb substantial amounts of water. In general, hydrogels have a good biocompatibility and are capable of resembling natural tissues, allowing for a favourable controlled interaction with living systems (Vyavahare and Kohn 1994). For this reason, these materials have found widespread application in the biomedical, biotechnological, and pharmaceutical fields. Hydrogels can be used in tissue engineering, as scaffolds to support and promote tissue regeneration (Peppas 1996, Kane et al. 1996, McCulloch et al. 1998, Peppas et al. 2000). In addition, hydrogels are gaining increasing attention as attractive systems for the controlled release of pharmaceutically active molecules (Okay and Gurun 1992, Walther et al. 1995). An important class of hydrogels is obtained by the polymerization of acrylate esters (e.g. vinyl acrylate, methacrylate, hydroxyethyl methacrylate, HEMA) through the polymerization of the water-soluble functionalized polymers (Van Dijk-Wolthuis et al. 1995, Hennink et al. 1996, Van Dijk-Wolthuis et al. 1997a, Ferreira et al. 2002 (a), Ferreira et al. 2002 (b)). In previous work, we have described a new dextrin based hydrogel in which the polysaccharide functionalized with reactive double bonds was cross-linked by free radical polymerization in aqueous solution (Carvalho et al. 2007). In these polymeric networks, ester bonds are present in the cross-links and may undergo hydrolytic cleavage, which can ultimately affect both the diffusion and the release profiles (Peppas et al. 2000). Recently, we reported on the diffusion and release of two different molecules from dextrin hydrogels (Carvalho et al. 2008). Further investigation has shown that the hydrolysis of the ester groups is very slow under physiological conditions. However, the hydrogels could be rendered degradable through the incorporation of the enzyme amyloglucosidase, which could prove to be an effective route to modulate the release profiles. An alternative approach is the functionalization of the polymer with a methacrylate ester (HEMA). As shown in several studies, hydrogels obtained by polymerization of this compound, have desirable degradation kinetics (Van Dijk-Wolthuis et al. 1997b, c, d, Stubbe et al. 2002).

In this study we report on the production of dextrin-HEMA hydrogels and perform a comparative study of the stability of the two different acrylated dextrins (dextrin-vinylacrylate (VA) and dextrin-HEMA). It is reasonable to expect that distinct mechanical properties resulted from the functionalization with the two ester functions. In this context, a rheologic analysis allowed exploring the time evolution of the viscoelastic behavior of the hydrogels, providing a qualitative characterization of the crosslinking processes, as well as the structural changes occurring in the network during degradation. Moreover, as the erosion process can play an important role in determining hydrogel properties such as the release profiles, a mass loss evaluation was also performed.

Materials and Methods

Materials

Dextrin - Koldex 60 starch was a generous gift from Tate & Lyle (Decatur, IL, USA). Vinyl acrylate (VA) was from Aldrich, N,N,N',N'-tetramethylenethylenediamine (TEMED) and ammonium persulfate (APS) were purchased from BioRad, dimethylsulfoxide (DMSO) and acetone were from AppliChem. 2-hydroxyethyl methacrylate (HEMA), hydroquinone monomethyl ether (>98%), 4-(*N,N*-dimethylamino)pyridine (DMAP, 99%), *N,N'*-carbonyldiimidazole (CDI, 98%) and tetrahydrofuran (THF) were from Fluka. Both DMSO and THF were dried with 0.4 nm molecular sieves at least overnight before use. Regenerated cellulose tubular membranes with 3500 molecular weight cutoff (MWCO) were obtained from Membrane Filtration Products (Seguin, USA).

Synthesis of HEMA-imidazolyl carbamate (HEMA-CI)

The synthesis of HEMA-CI was performed as described by Van Dijk-Wolthuis et al. (1997c). CDI (1.62 g, 10 mmol) was dissolved in about 15 ml anhydrous THF in a nitrogen atmosphere and HEMA (1.30 g, 10 mmol) was added. The reaction mixture was stirred for 16 h at ambient temperature. After addition of a small amount of hydroquinone monomethyl ether (60 mg), the solvent was evaporated and the crude product was dissolved in ethyl acetate, extracted with water and dried on anhydrous $MgSO_4$. After filtration, hydroquinone monomethyl ether (60 mg) was added once more and the solvent evaporated.

Synthesis of dextrin-HEMA

The modification of dextrin with HEMA was performed as described by Van Dijk-Wolthuis et al. (1997c) with few modifications. Briefly dextrin (10.0 g) was dissolved in dried DMSO (90 mL) in a nitrogen atmosphere. After dissolution of DMAP (2.0 g), HEMA-CI was added, in the molar ratios of 0.25 and 0.50 of HEMA-CI to glucopyranose residues in dextrin. The solution was stirred at room temperature for 4 days, after which the reaction was stopped by adding 2 ml of concentrated HCl to neutralize DMAP and imidazole. The reaction mixture was dialyzed for 2-3 days against demineralized water at 4°C. The methacrylated dextrin was lyophilized and the final white fluffy product was stored at - 20°C until use. The degree of acrylate substitution (DS) of the methacrylated dextrin was determined by proton nuclear resonance spectroscopy (^{1}H-NMR) in D_2O. Briefly, ^{1}H-NMR spectra were recorded in D_2O (10 mg in 1 mL) and DS was calculated using equation 1:

$$DS\% = \frac{x}{y} \times 100 \quad (1)$$

in which *x,* is the average integral of the protons at the double bond (δ at 5.8 and 6.2 ppm) and *y* is the integral of the anomeric proton (δ around 5.4 ppm).

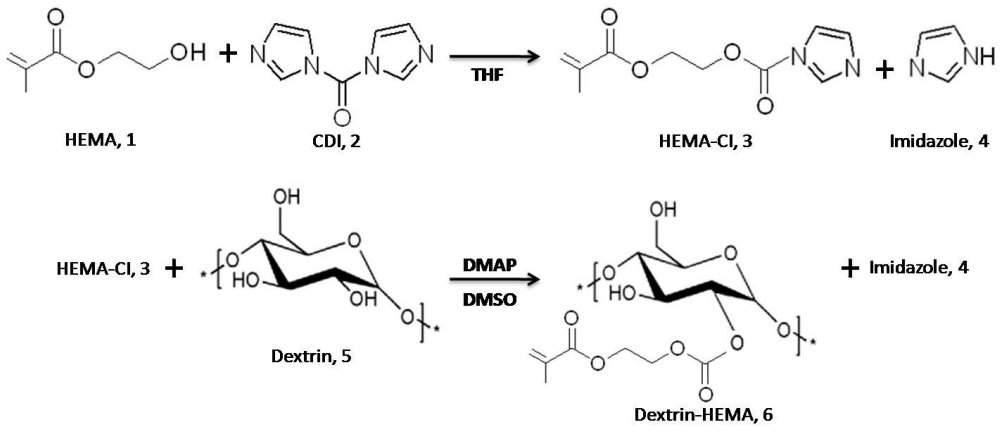

Figure 1. Synthesis of dextrin-HEMA.

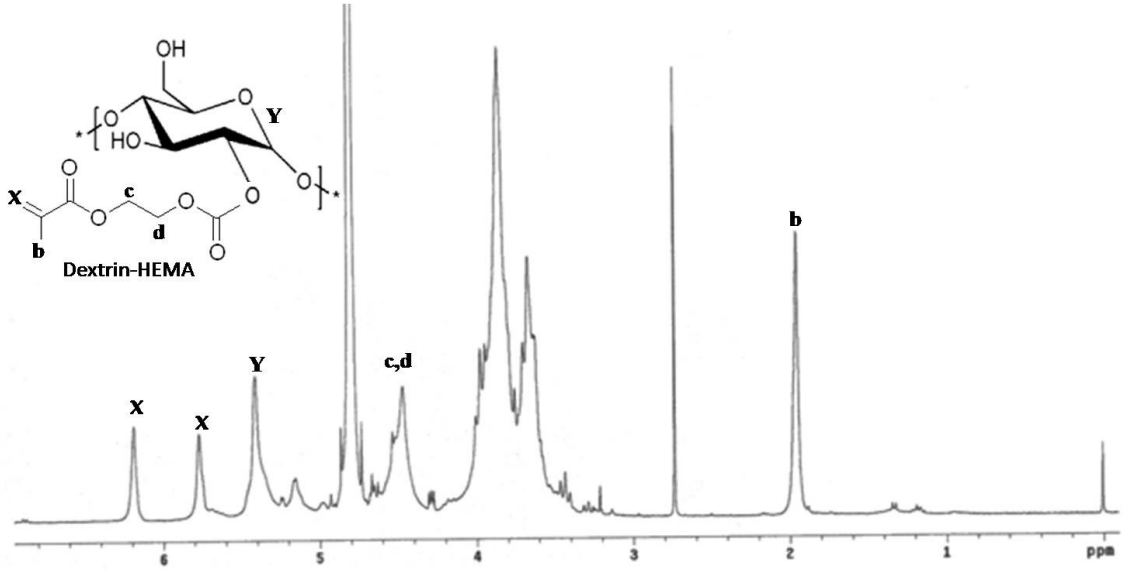

Figure 2. Dextrin-HEMA ^{1}H NMR spectrum.

General procedure for the preparation of dextrin-VA and dextrin-HEMA hydrogels

Dextrin-VA monomers were synthesized from dextrin in DMSO in the presence of different amounts of vinyl acrylate and the degree of acrylate substitution (DS) was determined by proton nuclear resonance spectroscopy (^{1}H-NMR) in D_2O as

previously described (Carvalho et al. 2007). The hydrogel slabs with different DS (20 and 40%) were prepared by radical polymerization of aqueous solution of either dextrin-VA or dextrin-HEMA (300 mg/mL). The gelation reactions were initiated by adding 90 µL APS (80 mg/ml in phosphate buffer) and 90 µL TEMED (13.6 % (v/v) in water, pH adjusted to 8.0 with HCl) and allowed to occur for 30 min at room temperature.

Standard degradation conditions

Two sets of hydrogels were used. After the polymerization reaction, gels were removed from the casting recipients. One set of hydrogels was immediately liofilized to determine the initial dry weight (*W*i). The other one was immersed in 50 mL of phosphate buffer saline (PBS) pH 7.4 and kept in a thermostated room at 37 °C. At set time intervals, gels were removed, blotted with filter paper to remove water excess, weighted (*W*s – weight in the swollen state) and lyophilised to determine the dry weight, *W*d. The swelling ratio (SR) and the mass loss (m_{loss}) were calculated according to equation 2 and 3, respectively:

$$SR = \frac{Ws - Wd}{Wd} \quad (2) \qquad m_{loss} = 100 - \left(\frac{Wi}{Wd} \times 100\right) \quad (3)$$

The hydrogel degradation time is defined as the time needed for complete degradation (*W*=0).

Rheological measurements

The rheological measurements were performed in a controlled-stress *Rheologica* rheometer at a controlled temperature (25ºC). A plate-plate measuring system was used (Ø 25 mm) and the gap between the plates was 1 mm. For the experiments, polymerized hydrogels with 25 mm diameter and 1 mm of thickness, were immersed in 50 mL of PBS at pH 7.4 and kept in a thermostated room at 37 °C to

allow degradation, with the exception of the initial time measurements (t=0), in which hydrogels were used immediately after polymerization. At set time intervals, gels were removed from the containers and placed between the plates of the measuring system. Two sets of experiments were performed:

a) Stress sweep experiments were carried out to optimize the applied stress used in the frequency-oscillation experiments. The lower limit of the applied stress is determined by the sensitivity of the instrument. On the other hand, the upper limit of the applied stress will be influenced by the linear viscoelastic range and the presence of slip effects.

b) Once the range of linear viscoelastic response was determined, experiments were performed in Small Amplitude Oscillatory Shear, SAOS, at a fixed frequency of 1 Hz and a stress known to yield a good quality signal in the linear viscoelastic regime.

Results and Discussion

In previous work, we have demonstrated the production of a new dextrin hydrogel, through the transesterification of the polymer with vinyl acrylate (VA) (Carvalho et al. 2007). However, these hydrogels were shown to degrade slowly under physiological conditions, which is a limitation in light of their application as controlled delivery systems. Several studies reported the successful functionalization of dextran with a methacrylate ester (HEMA) and it was shown that hydrogels obtained by polymerization of this compound, are able to degrade in desirable timeframes (Van Dijk-Wolthuis et al. 1997b, c, d, Stubbe et al. 2002). In this work, a similar dextrin modification was attempted using the same chemistry.

Activation of HEMA with CDI and synthesis of dextrin-HEMA

As previously described for dextran (Van Dijk-Wolthuis et al. 1997c), it was also possible to couple HEMA to the hydroxyl groups of dextrin via a mixed carbonate ester. This linkage can be effectively introduced by using N,N'-carbonyldiimidazole (CDI) as an intermediate activation step. In this activation reaction, HEMA reacts with an equimolar amount of CDI, in THF, to produce HEMA- imidazolyl carbamate, hereafter referred to as HEMA-CI (Figure 1). In the next step, HEMA-CI reacts with dextrin, using DMSO as reaction medium. ^{1}H RMN analysis shows that dextrin-HEMA with different DS (ranging from ca. 10% to 40%) can be obtained, by varying the molar ratio of HEMA-CI to dextrin (Figure 2). In addition, the modified polymer was successfully recovered by liofilization, after dialysis.

Swelling and degradation behaviour of dextrin-VA and dextrin-HEMA hydrogels

Upon exposure to an aqueous solution, the water molecules are drawn into the hydrogel network. The initial and relaxed state of the hydrogel (dry state) is, thereby, replaced by a swollen state. Since the water molecules are going to occupy some space, the meshes of the network will start expanding, allowing for the uptake of more water molecules. The stretching caused by the swelling process is counterbalanced by the covalent or physical crosslinking of the network, preventing its destruction and creating an equilibrium swelling state (Flory 1953). The functionality and number of linkages in the hydrogel, as well as the monomer concentration, influence not only the swelling ratio, but also the erosion profile and the overall time for degradation (Peppas et al. 2000). In this work, a comparative study on the swelling and degradation behaviour, was perfomed in hydrogels with two distinct ester functions [acrylate (dextrin-VA) and methacrylate (dextrin-HEMA)], and the influence of the DS on these parameters was also evaluated. Dextrin-VA hydrogels with DS 20 or DS 40 were compared with dextrin-HEMA with the same DS.

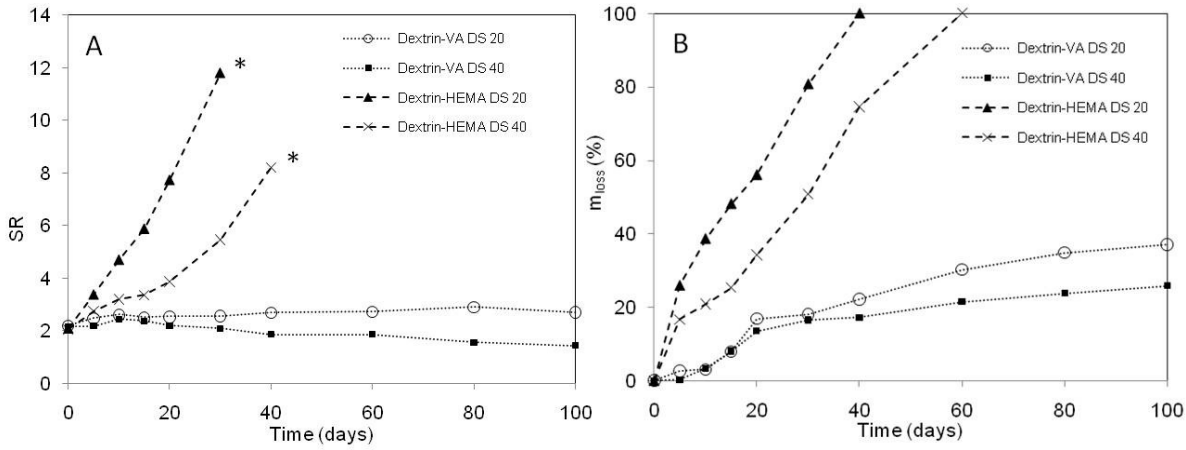

Figure 3. Degradability of dextrin-based hydrogels. Swelling ratio (A) and mass loss as a function of time, when immersed in PBS pH 7.4, at 37 ºC (average ±SD, n=3). (* from day 40 the hydrogel structure was severely damaged, preventing the SR measurements).

As can be seen in figure 3A, for dextrin-VA, at physiological pH, in the initial period of the incubation (around 5 days), a very slight increase in the swelling ratio is detected for both degrees of substitution (DS 20 or DS 40). Nevertheless, neither gel showed a significant increase in swelling over 100 days, indicating that no significant hydrolysis of the polymerized acrylate esters occurred. As expected, greater swelling values (in the range 2.5 to 3) were found for the DS 20 networks, compared with those (in the range 1.6 to 2.1) obtained with the higher DS, since in highly crosslinked hydrogels the mobility of the polymer chains is hindered, lowering the ability to swell.

Contrarily to the dextrin-VA hydrogels, in which the SR remains almost constant during the time course of the experiment, in the case of dextrin-HEMA, a progressive swelling occurred over the time, until the complete dissolution of the hydrogels is accomplished. The increased swelling is likely a consequence of the hydrolysis of the methacrylate bonds in the crosslinked structure. The cleavage of the ester bonds leads to a decrease in the crosslinking density, allowing for the network to expand and swell, absorbing more water.

As can be seen in figure 3B, the swelling ratio values reach a maximum at the time that the gels starts to dissolve, and dextrin chains most likely bear few crosslinks per chain. As also expected, while for a DS 20 dextrin-HEMA hydrogel, the dissolution took place in approximately 35 days, in case of DS 40 hydrogels, further time was needed to accomplish dissolution (60 days).

The mass loss of dextrin-VA/HEMA hydrogels with different DS (DS 20 and DS 40) was also monitored as a function of time. Figure 3B summarizes the degradation times of all dextrin gels investigated. This figure shows that for both gel systems (dextrin-VA or dextrin-HEMA), the degradation time increased with an increasing DS. It seems obvious that an increasing DS at a fixed initial water content (all the hydrogels have the same initial water content of about 70%), results in a network with a higher crosslinking density. In these cases, to accomplish the network dissolution, more cross-links have to be hydrolyzed, which requires more time. When comparing the two hydrogels systems, a linear mass loss profile can be observed in dextrin-VA, until day 20, with a rate of roughly 0.5% of gel solubilized per day. However, following this initial period, the rate slows down, and at day 100, only about 25% and 40% of mass loss is detected for DS 20 and DS 40 hydrogels, respectively. Probably, the mass losses observed correspond to the release of non-polymerized material.

Contrarily, as shown in figure 3B, for dextrin-HEMA hydrogels, the mass loss was dramatic, reaching about 30% for DS 20 and 50% for DS 40, after 20 days of incubation. In this case, hydrogels start to disintegrate quickly, until a complete dissolution is achieved. These results suggest that, in the case of dextrin-HEMA hydrogels, the degradation occurs via a homogeneous bulk erosion process. In this case the swelling ratio would be expected to increase with the incubation time, because of the loosened polymer network since, although hydrolyzed, the polymer chains remain in the matrix, allowing the network to become looser and to continuously swell, until the dissolution is complete (Wong and Mooney 1997, Suggs et al. 1998, Martens et al. 2002). In the case of dextrin-VA hydrogels, it

seems that the initial mass loss can be attributed to the release of a fraction of unbound molecules which were physically entrapped in the matrix at the time of polymerization. As diffusion occurs, the small molecules are released out of the network. A stabilization of the swelling values is, thus, observed at the time that all the unbound molecules are released. Contrarily to dextrin-HEMA, which are hydrolytically labile, dextrin-VA hydrogels are very stable under physiological conditions.

Mechanical Properties

Mechanical properties of hydrogels are very important for pharmaceutical applications. For example, the integrity of a drug delivery device during the lifetime of the application is crucial. The system must protect a sensitive therapeutic agent, such as protein, maintaining its integrity until it is released out of the system. Changes in the crosslinking density of the hydrogels have been routinely applied to achieve the desired mechanical properties. Increasing the crosslinking will result in a stronger gel with a more brittle structure, which sometimes is not desirable since it shall interfere with the release profiles. Hence, a compromise must be achieved between a relatively strong but yet elastic structure (Anseth et al. 1996). It is well established that the release of the proteins from a hydrogel is clearly influenced by its degradation rates (Meyvis et al. 2000). Dynamic mechanical analysis, performed on the hydrogel, provide quantitative information on the viscoelastic and rheological properties of the material, by measuring the mechanical response of the samples as they are deformed. The elastic (also real or storage) modulus G' and the viscous (also imaginary or loss) modulus G'' are presented. The storage modulus (G', Figure 4A) of a hydrogel is proportional to the amount of elastic chains within the polymer network (Ferry 1980). Figure 3 shows the rheological results for all the hydrogels and it is clear that the results are in good agreement with the degradability studies above. Dextrin-HEMA hydrogels show lower G' values, as compared with the dextrin-VA ones, indicating that physical structuring is less pronounced when dextrin is

modified with methacrylate side groups. Overall, it is possible to broadly correlate an increase in swelling ratio with a decrease in both the elastic and viscous responses, G' and G'' respectively, since the gels become softer as they swell, which tends to decrease G', whilst the increased water absorption, due to the hydrolysis of the methacrylate bonds in the crosslinked structure, leads to a decrease in G'' (Figure 4B).

In the case of the dextrin-VA gels, G' and G'' decrease at short times and stabilize after approximately 30 days. Since there is a large uptake of water and these gels do not degrade, the viscous response increases slightly relatively to the elastic one, as reflected by the slight increase in Tan(α), but the viscoelastic behavior clearly remains that of a soft solid (Figure 4C). The DS has an appreciable impact on the storage modulus. Dextrin-VA hydrogels with a higher DS (DS 40) has more crosslink sites, which results in a denser mesh structure, more network chains, and an associated higher G', compared to the lower DS (DS 20).

In the case of the dextrin-HEMA gels, the degradation is very severe, with the viscoelastic response quickly approaching that of a inelastic low-viscosity liquid, *i.e.*, low G' and G'' and Tan(α)>>1. This degradation culminated in the impossibility to perform any tests on the dextrin-HEMA gels, due to their extreme fragility above 20 days of rest.

From these results, it could be concluded that a good correlation exists between the influence of the ester function (methacrylate/acrylate) used in the polymer derivatization, as well as the grafted group percentage (DS), on the rheologic degradation rates. In addition, this finding evidences that a large dependence exists between the release rates and the evolution of the network structure, since it was proved that this last largely controls the degradation process. By knowing the degradation mechanism, rheological data can help to interpret release profiles of bioactive molecules from degrading hydrogels.

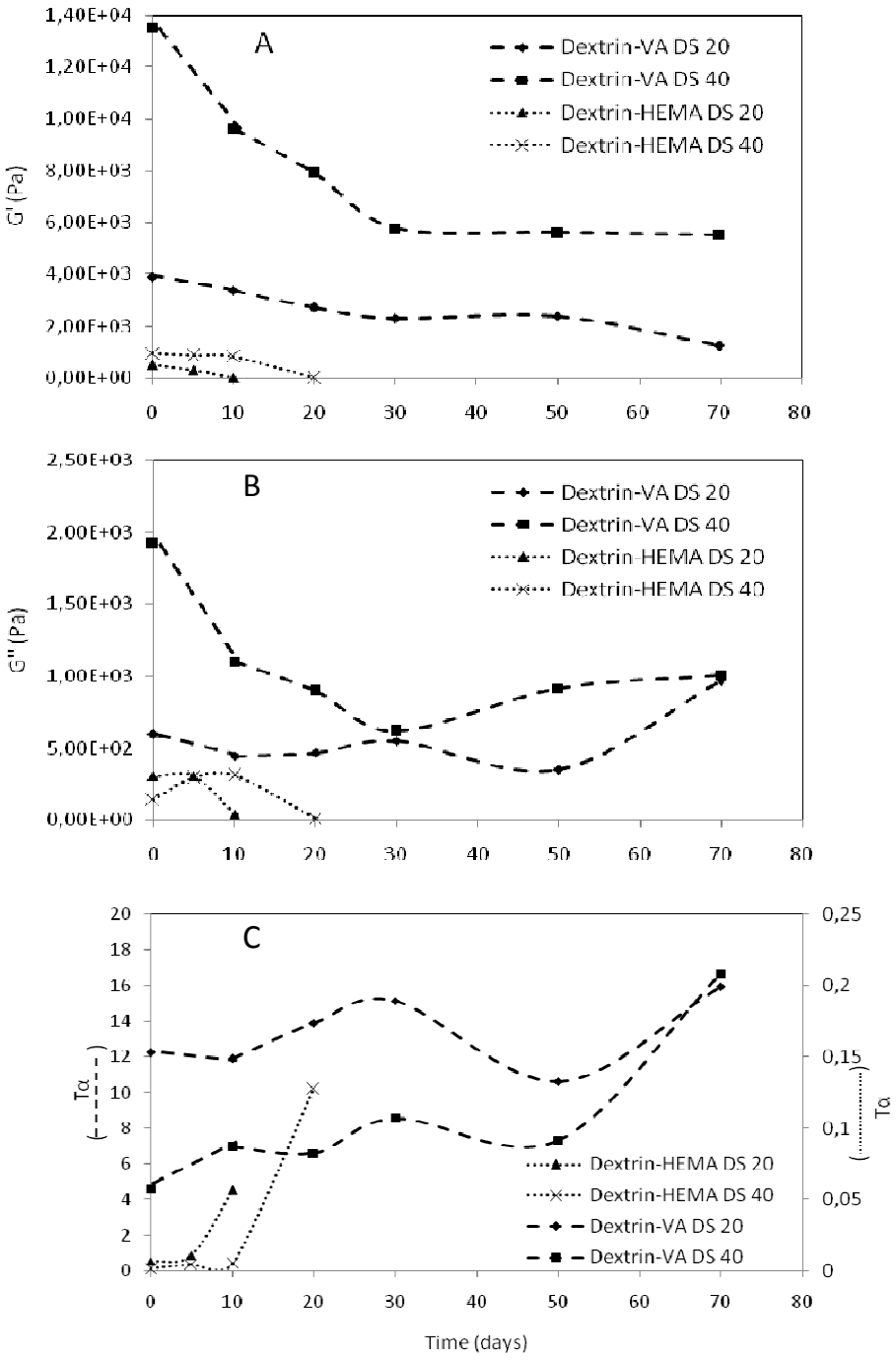

Figure 4. Rheological measurements of the different hydrogel samples. Frequency is 1 Hz and stress varies in order to keep the response in the linear viscoelastic regime.

CONCLUSIONS

It was possible to obtain a new class of polymerizable dextrins (dextrin-HEMA) by using a method based on the activation of the hydroxyl groups of HEMA with CDI, followed by their coupling with the polymer, yielding a derivatized material that can be polymerized in aqueous solution to form hydrogels. The comparative study of the stability of these new hydrogels and the previously described non-degradable dextrin-VA ones revealed that the firsts are effectively hydrolyzed under physiological conditions. Contrarily to the dextrin-VA hydrogels, in which the SR remains almost constant during storage, in the case of dextrin-HEMA, a progressive swelling accompanied by a severe mass loss process, occurred over the time, culminating in the complete dissolution of the hydrogels. Accordingly, the comparative study, extended to the rheological analysis of the viscoelastic behavior, revealed that dextrin-HEMA hydrogels have lower storage module (G') values, compared with the dextrin-VA ones, indicating that physical structuring is less pronounced when dextrin is modified with methacrylate side groups.

It can be concluded that the dextrin functionalization with HEMA, may be an effective route to modulate the hydrogel characteristics, namely the degradation kinetics, tailoring the release profiles to fit the purpose of the controlled delivery.

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CHAPTER 6

Modification of pulp and paper using CBD-PEG conjugates

Summary

In this work, the effect of recombinant cellulose-binding domains (CBD) on the properties of secondary paper fiber was evaluated. Two recombinant family 3 CBDs, from *Clostridium thermocellum* scaffolding protein CipA (CBD_{CipA}) and Cellobiohydrolase A (CbhA), were selected for these studies. The CbhA CBD was used either alone (CBD_{CbhA}) or fused with the internal fibronectin ($FN3_{1,2}$) module ($FN3_1$-$FN3_2$-CBD_{CbhA}). It was confirmed that although classified as a family 3 CBD, commonly assumed as a cellulose-binding module, CBD_{CbhA} lacks the binding function to microcrystalline cellulose. Additionally, the CBDs were chemically conjugated with an activated PEG. The data showed that the CBD_{CipA}-PEG conjugate leads to a change on the properties of secondary fibers, as revealed by the improvement in both pulp drainage (°SR decreased up to 15%) and paper tensile strength. This effect is attributed to the presence of the PEG molecule, since CBDs lacking PEG were unable to modify pulp and paper properties. It is suggested that PEG mimetizes the glycosidic fraction of fungal CBDs, which is absent in the highly purified bacterial modules used here. It is concluded that the improved drainability of the pulp is attributed to the hydration and stabilization of the fibers

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EMT | 2008 | Submitted

Introduction

Enzymes have long been applied in paper production, for the modification of both fiber and paper properties. These enzymes include xylanases, endo-glucanases, cellulases and hemicellulases (Suurnäki et al. 1994, Senior and Hamilton 1993, Gen and Li 2003, Seo et al. 2000, Jackson at al. 1993, Pala et al. 2001). However, several drawbacks result from enzyme utilization in paper treatment, such as the extensive hydrolysis of polysaccharides that leads to a reduction of both fiber strength and mass. To avoid these side effects that result from enzyme utilization, recently the use of cellulose-binding domains (CBDs) in paper treatment has been evaluated. CBDs are non-catalytic modules present in several cellulases and hemicellulases and exhibit high affinity for cellulose. These modules are produced by a large number of fungi and bacteria and potentiate the catalytic efficiency of the associated catalytic domains. The ability to fuse CBDs with other molecules of different sizes, charges, hydrophobicities, catalytic activities or bioactivities, allows these molecules to be potentially interesting and useful on the activation and modification of cellulose fibers (Kitaoka and Tanaka 2001, Levy and Shoseyov 2002, Pinto et al. 2004, Pinto et al. 2006). CBDs may be obtained through the proteolysis of cellulases, as described earlier, providing large amounts of peptides in a rather rapid procedure, or using the recombinant DNA technology (Creagh et al. 1996, Bothwell et al. 1997, Linder et al. 1999, Bayer et al. 2000). This last method allows overcoming the purity limitations of the proteolysis approach, although the CBDs obtained through these methods are not glycosylated (Pinto et al. 2006).

Cellobiohydrolase A (CbhA) is one of the most important catalytic components of the multi-enzyme complex produced by the anaerobic thermophilic bacterium *Clostridium thermocellum*, which was termed cellulosome. This complex is specialized in the hydrolysis of insoluble plant cell wall polysaccharides. It is composed of a cellulosome-integrating protein, termed CipA, containing nine repetitive cohesin domains which bind to the dockerin domains located in the

cellulosomal catalytic subunits that can express glycoside hydrolase, carbohydrate esterase or pectate lyase activities (Bayer et al. 1998, Beguin and Alzari 1998, Blum et al. 2000, Felix and Ljungdahl 1993, Kataeva et al. 2002, Kataeva and Ljungdahl 2003, Kurokawa et al. 2001, Shoham et al. 1999, Zverlov et al. 1998). The cellulosome is anchored to the plant cell wall primarily through the action of the internal family 3 CBM located in CipA. *C. thermocellum* cellulosomal CbhA is composed of an N-terminal family 4 CBD, an immunoglobulin-like domain, a family 9 glycosyl hydrolase catalytic domain (GH9), two Fn3-like repeats ($Fn3_{1,2}$), a family 3 CBD and a C-terminal dockerin domain (Kataeva et al. 2002). Previous work reported the ability of CbhA family 3 CBD to enhance the surface modification and hydrolysis of cellulose fibers (Kataeva et al. 2002). Additionally, it has been shown that CBDs obtained through the proteolysis of *Trichoderma reesei* cellulases, are capable of modifying the drainability of recycled paper pulps (Pinto et al. 2004). In the present work, we analyzed the potential of two recombinant family 3 CBDs (belonging to CipA and CbhA of the *Clostridium thermocellum*) and a fusion protein with the $FN3_{1,2}$ module ($FN3_1$-$FN3_2$-CBD_{CbhA}) to modify secondary paper fibers. Furthermore, the CBDs were chemically conjugated with an activated PEG, as this molecule mimetize the glycosidic fraction that is lacking in the highly purified bacterial CBDs. Finally, the technical properties of the fiber papers and paper sheets resulting from the CBD treatment were analyzed.

Materials and Methods

Chemicals

Sodium acetate was obtained from Panreac (Barcelona, Spain) and acid-swollen cellulose (ASC) was prepared by treatment of Avicel PH-105 (Fluka, Philadelphia) with phosphoric acid (Klyosov and Sinitsyn 1981). Whatman No. 3 filter paper and dinitrosalicylic acid (DNS) were from Sigma (St. Louis, Mo.). SS-m Polyethylene glycol (PEG) (SUNBRIGHT MW5000) was purchased from NOF Corporation

(Belgium). Paper fibers (Kraftline Paper) were gently supplied by *Portucel Viana* company. All chemicals were of the highest purity available.

Bacterial strains, culture conditions, and plasmids.

C. thermocellum JW20 was used as a source of genomic DNA. The bacterium was grown anaerobically under a nitrogen atmosphere at 60°C in a prereduced medium with 1% (w v^{-1}) cellobiose as described earlier (Kataeva et al. 1999). *E. coli* BL21(DE3)pLys (Stratagene Cloning Systems, La Jolla, Calif.) was used as the cloning host for the T7 RNA polymerase expression vector pET-21a (Novagen, Madison, Wisc.). It was grown in Luria-Bertani medium supplemented with ampicillin (100 µg ml^{-1}). Isolation of genomic DNA from *C. thermocellum* was performed through the method described by Marmur (Marmur 1961).

Primer design, PCR, and cloning.

Flanking primers containing restriction sites were designed according to the DNA sequence of the gene encoding CbhA (Table 1). DNA fragments were amplified through PCR using purified genomic DNA from *C. thermocellum* as a template. The PCR reactions were carried out with Taq polymerase (New England Biolabs, Beverly, Mass.). PCRs were performed as follows: preheating at 95 ºC for 2 min, 40 cycles at 95 ºC for 45 s, 53 ºC for 45 s and 72 ºC for 45s, followed by an elongation at 72 ºC for 10 min. PCR products were separated by 1% agarose gel electrophoresis. The extracted DNA fragments were digested with the appropriated restriction enzymes and ligated into the pET-21a vector digested similarly. The ligation products were used to transform BL21(DE3)pLys competent cells. The integrity of each construct was verified by both restriction analysis and DNA sequencing.

Table 1. Oligonucleotides used for cloning.

Nº and primer	Sequence	Restriction Site	Direction
1.CBD_{CbhA}	5´- CTC **GCT AGC** GAT GTA AAA GTA GAG TAC TTG - 3´	*Nhe I*	Forward
2.CBD_{CbhA}	5´- CAC **CTC GAG** GGT TCC CCA AAC AAG TTC TCC - 3´	*Xho I*	Reverse
3.$FN3_1$-$FN3_2$-CBD_{CbhA}	5´- CTC **GCT AGC** ACA GAC AGT GAA ACC GAT AAG - 3´	*Nhe I*	Forward
4.$FN3_1$-$FN3_2$-CBD_{CbhA}	5´- CAC **CTC GAG** GGT TCC CCA AAC AAG TTC TCC - 3´	*Xho I*	Reverse
5.CBD_{CipA}	5´- CTA **GCT AGC** AGA GGT GAT ACA CCG ACC AAG GGA G - 3´	*Nhe I*	Forward
6.CBD_{CipA}	5´- CAC **CTC GAG** TTC TTT ACC CCA TAC AAG AAC - 3´	*Xho I*	Reverse

Protein purification

All recombinant proteins were six-Histagged at the C terminus and were purified from BL21(DE3)pLys cultures (1 liter) harboring the appropriated pET-21a derivative. The cells were harvested 5 h after induction with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) at 37ºC. All purification steps were performed at 4°C. After collection, the cells were resuspended with 20 mM Na-phosphate buffer, pH 7.5, containing 0.5 M NaCl, and disintegrated with a sonicator. Cell debris was removed by centrifugation. Clear supernatant was applied onto a Ni-nitrilotriacetic acid (Ni-NTA) agarose (HisTrap, Amersham) column equilibrated with the 20 mM Na-phosphate, pH 7.5, containing 0.5 M NaCl. The column was washed with 20 mM Na-phosphate–0.5 M NaCl, pH 7.4. Proteins were eluted by a gradient of 0 to 0.3 M imidazole, pH 7.4. Fractions containing recombinant proteins were combined and dialyzed against PBS. Dialyzed proteins were further used in the adsorption assays. Additionally, the glycohydrolase GH9 from CbhA and the endoglucanase Cel5B from *Cellvibrio mixtus* were produced as described previously (Fontes et al. 1998).

CBD-PEG Conjugation

Briefly, the protein to be pegylated was dissolved in 0.1 M Sodium Phosphate, pH 7.5, at a concentration of 2 mg mL^{-1}. A designated quantity of succinimidyl succinate (SS)-mPEG (Figure 1) (at the molar ratio of polymer to primary amines

of the protein of 3%) was added to the protein solution, with magnetic stirring, and allowed to react for 1 hour at 25ºC (Abuchowski et al. 1977, Bailon and Berthold 1998). The PEG-modified protein was analyzed by SDS-PAGE and stored at -20ºC before use.

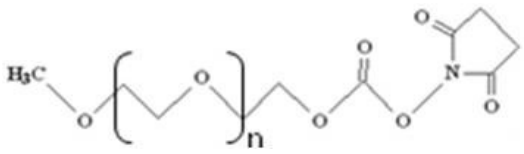


Figure 1. Structure of SS-mPEG, showing the activating succinimidyl succinate terminal group, highly reactive toward nucleophile amines.

MALDI-TOF

Mass spectrometry measurements were performed on a Mass Spectrometer *MALDI-TOF*. The samples were diluted on the matrix reagent, sinapinic acid. Calibration was performed using the Promix 3 (Applied Biosystems) peptide mix.

Cellulose-binding assay

Adsorption assays were performed at a room temperature in 2 ml microcentrifuge tubes. Proteins were mixed with cellulose (50 g L^{-1}) in PBS buffer (pH 7.4) in a final volume of 1 ml. Tube contents were continuously mixed by rotation. After equilibration for 2 h, cellulose with bound protein was removed by centrifugation at 10000 g for 10 min. Centrifugation and washing was repeated twice to ensure the removal of all unbound protein. The amount of unbound protein was determined allowing for the estimation of the total bound. Data presented are from three replicate experiments.

Enzyme assays

The cellulolytic activity of the truncated variants CBD_{CbhA} and $FN3_1$-$FN3_2$-CBD_{CbhA} were assayed isolated and in the presence of the enzymes GH9 and Cel5B (at the optimum enzyme temperature, 60 and 50°C for GH9 and Cel5B, respectively) was determined in a 50 mM sodium citrate buffer, pH 6.0, containing 5 % of the substrate. Control assays were performed in the same conditions, only with buffer. The activity was monitored by measuring the release of reducing sugars. Reducing sugars were determined with DNS (Miller 1959). Activity was expressed as micromoles of product (cellobiose equivalent) released per minute per milligram of enzyme. Efficiency of cellulose hydrolysis was determined with ASC and filter paper as substrates.

Treatment with CBDs and CBD-PEG conjugate

Cellulose fibers, at 10% (w w^{-1}) consistency, were beated in a PFI mill for 3000 revolutions. Afterwards, CBDs (CBD_{CipA}, CBD_{CipA}-PEG, CBD_{CbhA} and $FN3_1$-$FN3_2$-CBD_{CbhA}) were adsorbed by mixing the protein at different concentrations (0.1, 1 and 2 mg per gram of fiber, dry weight) with 30 g of fibers (dry weight) in acetate buffer (50 mM, pH 5.0 and final volume of 2 L) for 30 minutes at room temperature. Control experiments were performed by adding buffer without the protein.

Analysis of the Pulp and Paper Properties

The pulp and handsheet properties were analyzed using standard procedures: ISO 5267/1 (Shopper-Riegler); ISO 5636/3 1992 (F) (permeability); ISO 1924/2 1985 (F) (tensile strength and break length); ISO 1947 (F) (tearing); ISO 2758 1983 (F) (bursting strength).

Results and Discussion

Protein production and purification

All proteins were expressed in the soluble fraction and purified close to homogeneity according to SDS/PAGE analysis (Figure 2). Since all polypeptides interact with the Ni-NTA affinity columns and have molecular masses close to those calculated from the deduced amino acid sequences ($FN3_1$-$FN3_2$-CBD_{CbhA} ≈ 37.35 kDa; CBD_{CbhA} ≈ 16.11kDa and CBD_{CipA}≈ 22.90 kDa), it was conclude that the proteins were purified in the intact form.

Additionally, the MALDI-TOF analysis of the purified $FN3_1$-$FN3_2$-CBD_{CbhA} showed that the protein sample is highly homogeneous, with a main peak detected at 37.42 kDa - the deduced molecular mass of $FN3_1$-$FN3_2$-CBD_{CbhA} is of 37.35 kDa. Furthermore, N-terminal sequence analysis revealed the expected amino acid sequence (^{1}ASTDSET^7) for $FN3_1$-$FN3_2$-CBD_{CbhA}. Taken together, the data suggests that $FN3_1$-$FN3_2$-CBD_{CbhA} was not subjected to proteolysis during expression and/or purification.

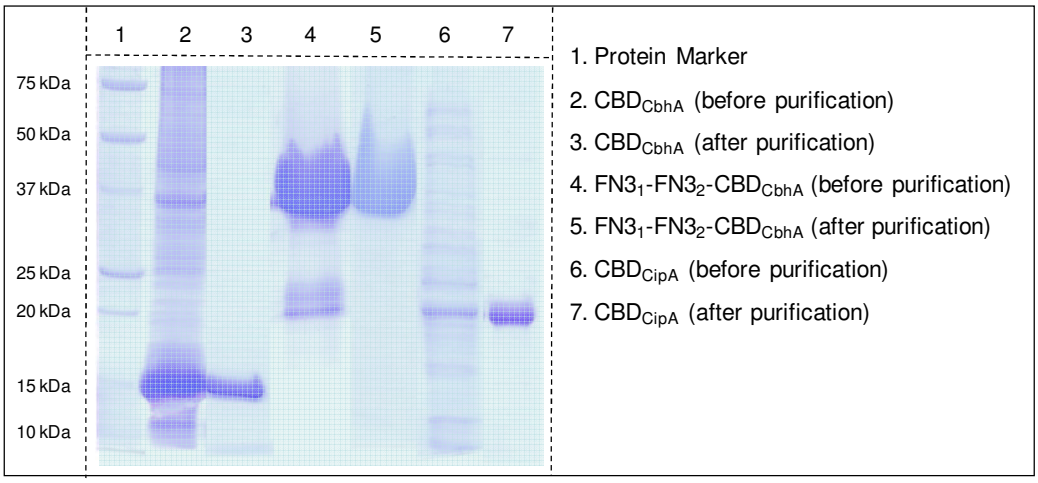


Figure 2. SDS/PAGE analysis of unpurified and purified extracts of CBD_{CbhA}, $FN3_1$-$FN3_2$-CBD_{CbhA} and $CBD_{CipA.}$. Proteins were purified through nickel-affinity chromatography as described in Materials and Methods.

Binding and Hydrolysis of Cellulose

Several studies demonstrated that isolated CBDs are capable of disrupting the surfaces of cellulose fibers. This non-hydrolytic disruptive activity has also been ascribed to the $Fn3_{1,2}$ module of CbhA from *C. thermocellum* (Din et al. 1991, Kataeva et al. 2002). Additionally, it was suggested that $Fn3_{1,2}$ affects hydrolysis more significantly when it is attached to its appended catalytic domain in what seems to be a synergistic process. Therefore, in order to clarify this issue the hydrolytic activities of the truncated forms of CbhA, CBD_{CbhA} and $FN3_1$-$FN3_2$-CBD_{CbhA}, were evaluated. In addition, the capacity of the truncated derivatives of CbhA to promote the activity of Cel5B and GH9 cellulases from *C. thermocellum* CbhA *in trans* was determined. The data, presented in Table 1, confirm that both GH9 and Cel5B are efficient degraders of ASC and filter paper, producing cellobiose and glucose, respectively, as the main hydrolysis products. In contrast, mixing of GH9 or Cel5B, *in trans*, with $CBD_{CbhA,}$ or $FN3_1$-$FN3_2$-CBD_{CbhA} resulted in only a very slight increase in the levels of reducing sugars released, suggesting that $FN3_1$-$FN3_2$ has a marginal capacity to affect the activity of non-attached catalytic domains during the hydrolysis of insoluble forms of cellulose.

Table 2. Hydrolysis of ASC and filter paper by truncated forms of CbhA and combinations of GH9 and Cel5B with CBD_{CbhA} and $FN3_1$-$FN3_2$-$CBD_{CbhA.}$

Protein Used	**Reducing sugars produced (mg of glucose/mL)**	
	ASC	**Filter Paper**
Gh9	2.46	1.10
Gh9 + CBD_{CbhA}	2.51	1.13
Gh9 + $FN3_1$-$FN3_2$-CBD_{CbhA}	2.58	1.21
CBD_{CbhA}	0.18	0.02
$FN3_1$-$FN3_2$-CBD_{CbhA}	0.22	0.06
Cel5B	0.79	0.33

Cel5B + CBD_{CbhA}	0.83	0.34
Cel5B + $FN3_1$-$FN3_2$-CBD_{CbhA}	0.84	0.36
Control	0.19	0.02

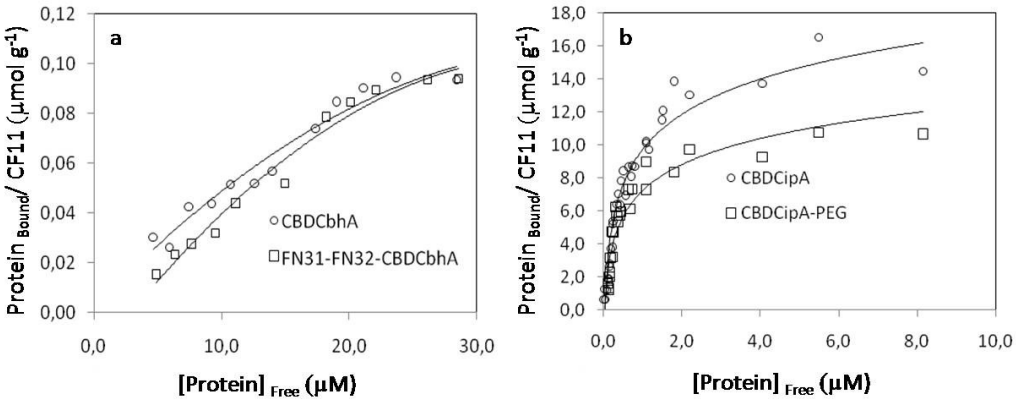
The capacity of the proteins expressed in this study to bind insoluble forms of cellulose was evaluated. The data, presented in figure 3a, revealed that both CBD_{CbhA} and $FN3_1$-$FN3_2$-CBD_{CbhA} present a low affinity to cellulose, as shown by the analysis of the adsorption isotherms. Non-linear regression analysis was used to calculate the parameters of the adsorption isotherm, using the expression:

$$CBD_{Bound} = \frac{CBD_{Max} \times K_a \times [CBD]_{Free}}{1 + K_a \times [CBD]_{Free}}$$

where CBD_{Bound} is the molar amount of protein adsorbed, per unit weight of cellulose, $[CBD]_{Free}$ is the molar protein concentration in the liquid phase at the adsorption equilibrium, CBD_{Max} and K_a are the maximum molar amount of protein adsorbed, per unit weight of cellulose, and the adsorption equilibrium constant, respectively.

Both proteins bound weakly to cellulose, since the values of CBD_{Max} and K_a were, respectively, 0.27 µmol g^{-1} and 0.01 L µmol^{-1} (0.94 L g^{-1}) for CBD_{CbhA} and 0.14 µmol g^{-1} and 0.07 L µmol^{-1} (1.83 L g^{-1}) for $FN3_1$-$FN3_2$-CBD_{CbhA}. These values are lower than those previously referred for $CBD_{CbhA,}$ by Kataeva and colleagues (2002), which described the domain as a cellulose binder with a CBD_{Max} of 2.45 µmol g^{-1} and a K_a of 1.12 L g^{-1}. Taken together, the data suggest that, although surprisingly, the $FN3_1$-$FN3_2$-CBD_{CbhA} exhibits poor affinity for cellulose fibers, and its lower affinity cannot be explained by proteolysis, as described above. The family 3 CBDs are found associated with GH9, which are known to act in cellulosic substrates (Bayer et al. 2000). There are three major subgroups in the family 3 CBDs; a, b and c, based on sequence similarity (Morag et al. 1990). In biochemical studies, the CBDb subgroup (to which belongs the CBD_{CbhA} used in this work), have been shown to bind strongly to the surface of microcrystalline cellulose and were proposed to promote the cellulolytic activity by concentrating the enzyme near the cellulose surface (Tormo et al. 1996, Gilad et al. 2003).

However, recent studies question its apparently clear functions as a cellulose-binding module (Jindou et al. 2006, Jindou et al. 2007). Jindou and colleagues have demonstrated that certain atypical family 3 CBDbs, Cel9V and Cel9U from *C. thermocellum*, although properly folded, lack some of the highly conserved residues (of the 'classic' CBDs) and also fails the ability to bind microcrystalline cellulose. In addition, further investigation on $CBD_{CbhA,}$, have shown that it diverges from the standard CBDb and lacks several of the conserved residues from its putative cellulose-binding surface. In accordance with our results, Jindou and co-workers have also found that CBD_{CbhA} lacks the binding function to microcrystalline cellulose and have started to explore a structural explanation for this inconsistency. Taken together with our results, these findings suggest that the status of the family3 CBDs is much more complicate and diverse than it could be expected. Contrarily to the CBD_{CbhA}, under the present experimental conditions CipA CBD was shown to bind efficiently to cellulose, expressing CBD_{Max} and K_a values of 16.80 µmol g^{-1} and 1.54 L µmol^{-1} (Figure 3b).



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