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NANOPARTÍCULAS COM SUPERFÍCIE MODIFICADA DO TIPO NÚCLEO-COROA DE NOVOS COPOLÍMEROS DE DEXTRANA-POLICAPROLACTONA PARA CONJUGAÇÃO DE LIGANTES DE RECONHECIMENTO

JAQUELINE RODRIGUES DA SILVA

Recife, Brasil

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Ao meu pai

Manoel José da Silva

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RESUMO

A vetorização de fármacos para órgãos específicos é extremamente complexa devido a necessidade do carreador desempenhar simultaneamente diferentes funções. Primeiramente, as nanopartículas devem ser capazes de conduzir uma quantidade significativa de fármacos e promover a sua liberação com perfil cinético adequado. Numa segunda fase, após a administração o fármaco deve ser capaz de atingir o sítio alvo de uma maneira específica e subsequentemente liberar a droga. O *Estado da Arte* demonstra que as ferramentas para atingir estes objetivos devem ser aprimoradas.

Objetiva este trabalho investigar a preparação e caracterização de nanopartículas como carreadores de fármacos utilizando um novo copolímero de dextrana-policaprolactona (Dex-PCL_n). Duas abordagens destintas foram investigadas. Inicialmente, foi verificada a possibilidade da aplicação das nanopartículas para o encapsulamento de substâncias de interesse farmacêutico. Dentre essas substâncias, peptídeos e proteínas são de considerável interesse devido a difículdade no encapsulamento de quantidades importantes e na manutenção da estabilidade durante o processo de fabricação. Diferentes proteínas foram utilizadas como substâncias modelo, incluindo a albumina sérica bovina (BSA), lectina de folha de *Bauhinia monandra* (BmoLL), a qual demonstrou uma atividade hipogliciemiante, e lectina de *Lens culinaris* (LC). Várias matrizes poliméricas foram utilizadas para o encapsulamento de BmoLL em nanopartículas. Polímeros convencionais de ε-caprolactona (PCL), ácido lático (PLA) e copolímero de ácido lático e glicólico (PLGA) e novos copolímeros sintetizados a partir de dextrana e policaprolactona foram utilizados para atingir este objetivo. A outra estratégia consistiu na preparação de nanopartículas do tipo núcleo-coroa com novos copolímeros anfifílicos de dextrana e policaprolactona. Estas partículas apresentam uma superfície hidrofílica com alta densidade de grupos hidroxilas que podem ser utilizados para conjugação de ligantes de reconhecimento de sítios específicos no organismo. No presente trabalho, nanopartículas foram preparadas e caracterizadas na tentativa preliminar de adsorção ou conjugação das lectinas BmoLL e LC na superfície das nanopartículas.

A tese consiste em três partes experimentais. A primeira parte da tese diz respeito ao estudo da estabilidade de BmoLL e sua encapsulação em nanopartículas. Inicialmente, foram avaliados os efeitos das condições de armazenamento de BmoLL e da preparação de nanopartículas na atividade hemaglutinante da BmoLL. Em seguida, BmoLL foi

encapsulada em nanopartículas preparadas com PCL, PLA e PLGA. A caracterização físico-química das nanopartículas foi efetuada através da determinação do tamanho médio das partículas, do potencial zeta, da taxa de encapsulação de BmoLL e análise morfológica por microscopia eletrônica de varredura. A cinética de liberação *in vitro* de BmoLL a partir das nanopartículas foi avaliada e a cinética de adsorção de BmoLL na superfície das partículas foi estudada.

A segunda parte da tese está relacionada com a síntese de novos copolímeros que permitam a fabricação de nanopartículas com possibilidade de conjugação de lectinas na superfície. Neste contexto, diferentes copolímeros de dextrana e policaprolactona (Dex-PCL_n) foram sintetizados por ligação química entre grupos funcionais carboxílicos presentes na policaprolactona e grupos hidroxilas presentes naturalmente no polissacarídeo. A composição de uma série de copolímeros Dex-PCL_n com uma média de 3, 5,5 e 7,1 cadeias de PCL conjugadas em dextrana foi determinada por cromatografia de permeação em gel e espectroscopia de ressonância magnética nuclear de prótons (RMN^1H) e de infravermelho (FTIR).

A terceira parte da tese está dedicada à aplicação de novos copolímeros Dex-PCL_n na obtenção de nanopartículas do tipo *núcleo–coroa* com matriz polimérica de PCL e coroa de dextrana. As nanopartículas de Dex-PCL_n foram desenvolvidas utilizando o método de emulsão múltipla seguida de evaporação de solvente e caracterizadas pelos métodos citados anteriormente para as nanopartículas convencionais. Um estudo da citotoxicidade dos constituintes e das nanopartúculas foi efetuado com células de carcinoma humano de cólon (Caco-2). Adicionalmente, a resistência transepitelial (TEER) e o potencial bioadesivo das nanopartículas radiomarcadas foram avaliados em células Caco-2.

Os resultados relacionados ao estudo de estabilidade de BmoLL demonstraram que a forma liofilizada mantém a atividade hemaglutinante após submissão aos ultra-sons, à agitação mecânica e aos solventes orgânicos. BmoLL foi eficientemente encapsulada em nanopartículas de polímeros convencionais. A cinética de liberação de BmoLL a partir das nanopartículas foi de forma controlada e a adsorção de BmoLL na superfície das nanopartículas foi comprovada.

Dentre os copolímeros testados, o Dex-$PCL_{5,5}$ produziu nanopartículas de menor diâmetro e de maior estabilidade. A caracterização físico-química das nanopartículas Dex-PCL_n demonstrou um diâmetro médio das partículas inferior a 200 nm e carga de superfície, medida através do potencial Zeta, elevada com relação as nanopartículas constituídas de

polímeros convencionais. O potencial Zeta elevado das nanopartículas de Dex-PCL_n confirmou a hipótese de formação da uma cobertura com carga proporcionada pelas cadeias de dextrana organizadas em forma de *coroa* em torno do núcleo constituído de PCL. A taxa de encapsulação de BmoLL e de lectina de *Lens culinaris* nas nanoparticulas de Dex-PCL_n foi elevada. Os resultados do estudo de bioadesão demonstraram uma maior interação não específica das nanopartículas de Dex-PCL com as membranas de células Caco-2, comparada às nanopartículas de PCL.

Concluindo, sistemas com superfície modificada do tipo núcleo coroa, preparados com copolímeros anfifílicos foram obtidos e caracterizados. Níveis razoáveis de encapsulação de proteínas foram atingidos e a modificação na superfície oferece a possibilidade de produção de sistemas com ligantes conjugados para a aplicação na vetorização de moléculas de interesse biofarmacêutico.

RÉSUMÉ

Le ciblage de sites spécifiques de l'organisme au moyen de nanoparticules représente un défi d'une grand complexité dans la mesure où le transporteur du principe actif doit assurer simultanément un certain nombre de tâches dans l'organisme. En premier lieu, les nanoparticules doivent être capables de transporter une quantité importante de principe actif et de le libérer selon un profil de libération adéquat. En second lieu, le système d'administration doit être capable de rejoindre le site particulier de l'organisme (un organe, une cellule, un compartiment subcellulaire) où on souhaite effectuer la libération. L'étude détaillée de la littérature montre que les outils qui sont actuellement à notre disposition doivent encore être améliorés. Le but du présent travail était de développer et de caractériser des nanoparticules cœur-couronne à base de nouveaux copolymères amphiphiles afin de disposer de systèmes mieux adaptés aux contraintes de la vectorisation.

Deux approches ont été envisagées au cours du travail expérimental. En premier lieu, la possibilité de préparer des nanoparticules à partir de ces polymères et de les charger avec des substances modèles d'intérêt pharmaceutique a été étudiée. Dans ce cadre, les peptides et les protéines sont des candidats particulièrement intéressants, dans la mesure où ces substances sont habituellement difficiles à encapsuler en grande quantité et où se posent fréquemment des problèmes de stabilité dans le cours du procédé de fabrication. Différentes protéines ont été utilisées en tant que substances modèles, notamment la sérum albumine bovine, la lectine de feuille de *Bauhinia monandra* (BmoLL) qui a montré une activité hypoglycémiante, et la lectine de *Lens culinaris*. L'emploi de différents polymères a été envisagé, notamment l'emploi de polyesters tels que la poly(epsiloncaprolactone) (PCL), le poly(acide lactique) (PLA), les copolymères de l'acide lactique-co-glycolique (PLGA), et une série de nouveaux copolymères du dextran et de l'epsilon-caprolactone.

En second lieu, la possibilité d'ajuster sur mesure les propriétés de surface des particules a été envisagée. La stratégie a consisté à préparer des nanoparticules cœur-couronne basées sur l'emploi de copolymères amphiphiles de dextran et de poly(epsiloncaprolactone). Comparativement à des particules conventionnelles basées sur des polymères classiques de type PLA ou PLGA, ces nouvelles particules présentent une surface très hydrophile qui permet notamment la stabilisation des suspensions. Par ailleurs, et de manière

extrêmement intéressante, la densité importante de groupements hydroxyles à la surface des particules peut être utilement mise à profit pour obtenir le greffage de ligands destinés à la reconnaissance spécifique de différentes cibles dans l'organisme. Dans ce travail, des essais préliminaires d'adsorption ou de couplage covalent de ligands sur ces surfaces ont été menés. Deux lectines ont été sélectionnées en tant que ligands capables de reconnaître spécifiquement certains sucres, la lectine de *Bauhinia monandra* (BmoLL) et la lectine de *lens culinaris* (LC). Ces lectines sont respectivement spécifiques du D-galactose et du D-mannose, deux sucres présents dans les glycoprotéines disposées en quantité variable au niveau des membranes celluaires. Idéalement, il est possible d'envisager l'emploi de ces particules conjuguées à ces lectines en vue d'assurer *in vivo* le ciblage de ces entités chimiques.

Le manuscrit comprend trois parties. Dans une première partie, la stabilité de BmoLL dans les conditions d'emploi et de préparation des nanoparticules a été étudiée. Dans un premier temps, les effets des conditions de préparation des particules sur l'activité hémaglutinante de la BmoLL a été évaluée, notamment en soumettant la lectine a des étapes de sonication (30s) ou après agitation mécanique, et également lorsque la lectine se trouve sous forme lyophilisée et sous forme de solution dans un tampon citrate-phosphate. Ces études ont montré que l'activité hémaglutinante était maintenue dans ces différentes conditions. Des nanoparticules d'un diamètre d'environ 160 nm, chargées en BmoLL ont été préparées avec de la PCL, du PLA et du PLGA au moyen de la méthode de double émulsion. Les conditions de préparations ont été optimisées afin d'accroître le taux d'encapsulation. L'encapsulation la plus élevée a été obtenue avec le PLGA (50/50) (68,5% +- 5%). Les modalités d'adsorption, et en particulier les cinétiques d'adsorption, de la BmoLL ont été déterminées, montrant que l'absorption était maximale après deux heures d'incubation et qu'elle restait adsorbée au moins pendant 24 heures. Enfin, les profils de libération de la BmoLL à partir des nanoparticules ont permis de montrer que 40% de BmoLL étaient libérés après 24 heures.

La seconde partie, décrit la préparation de nouveaux polysaccharides amphiphiles possédant une structure contrôlée, obtenus par le couplage entre la fonction carboxylique de chaînes polyesters préformées et un groupe hydroxyle naturellement présent sur les polysaccharides. En premier lieu, la synthèse chimique de poly(epsilon caprolactone) monocarboxylique a été conduite par polymérisation du monomère et ouverture de cycle en présence d'un acide carboxylique (RCOOH). Le polymère obtenu (R -PCL-COOH) a

été activé par le carbonyle diimidazole de manière à former un intermédiaire imidazoline que l'on a ensuite fait réagir avec le dextran dans différents rapports molaires afin d'obtenir des copolymères amphiphiles présentant une balance hydrophile-lipophile variable (Dex-PCL_n). Les différents copolymères ont été caractérisés par la suite par GPC, ^1H NMR et FTIR. Enfin, la capacité des différents copolymères (Dex-PCL_n) à former des nanoparticules a été évaluée, puis comparée à celle de différentes poly(epsilon-caprolactone) conventionnelles (PCL 2K, 10K et 40K). Deux méthodes différentes de préparation ont été mises en œuvre pour tester la capacité d'encapsulation de la sérum albumine bovine et des lectines de *Bauhinia monandra* et de *Lens culinaris* dans ces particules. Ces essais ont permis de montrer que la lectine pouvait être efficacement encapsulée dans les particules constituées de ces polymères hydrophiles. L'aspect microscopique des nanoparticules, leur diamètre et leur potentiel zéta ont été déterminés, ainsi que les cinétiques de libération. Enfin, leur cytotoxicité vis-à-vis des cellules Caco-2 a été évaluée. Ces cellules ont été sélectionnées dans la mesure où des applications par la voie orale peuvent être envisagées.

Dans une troisième partie, la capacité de fixer différents ligands à la surface des nanoparticules composées des polymères amphiphiles a été envisagée. L'adsorption de la lectine de *Bauhinia monandra* à la surface de nanoparticules radiomarquées a permis de montrer l'affinité très importante de ces particules pour les cellules Caco-2, porteuses de glycoprotéines contenant du D-galactose, comparativement à des particules ne comportant pas ces lectines en surface. Ces résultats préliminaires démontrent ainsi le potentiel important de ces nanoparticules dans le cadre de la vectorisation et sont donc extrêmement prometteurs pour le futur.

ABSTRACT

Targeting desired organs in the body following drug administration is a very complex task, because it is necessary for the drug carrier to achieve simultaneously different tasks. Firstly, the nanoparticle have to be able to carry a significant amount of drug and to release this drug accordingly to a desired release profile. Secondly, following administration, the drug have to be able to reach the site of delivery of the drug, i.e. the targeted organ in the body, in a specific way and to subsequently deliver the drug. At present, a detailed examination of the literature demonstrates that the tools for achieving these objectives are yet to be improved. The purpose of the present work was to develop and characterize biodegradable core corona nanoparticles based on new amphiphilic copolymers for such targeting applications.

Two approaches were foreseen during the experimental work. Firstly, the possibility of loading nanoparticles with model substances of pharmaceutical interest has been investigated. Among various chemical structures, peptides and proteins are of considerable interest, since it is usually difficult to encapsulate significant amounts of drugs and to address stability problems during the preparation process. Different proteins were used as model substances including bovine serum albumin (BSA), *Bauhinia monandra* leaf lectin (BmoLL), which had demonstrated an antidiabetic activity, and *Lens culinaris* lectin (LC). Several polymeric matrixes were investigated for the entrapment of BmoLL in nanoparticles. Conventional polymers of ε-caprolactone (PCL), lactic acid (PLA), lactic acid-co-glycolic acid (PLGA), and new synthesized copolymers of dextran and caprolactone were used to achieve this goal. Secondly, the possibility of tailoring the surface properties of the particles has been investigated. The strategy consisted in preparing core/corona nanoparticles based on new amphiphilic copolymers of dextran and polyepsilon caprolactone. Compared to conventional particles based on classical polyesters such as PLA or PLGA, particles exhibiting a hydrophilic surface can be obtained which stabilizes the colloidal suspensions. Interestingly, the high density in hydroxyl groups in the surface of the particles can be used for grafting ligands intended for the recognition of targets in the body. In the present work, nanoparticles were prepared and characterized and preliminary attempts were made either to adsorb or to link covalently ligands on these surfaces. Selected ligands were BmoLL and LC lectins. These lectins are specific for D-galactose and D-mannose. These sugars are present in

glycoproteins bearing by certain cells membranes. Ideally, it is thought that the lectins conjugated particles can be used for targeting specifically the cells bearing these chemical moieties in vivo.

The manuscript consists in three experimental parts. In a first part, the stability of BmoLL concerning its conditioning forms and under manufacturing conditions of nanoparticles was verified. Initially the effect of parameters involved in the preparation of nanoparticles on the haemagglutinating activity (HA) of BmoLL was evaluated for its both forms of storage (lyophilized and citrate-phosphate buffered solution). Then, BmoLL-loaded nanoparticles were prepared with PCL, PLA, and PLGA copolymers by using a modified double emulsion method. Parameters involved in the manufacturing conditions of nanoparticles were optimized to improve BmoLL entrapment. Following, i*n vitro* kinetic release of BmoLL from nanoparticles and kinetic adsorption studies were carried out.

In a second part, new amphiphilic polysaccharides with controlled structures were synthesized by coupling between a carboxylic function of preformed polyesters chains and a hydroxyl group naturally present on polysaccharides. Firstly, the synthesis of poly (ε-caprolactone) monocarboxylic acid (R-PCL-COOH) was carried out by ring-opening uncatalyzed polymerization of monomer in the presence of a carboxylic acid (R-COOH). R-PCL-COOH was reacted with carbonyl diimidazol and the resulting activated intermediate (imidazoline) was further reacted with dextran (Dex) at different molar ratios to obtain amphiphilic copolymers with various hydrophilic-lipophilic balances (Dex-PCL_n).

Then the Dex-PCL_n copolymers were further characterized by GPC, ^1H NMR and FTIR. Finally, the ability of new Dex-PCL_n copolymers to form nanoparticles was evaluated in comparison with some conventional caprolactone polymers (PCL 2K, 10K and 40K). Two different preparation methods were tested for evaluating the encapsulation efficiency of Bovine Serum Albumine (BSA), BmoLL and LC lectins into Dex-PCL_n nanoparticles. The characterization of unloaded and loaded-Dex-PCL_n nanoparticles was evaluated through the examination of microscopic aspects, mean diameter and zeta potential measurements, *in vitro* kinetic studies, and cytotoxicity against Caco-2 cells. Additionally, the transepithelial resistance (TEER) of Caco-2 cells monolayers was measured on Transwell® filters and the bioadhesive potential of the nanoparticles was determined by incubating radiolabelled nanoparticles in the presence of Caco-2 cells.

Results concerning BmoLL stability showed no difference in the HA of lyophilized BmoLL after sonication steps of 30 sec, or after submitting it to mechanical agitation. Moreover, the HA was preserved after mixing resuspended lyophilized BmoLL in buffered solution with organic solvents. Nanoparticles with a mean diameter around 160 nm were obtained whatever the nature of the used polymer. The highest rate of encapsulation (68.5% ± 5%) was obtained by using PLGA 50/50. In vitro kinetic profile of BmoLL from nanoparticles revealed a releasing of 40% at 24h. Furthermore BmoLL was adsorbed onto nanoparticles at the first two hours and remained adsorbed over 24 h.

New biodegradable Dex-PCL_n nanoparticles presenting a hydrophobic PCL core and a dextran hydrophilic corona with potential interest for oral controlled release of bioactive compounds were successfully developed and characterized. BmoLL was successful loaded in Dex-PCLn nanoparticles. Furthermore, BSA, BmoLL and LC lectin were adsorbed into the surface of Dex-PCLn nanoparticles. Results of bioadhesion evaluation showed that the non-specific interactions of the particles with the Caco-2 cells membranes was favored in the case of the Dex-PCL particles, compared to the plain PCL particles, as result of a time dependent phagocytosis of the particles by the Caco-2 cells, following their adhesion to the cytoplasmic membrane of the cells.

In conclusion, a core/corona system based on amphiphilic copolymers has been prepared and characterized. Reasonable encapsulation levels can be obtained for proteins and surface modifications offer the possibility of preparing ligands conjugated systems of interest for targeting applications.

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1. INTRODUÇÃO

A concepção de sistemas que carreiam fármacos ou substâncias ativas para sítios de ação específicos no organismo parte do princípio que eles manteriam a estabilidade do fármaco na circulação, permitindo assim uma maior concentração do mesmo no local de ação. Esta visão, vinda desde a antiguidade, incluía os inalantes de polens de plantas medicinais, aplicação de cataplasma na pele e administração de fármacos na corrente sanguínea utilizando espinhos de plantas e bicos de pássaros, como materiais perfurantes. Além das formas farmacêuticas populares como ingestão oral de soluções, comprimidos e cápsulas (Yang & Robinson, 1998).

Os fármacos são raramente administrados como uma substância química pura, mas sempre em preparações formuladas. Há numerosas formas farmacêuticas não convencionais nas quais uma substância pode ser incorporada para o tratamento conveniente e eficaz de uma doença, promovendo interesse no desenvolvimento de sistemas de liberação controlada e sustentada de fármacos. Sistemas estes, desenvolvidos através da investigação de novos polímeros ou copolímeros e também de substâncias que promovam um melhor reconhecimento pelas células dos tecidos alvos.

As preparações farmacêuticas podem variar de uma simples solução a um complexo sistema de liberação pelo uso de aditivos apropriados ou excipientes constituintes da formulação, os quais fornecem variadas e especializadas formas farmacêuticas.

Durante as décadas de 80 e 90 vários sistemas de liberação de fármacos foram desenvolvidos com o objetivo de aumentar a eficiência do fármaco e minimizar os efeitos colaterais. Lipossomas, vesículas lipídicas, têm sido usados como potenciais carreadores de fármaco por apresentarem tais vantagens, entretanto suas aplicações são um pouco limitadas devido a problemas como baixa eficiência de encapsulação e rápida liberação de

fármacos solúveis em água na presença dos componentes sangüíneos (Soppimath *et al*., 2001). Por outro lado, nanopartículas poliméricas oferecem algumas vantagens sobre os lipossomas por aumentarem a estabilidade do fármaco e terem a capacidade de criar uma liberação controlada dependente da degradação da matriz polimérica (Hans & Lowman, 2002).

1.1 Sistemas Nanoparticulados Convencionais Carreadores de Fármacos

Nanopartículas são definidas como um sistema coloidal submicrônico (<1 μm) formadas por polímeros biodegradáveis ou não. Conforme a metodologia de preparação utilizada e as características do produto final obtido podem ser denominadas de nanoesferas ou de nanocápsulas. As nanoesferas são nanopartículas constituídas de uma matriz polimérica em que a droga está dispersa por toda a partícula (Figura 1a). Enquanto que as nanocápsulas são constituídas de um sistema reservatório na qual uma cavidade oca ou oleosa está circundada por uma fina parede polimérica, onde o fármaco encontra-se confinado na cavidade interna (Figura 1b) (Martin *et al*., 1993; Barrat, 2000).

As nanopartículas foram introduzidas nos anos 70 como potenciais carreadores para liberação controlada de fármacos por Kreuter e Speiser (1976) utilizando, na época, polímeros não biodegradáveis. A aplicação de nanopartículas no desenvolvimento de sistemas de liberação controlada de fármacos foi reavaliada na década de 80, após o advento da síntese de polímeros biodegradáveis pela equipe de Couvreur e colaboradores (Couvreur *et al*., 1978; Marty *et al*., 1978; El-Samaligy *et al*., 1985; Couvreur *et al*., 1985;Couvreur *et al.*, 1989). Posteriormente, outras equipes de pesquisas desenvolveram sistemas nano e microparticulados contendo diferentes substâncias ativas tais como proteínas, peptídeos, ácidos nucléicos, partículas virais e vacinas (Damgé *et al.*, 1997; Ferdus *et al.*, 1998; Benns & Kim, 2000); e bem como fármacos como antibióticos,

analgésicos, imunossupressivos e anticancerígenos (Fattal *et al.*, 1998; Tóbio *et al.*, 1998; Yoo *et al.*, 1999; Molpeceres *et al*., 2000).

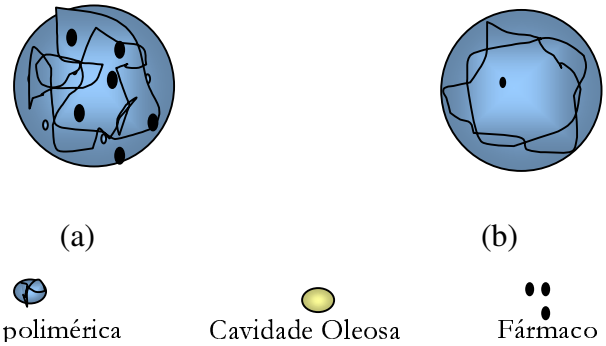

Figura 1. Tipos de nanopartículas poliméricas: (a) nanoesfera; (b) nanocápsula.

As nanopartículas podem se formuladas para liberação direcionada ao sistema linfático, cérebro, fígado, baço ou preparadas para serem administradas na circulação sistêmica prolongada. Numerosos protocolos existem na produção de nanopartículas baseado no tipo de fármaco usado e na rota de liberação. A escolha do polímero é um dos fatores mais importantes, sendo os biodegradáveis os mais indicados para compor matrizes ou cápsulas de sistemas nanoparticulados. Uma vez escolhido o protocolo, os parâmetros devem ser avaliados com o objetivo de criar as melhores características para as nanopartículas. Entre uma variedade de características quatros são considerados mais importantes: o tamanho, a eficiência de encapsulação, o potencial zeta (carga da superfície) e características de liberação do fármaco (Barrat, 1999; Hans, 2002)

1.1.1 Polímeros Utilizados no Desenvolvimento de Sistemas Nanoparticulados

Os polímeros mais utilizados são os poliésteres biodegradáveis, aprovados pela agência de vigilância sanitária americana *Food and Drug Administration* (FDA), tais

como derivados do ácido lático (PLA), do ácido glicólico (PGA), polietilenoglicol (PEG), derivados da caprolactona (PCL) e especialmente os copolímeros de ácido lático e glicólico (PLGA).

Copolímeros são polímeros compostos de várias unidades monoméricas diferentes e classificados em quatro tipos de acordo com o arranjo dos monômeros na cadeia polimérica: copolímeros de arranjo aleatório, de arranjo alternado, enxertado e em bloco (Kumar *et al*., 2001). Os polímeros de arranjo aleatório são os copolímeros que apresentam unidades repetidas de monômeros colocados de forma aleatória na cadeia polimérica. Copolímeros de arranjo alternado são sintetizados pela adição de monômeros de forma alternada. Ambos copolímeros em bloco e enxertados são compostos de vários segmentos que diferem no segmento do sítio de interligação: os copolímeros enxertados são definidos como sendo pares de homopolímeros ligados quimicamente, enquanto que os copolímeros em bloco são constituídos de estruturas ligadas nas conexões terminais (Figura 2).

Os copolímeros PEG são hidrofílicos, flexíveis e não iônicos, apresentando, portanto, excelente biocompatibilidade. Podem ser ligados covalentemente à superfície de nanopartículas, criando um revestimento hidrofílico e, por conseguinte, impedindo a captura rápida das nanopartículas pelo sistema mononuclear fagocitário (SMF). Nanopartículas revestidas com PEG são nomeadas de *furtivas*. Uma atenção crescente vem sendo demonstrada para nanopartículas furtivas como carreadores injetáveis de fármacos, os quais permitem um maior tempo de circulação sistêmica das nanopartículas (Gref *et al.*, 1994 e 1995; Peracchia *et al.*, 1997; Quellec *et al.*, 1999; Mosqueira *et al*., 2001). A síntese de novos copolímeros utilizando PEG e óxido de etileno (PEO) permitiu a utilização como revestimento hidrofílico de nanopartículas formadas com matriz de

policaprolactona (Allen *et al*., 1998, Chull Ha *et al*., 1999; Kim *et al*., 2000) ou PLA (Otsuka *et al*., 2000).

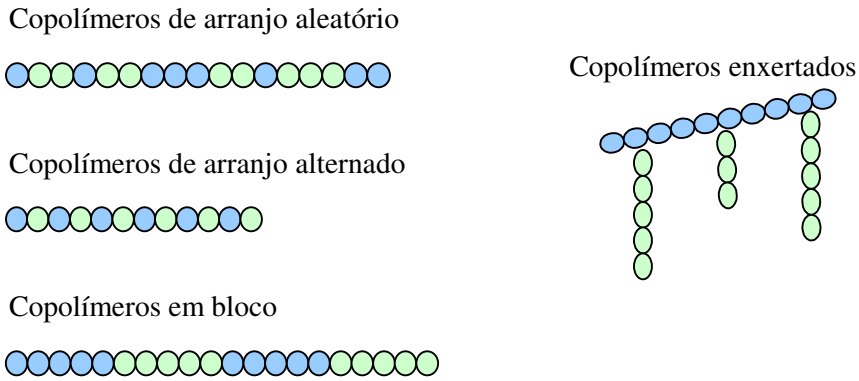

Figura 2 - Esquema da arquitetura de diferentes tipos de copolímeros utilizando duas unidades de monômeros definidas como: A = ● e B = ○ (Kumar *et al*., 2001).

1.1.2 Métodos de Preparação de Sistemas Nanoparticulados

Os métodos de preparação das nanopartículas são classificados dentro de duas categorias: formação das nanopartículas por polimerização *in situ* de unidades monoméricas ou diretamente a partir do polímero pré-formado. A polimerização segue um mecanismo aniônico, iniciada na presença de iniciadores nucleofílicos como OH^-, CH_3O^- e CH_3COO^- que levando a formação de nanopartículas com baixa massa molecular e de rápida degradação. Para resolver este problema e produzir nanopartículas de alto peso molecular e com paredes mais estáveis, a polimerização é realizada em meio ácido (pH 1,0-3,5) utilizando vários estabilizadores como dextrana-70, dextrana-40, dextrana-10 e polaxamer-188, polaxamer-184, polaxamer-237. O tamanho e a massa molecular das nanopartículas dependem do tipo e concentração do estabilizador e/ou tensoativo utilizados no processo de polimerização (Soppimath *et al*., 2001).

As primeiras nanopartículas obtidas pela polimerização de monômeros foram propostas por Birrenbach e Speiser (1976), utilizando a acrilamida e a N,N'-metileno-bis-

acrilamida, tendo como inicializador da polimerização a radiação gama. Em 1989, Couvreur e colaboradores desenvolveram nanoesferas com polímero de polialquilcianoacrilato (PACA) preparadas por polimerização de monômeros de cianoacrilato dispersos em uma fase aquosa ácida. As nanopartículas obtidas apresentaram um tamanho de aproximadamente 200 nm. Posteriormente nanopartículas foram obtidas com diâmetro reduzido de 30 a 40 nm pelo uso de tensoativos não iônicos durante a polimerização (Seijo *et al.*, 1990 *Appud* Fattal *et al*., 1998).

Vários métodos têm sido sugeridos na preparação de nanopartículas biodegradáveis de PLA, PLG, PLGA e PCL por dispersão de polímeros pré-formados. O método mais empregado é o de emulsificação seguida de eliminação de solventes. Neste método o polímero é dissolvido em um solvente orgânico como o diclorometano, clorofórmio ou acetato de etila. A droga é dissolvida ou dispersada dentro da solução polimérica e esta mistura é então emulsificada dentro de uma solução aquosa formando uma emulsão óleo / água (O/A), pelo uso de um tensoativo como gelatina, álcool polivinil, polisorbato 80, poloxamer-188 e outros. Após a formação de uma emulsão estável, o solvente orgânico é evaporado ou pelo aumento da temperatura / pressão reduzida ou por agitação constante. O método de múltipla emulsão (A/O/A) também é usado para preparar nanopartículas carregadas de fármacos solúveis em água Os dois métodos usam homogenização em alta velocidade ou sonicação (Zambaux *et al*., 1998 e 1999; Quellec *et al*., 1998; Lamprecht *et al.*, 1999).

Uma versão modificada do método de evaporação de solvente é o método de emulsificação espontânea / difusão de solvente. Uma fase oleosa é formada pela mistura de dois tipos de solventes orgânicos um solúvel em água como a acetona ou metanol, e um outro insolúvel como o diclorometano ou clorofórmio. Devido à difusão espontânea do solvente solúvel em água, uma turbulência interfacial é criada entre as duas fases levando

a formação de pequenas partículas. Como a concentração de solvente solúvel em água aumenta, uma diminuição no tamanho da partícula é observada (Gref *et al*., 1994; Peracchia *et al*., 1997).

1.1.3 Mecanismos de Liberação de Fármacos a partir de Sistemas Nanoparticulados

Os mecanismos de liberação de um determinado fármaco a partir de matrizes poliméricas são governados predominantemente por três fatores: pelo tipo e morfologia do polímero, bem como pela presença de excipientes no sistema. Várias revisões foram relatadas na literatura sobre os mecanismos e os aspectos matemáticos que regem a liberação de fármacos a partir de matrizes poliméricas (Peppas, 1997; Brazel and Peppas, 2000). De acordo com estes estudos, a liberação contínua do fármaco de uma matriz polimérica pode ocorrer por difusão, pela erosão do polímero (devido à degradação) ou pelo entumescimento. A difusão acontece quando o fármaco ou outro agente ativo passa da matriz polimérica para o meio externo, podendo ocorrer em escala macroscópica, quando acontece pelos poros da matriz ou ao nível molecular passando entre as cadeias do polímero. A maioria dos polímeros biodegradáveis é projetada para degradar como resultado da hidrólise de suas cadeias em produtos biologiamente metabolizáveis. Os polilácticos, poliglicólicos e seus copolímeros, sofrem degradação transformando-se em ácido láctico e ácido glicólico, os quais posteriormente entrarão no ciclo de Krebs, e serão degradados a carbono e água, sendo excretados pelas vias normais de eliminação. A degradação pode ocorrer de uma maneira uniforme ao longo da matriz (Figura 3a) ou em alguns polímeros, como no caso os polianidridos e os poliortoésteres, pode acontecer somente na superfície da matriz resultando numa taxa de liberação proporcional a área da superfície do sistema nanoparticulado restante (Figura 3b).

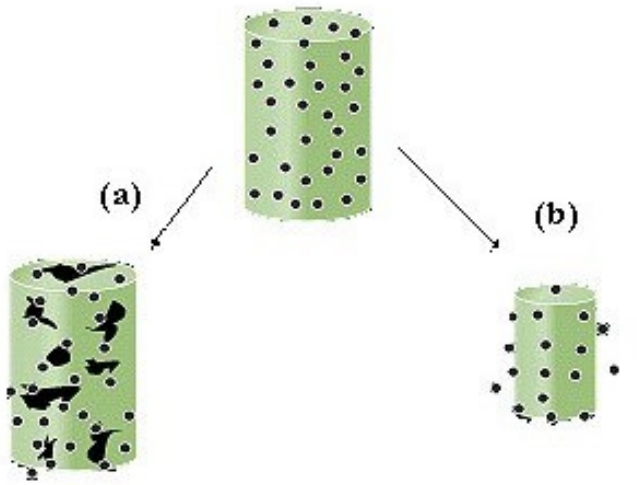

Figura 3 - Esquema da liberação de fármaco a partir de matrizes poliméricas: (a) degradação da matriz e (b) degradação da superfície do sistema (Peppas, 1997).

1.1.4 Biodistribuicão de Sistemas Nanoparticulados

É sabido que após a administração intravenosa nanopartículas são rapidamente recobertas por proteínas plasmáticas, fenômeno conhecido como opsonização. Proteínas adsorvidas ou ligadas na superfície de nanopartículas promovem o reconhecimento e captura por células fagocitárias do sistema retículo endotelial (SRE), essencialmente macrófagos (células de Kupffer no fígado e células polimorfonucleares - PMN) (Passirani *et al*., 1998; Kumar *et al*., 2001). O desenvolvimento de sistemas nanoparticulados como eficientes transportadores de fármacos no organismo é, portanto, limitado devido a rápida captura pelo SER. Os possíveis benefícios terapêuticos dependem da via de administração e conseqüentemente da distribuição das nanopartículas no organismo, a qual está relacionada com as propriedades de superfície (natureza, comprimento e densidade da cadeia polimérica).

Devido à rápida captura das nanopartículas da corrente sanguínea pelas células do SRE e PMN, a vetorização de fármacos para sítios específicos que não sejam o fígado e o baço é extremamente difícil. Para manter as nanopartículas no sistema intravascular seria necessário bloquear a atividade do SRE antes da aplicação da partícula ou fazer com que elas escapem do reconhecimento por este sistema. Com esse objetivo vem sendo desenvolvida nanopartículas com superfície modificada (furtivas) que não seriam reconhecidas pelo sistema fagocitário e que poderiam ser direcionadas a uma célula alvo.

1.2 Sistemas Nanoparticulados com Superfície Modificada (Furtivas)

A idéia de obtenção de nanopartículas biodegradáveis com superfície modificada, chamadas nanopartículas furtivas, foi investigada e modelada por Jeon e colaboradores (1991) com a proposta da criação de uma barreira estérica ao redor das partículas capaz de repelir as proteínas e assim evitar o efeito de opsonização. Efeito este que estudados nestes últimos anos por vários autores, no caso de lipossomas (Woodle & Lasic, 1992) ou nanopartículas (Greff *et al*., 1994; Vittaz *et al*., 1996; Sahli *et al*., 1999).

Inicialmente, polímeros anfifílicos foram utilizados na criação de uma barreira estérica, através da adsorção ou incorporação na superfície dos carreadores por interações hidrofóbicas, desempenhando a função de âncora com sua metade hidrofílica exposta ao meio externo, protegendo as partículas das interações indesejáveis com as proteínas plasmáticas (Torchillin *et al.*, 1995).

Um dos métodos de obtenção de nanopartículas furtivas consiste na ligação de PEG a polímeros tais como PCL, PLA ou PLGA, produzindo nanopartículas do tipo "núcleo-coroa" (Figura 4). Neste tipo de nanopartículas, as cadeias hidrofílicas do PEG estão orientadas na superfície formando um revestimento que controla a adsorção de proteínas e regula a ação de células do SRE na superfície das partículas (Greff *et al*., 1994, Otsuka *et al*., 2000).

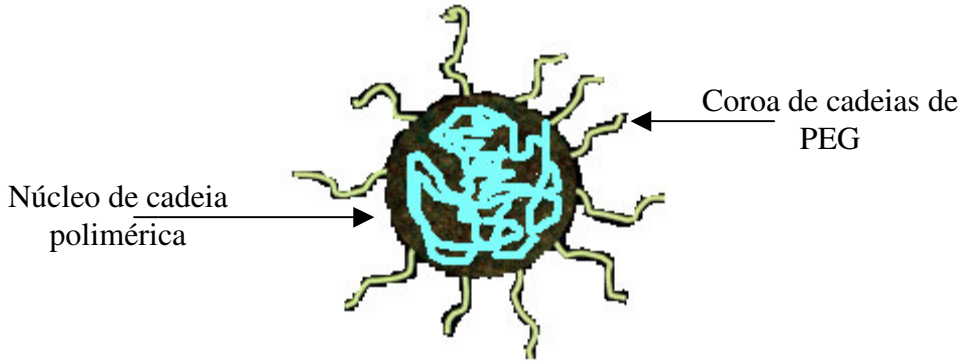

Figura 4. Esquema de nanopartícula do tipo núcleo-coroa formada por copolímero PEG-R, onde R é um polímero hidrofóbico constituinte do núcleo.

Polissacarídeos também são capazes de modificar a superfície dos sistemas micro e nanoparticulados modulando a ação do sistema complemento e mediando o reconhecimento, por meio de carboidratos e receptores específicos nas células. Um grande número de polissacarídeos são estudados como potencial aplicação em sistemas carreadores de farmacos, como a quitosana, pectina, ciclodextrina, dextranas e outros (Ohya *et al*., 2000; Sinha & Kumria, 2001). A dextrana tem sido usada combinada com PEG (Coombes *et al*., 1997), com poli (metacrilato de vinila) (Passirani *et al*., 1998) ou com PLA (Rouzes *et al*., 2000) na produção de micro e nanopartículas com capacidade de favorecer a interação de fármacos e vacinas com sítios específicos, reduzindo a adsorção de proteínas circulantes.

A modificação da superfície de nanopartículas furtivas por adsorção de proteínas, lectinas, anticorpos, carboidratos e vitamina B12, possibilitando assim interações específicas entre ligantes e receptores, é também relatada (Olivier *et al.*, 1995; Ponchel *et al.*,1998; Breitenbach *et al*., 2000). Interações bioadesivas com superfícies da mucosa podem ser construídas por interações não específicas dependendo das propriedades físico-

químicas do polímero ou no desenvolvimento das interações específicas. Os sistemas bioadesivos têm sido preparados por conjugação de ligantes aderidos ao polímero ou às partículas, incluindo nanopartículas (Irache *et al*., 1994; Ezpeleta *et al.*, 1996; Hussain *et al*., 1997).

De uma série de moléculas de reconhecimento as lectinas são um dos ligantes mais promissores, nos últimos anos as lectinas têm sido de grande interesse farmacêutico devido à sua inerente habilidade em promover ligação específica em superfícies biológicas, através de resíduos de carboidrato, localizados na superfície de células epiteliais. A superfície de todas as células é composta de lipídeos e proteínas, e em muitas células carboidratos ligados a estas proteínas e lipídeos participam dos processos celulares incluindo o reconhecimento célula-célula, as respostas aos sinais extracelulares e também o desenvolvimento celular(Irache, *et al*., 1994; Ezpeleta *et al.*, 1996; Chen, *et al*., 1996; Gabor, *et al*., 1997).

1.2.1 Lectinas como Moléculas Modificadoras de Superfície de Nanopartículas

Lectinas foram originalmente descritas em 1954 por Boyd e colaboradores como substâncias contidas em extratos de plantas, capazes de aglutinar células vermelhas. A promoção de aglutinação de eritrócitos por extratos de plantas foi verificada por Stillmark em 1888, estudando fatores de toxicidade em *Ricinus communis* e em proteína de *Abrus precatotrius* (*Appud* Kennedy *et al*., 1995). Em 1980, lectinas foram consideradas por Goldestein e colaboradores como proteínas de origem não imune que se ligam a carboidratos, capazes de aglutinar células e/ou precipitar glicoconjugados. Atualmente, o termo lectina é aplicado para descrever uma classe de proteínas estruturalmente muito diversa que não são enzimas nem anticorpos, caracterizadas pela sua habilidade em ligar à carboidratos com considerável especificidade (Rini, 1995; Vijayan & Chandra, 1999).

As fontes de obtenção de lectinas são bem diversificadas, podendo ser encontradas em microorganismos, animais e plantas. As primeiras lectinas descobertas foram de origem vegetal presente em grãos de legumes como as lentilhas e ervilhas. Nas últimas décadas, lectinas têm sido isoladas de bulbos, rizomas e sementes das famílias das monocotiledôneas Amaryllidaceae, Orchidaceae e Alliaceae; sendo subdivididas de acordo com a natureza do carboidrato inibido, incluindo manose/glicose, N-acetilglicosamina, N-acetilgalactosamina, galactose, fucose e ácido N-acetilneurâminico ou grupo do ácido siálico. Esta característica determina a especificidade dos diferentes tipos de lectina (Kennedy *et al*., 1995, Ponchel *et al*., 1998). A afinidade entre a lectina e seu receptor pode variar devido a pequenas mudanças na estrutura do carboidrato presente no receptor. Estas interações são muito importantes em diferentes funções biológicas, como reconhecimento, adesão e comunicação entre células (Yamazaki *et al.*, 1998).

Existem dois tipos de lectinas bem caracterizados. Lectinas Ca^{2+} -dependente, chamadas lectinas tipo-C, muitas das quais estão associadas a asiaglicoproteínas e as tiol-dependentes ou as chamadas lectinas tipo-S, as quais são específicas para β-galactosídeos e são solúveis sem detergentes (Yang & Robinson, 1998).

Uma das características conhecida das lectinas é a capacidade de reconhecer os resíduos de carboidratos presentes na superfície das células de mamíferos com um alto grau de especificidade. Utilizando-se dessa especificidade, a sua aplicação em estudos biológicos vem se desenvolvendo ao longo dos anos em diferentes áreas. Aplicações nos estudos de aglutinação de eritrócitos e grupos sanguíneos, estudos histoquímicos e experimentos cromatográficos, como reagentes para detecção citoquímica de glicoconjugados na superfície de células de mamíferos (Beltrão *et al*., 1998; Wang *et al.,* 2000), e em sistemas carreadores de fármacos, protegendo os fármacos degradáveis por

proteínas ou peptídeos da ação do suco intestinal e tendo como alvo as células M (Naisbett & Woodley, 1994; Chen *et al*., 1996 ; Hussain *et al*., 1997; Garbor *et al*., 1998).

Uma das lectinas mais estudadas é a lectina extraída do feijão *jack bean*, *Conavalia ensiformis* (Con A) (Agrawal & Goldstein, 1965; Edelman *et a*l., 1972). Em 1996 Leist & Wendel demonstraram que a Con A estimula receptores de membrana e com subsequente desestruturação do citoesqueleto de hepatócitos de ratos. Em 1998 Pryme e colaboradores estudando a lectina do feijão roxo *Phaseolus vulgaris*, observaram que esta lectina apresentou a capacidade de diminuir a massa de tumores (linfoma) em ratos, pela redução na disponibilidade dos fatores de crescimento e nutrientes necessários ao desenvolvimento do tumor.

Vários trabalhos na literatura demostram o uso de lectinas no campo da tecnologia farmacêutica como uma estratégia para a vetorização de fármacos mediada por lectinas (Wirth *et al*., 1998; Yamazaki *et al.*, 1998, Ezpeleta *et al*., 1999; Serizawa *et al*, 2001; Kompella & Lee, 2001). Diferentes técnicas são aplicadas nestes sistemas conjugados principalmente como bioadesivos e terapia do câncer. Irache e colaboradores (1994) ligaram covalentemente várias lectinas de plantas, incluindo a lectina do tomate (*Lycopersicon esculentum*), a nanopartículas modificadas de poli-estireno (látex), as quais se ligaram irreversivelmente à mucina gástrica de porco.

A conjugação de lectina à nanopartículas poliméricas se mostra eficiente por aumentar as interações com o muco (Florence *et a*l., 1997), e/ou com células epiteliais . Podendo ser usadas para atingir áreas espécíficas do tracto gastrointestinal, devido à distribuição heterogênea dos açúcares ao longo do intestino normal ou doente (Ponchel, *et al*., 1998).

Semelhantemente aos processos biológicos conduzidos pelas interações proteícas, como a sinalização celular, replicação de DNA, expressão gênica, e o tráfico de alguns

componentes celulares nos vários compartimentos da célula, proteínas poderiam então ser usadas no controle de liberação de fármacos em sítios específicos no organismo. A função central dessas interações proteícas poderia produzir um controle sobre a ligação, estabilização, e a liberação do fármaco (Yamazaki *et al*., 2000).

A associação de ligantes com lipossomas ou nanopartículas pode ser realizada por diferentes procedimentos, incluindo ligações covalentes e processo de adsorção. As ligações covalentes são ideais, pois são mais estáveis que os processos de adsorção e não afetam a especificidade do ligante. Dependendo da natureza química do grupo funcional localizado na superfície do carreador, os métodos mais utilizados para ligação de lectinas são técnicas com glutaraldeído e carbodiimida. (Irache *et al*., 1994; Ponchel *et al.*, 1998; Khopade et al., 1998).

A ligação com o glutaraldeído ocorre pela formação de uma base de Schiff entre um grupo amino, localizado na superfície da partícula e uma função aldeído fornecida pelo reagente. Assim, este grupo ativado tendo a função aldeído pode reagir com a lectina. A técnica com a carbodiimida envolve a ativação de grupos carboxilícos localizados na superfície das partículas os quais podem reagir com grupos amino livres da cadeia polipepitídica da lectina (Ponchel *et al.*, 1998).

Apesar do campo de sistemas de liberação de fármacos mediado por lectinas ser recente uma variedade de estudos confirmam a preferência por essa técnica. Trabalhos futuros terão como desafios aumentar a estabilidade *in vivo* do fármaco até que este atinja as células ou órgãos alvos. Numerosas técnicas em produzir lipossomas com superfície modificada foram desenvolvidas [Meynburg *et al*., 2000; Meyer et al., 1994; Ezpeleta et al., 1999; Forseen & Willis, 1998]. Em contraste, algumas dificuldades técnicas permanecem na tecnologia de nanopartículas biodegradáveis lectin-conjugada; as quais

poderiam ser solucionadas pelo desenvolvimento de novos polímeros biodegradáveis, capazes de melhorar a ligação covalente de lectinas à superfície das nanopartículas.

O interesse no desenvolvimento de sistemas de liberação controlada de fármacos preparados com polímeros biodegradáveis vem aumentando nos últimos anos, devido à possibilidade de fabricação de carreadores coloidais nanométricos para administração parenteral. Estes sistemas promovem uma liberação controlada, um aumento da eficácia e a redução da toxicidade de fármacos encapsulados ou dispersos em matrizes poliméricas. Carreadores coloidais de fármacos envolvem principalmente emulsões submicrônicas, lipossomas, complexos lipídicos e nanopartículas. Nanopartículas, sistemas poliméricos sólidos nanométricos, é o nome geral para descrever nanoesferas e nanocápsulas. Um dos objetivos em nanotecnologia farmacêutica é obter nanopartículas para direcionamento específico de fármacos para sítios alvo. Neste caso é requerida uma modificação da superfície das nanopartículas pela ligação com compostos tais como proteínas, lectinas, anticorpos e carboidratos que possibilitem interações específicas entre ligantes e receptores.

3. PUBLICAÇÕES

NANOPARTÍCULAS CONTENDO LECTINA DE FOLHA DE *BAUHINIA MONANDRA* (BmoLL) ENCAPSULADA

Trabalho submetido ao **International Journal of Pharmaceutics**

NANOPARTICLES LOADED WITH *Bauhinia monandra* LEAF LECTIN (BmoLL)

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ABSTRACT

The effect of parameters involved in the fabrication of nanoparticles on the haemagglutinating activity (HA) of Bauhinia monandra leaf lectin (BmoLL) was evaluated. BmoLL-loaded nanoparticles were prepared using different polymers. Parameters involved in nanoparticle preparation were optimized, and an in vitro kinetic study was carried out. No difference was observed in the HA of lyophilized BmoLL after sonication steps of 30 sec, or after submitting it to mechanical agitation. Moreover, the HA was preserved after mixing resuspended lyophilized BmoLL in buffered solution with organic solvents. Nanoparticles with a mean diameter around 160 nm were obtained whatever the nature of the used polymer. The highest rate of encapsulation (68.5% ± 5%) was obtained by using PLGA 50/50. In vitro kinetic profile of BmoLL from nanoparticles revealed a releasing of 40% at 24h. Furthermore BmoLL was adsorbed onto nanoparticles at the first two hours of incubation and remained strongly adsorbed over 24 h.

1. INTRODUCTION

Lectins are carbohydrate-binding proteins widely used in biochemical, immunochemical, and histochemical studies. They are proteins of recognition with a ubiquitous distribution in nature, which are involved in a variety of biological processes, such as cell-cell and host-pathogen interactions, serum glycoproteins turnover, and innate immune response (Kennedy *et al*., 1995; Vijayan and Chandra, 1999; Coelho and Silva, 2000). These characteristics have attracted the interest of pharmaceutical researchers especially due to their inherent ability to provide specific binding to biological surfaces bearing sugar residues located at the surface of cells (Irache *et al*., 1994; Ezpeleta *et al*., 1996; Ponchel and Irache, 1998; De Wolf and Bret, 2000). Lectins are found in microorganisms, animals and plants, and they have been purified from leaves, fruits, roots, tubers, and from seeds. Lectins can be classified according to the nature of the inhibitors sugar, mannose/glucose, N-acetylglucosamine, N-acetylgalactosamine, galactose, fucose and N-acetyneuraminic or sialic acid group (Kennedy *et al*., 1995; Ponchel and Irache, 1998; Yamazaki *et al*., 1998).

Their specificity, multivalent featured, as well as their non-immunogenic and bioadhesive properties make lectins appealing candidates for developing a third-generation of site-specific nanosystems as carrier for drug delivery (Kompella and Lee, 2001). Two approaches have been applied to bring proteins in therapy. In the first case, the protein itself presents biological activity and it can be encapsulated or incorporated into nanosystem such as liposomes or nanoparticles. In many conventional systems, the control of drug release is based on the encapsulation and/or non-specific reversible interactions between the carrier and the active agent. On the other hand, proteins, much specifically lectins, could also be binding at the surface of nanodispositives for site-specific drug targeting. One of the crucial and pervasive dilemmas in the human therapy

is to achieve a satisfactory balance between the drug toxicity and its therapeutic effect. In this way, site-specific delivery could prevent possible toxic effect at non-target sites and increase the efficacy of the therapeutic agent. Protein delivery from biodegradable polymeric systems has been a challenging area of research. In a first approach, nanospheres should be seemed inappropriate for encapsulating water-soluble compounds due to their reduced size and the core hydrophobic character. However it has been recently reported that protein-loaded "stealth" nanospheres containing human serum albumin (HSA) was obtained (Quellec *et al*., 1998, 1999). BSA-loaded nanospheres were prepared by using an adaptive double-emulsion method for the fabrication of microspheres (Blanco *et al*., 1997). Moreover, Tetanus toxoid, an antigen with a high molecular weight (150,000 g/mol), was also efficiently entrapped within nanospheres with a mean diameter of 130-150 nm (Tobío *et al*., 1998). However, one of the largest problems of protein-release technology is the loss of activity caused by denaturation and deactivation of protein during the formulation process of the nanoparticles (Hora *et al*., 1990). All preparation methods for production of nanoparticles involve the use of organic solvents and sonication steps, which can be deleterious for the biological activity of the entrapped proteins. When exposed to environmental changes, such as heating and exposure to ultrasounds and organic solvents, proteins are generally denatured, losing their biological activity (Hora *et al*., 1990).

Lectins might be implicated in the pharmacological activity of different genus of plants useful in the popular medicine for the treatment of diseases such as cancer and diabetes. For instance, the genus Bauhinia (Febaceae) contains a number of ornamental species, which are well distributed in Brazilian cities, have been used as forage, as human food and in popular medicine for the treatment of diabetes and as a diuretic agent. Recently, a leaf Bauhinia monandra lectin (BmoLL) was purified and showed

specificity for D (+)-galactose (6.25 mM), D (+)-raffinose (6.25mM) and methyl-β-D-galactopyranoside (25 mM). Native BmoLL was apparently homogeneous by PAGE (Coelho and Silva, 2000).

Bearing in mind a further application of BmoLL on pharmaceutical nanotechnology either as a bioactive agent or surface-specific ligand, the effect of manufacturing conditions involved in the fabrication of nanoparticles on the haemagglutinating activity of BmoLL was evaluated. In addition, the entrapment of BmoLL in nanoparticles made of biodegradable polyesters was optimized. Besides, in vitro kinetic pattern of BmoLL from different formulations of nanoparticles was determined.

2. MATERIALS AND METHODS

2.1 Materials

The nanoparticles were prepared with different biodegradable polymers such as poly D,L-lactic-co-glycolic acid (PLGA, 50/50 and 75/25; MW 21,000 and 26,000 Da, respectively), poly ε-caprolactone (PCL; MW 40,000, 10,000 and 2,000 Da) and poly lactic acid (PLA 50, MW 42,000 Da) were purchased from Birmingham Polymers (Alabama, USA), and Aldrich (Steinheim, Germany) respectively. Polyvinyl alcohol (PVA, MW 20,000), Sodium cholate (SC) (MW 430.6 Da), and Bovine Serum Albumin (BSA-fraction V, MW 65,000 Da) were furnished from Sigma (USA). The BmoLL lectin was purified and characterized as described by Coelho and Silva (2000). All the other chemical reagents were obtained from Merck (Darmstadt, Germany) and were of analytical grade.

2.2 Stability of BmoL

The influence of storage conditions on BmoLL heamagglutinating activity (HA) was evaluated. Two different samples of BmoLL, one in citrate-phosphate buffered solution at pH 6.5 (protein concentration of 0.45mg/ml) and other lyophilized (protein concentration of 1.6 mg/ml) were tested and the stability of the ascertained preparations.

A pre-formulation study was carried out before fabrication of nanoparticles for evaluating the influence of BmoLL exposure to sonication, ultra-turrax agitation and organic solvents on its heamagglutinating activity.

2.2.1 Haemagglutinating activity of BmoLL

The evaluation of the HA of BmoLL was performed with glutaraldehyde-treated rabbit erythrocytes as previously described (Bing *et al*., 1967). Briefly, samples of BmoLL lectin (50µL) were two fold serially diluted in 0.15M NaCl, in microtiter plates. An erythrocyte suspension (2.5% v/v in 0.15M NaCl, 50µL) was added and the titer was read after 45 min. The HA was defined as inverse of the lowest sample dilution that exhibited the last visible red cell agglutination (Correia and Coelho, 1995; Arangoa *et al*., 2000).

2.2.2 Effect of the exposition to nanoparticle manufacturing conditions on the Haemagglutinating activity of BmoLL

250 µl of BmoLL in water or citrate-phosphate buffered solution at pH 6.5 (41 µg/ml), were added to 2 ml of distilled water and submitted to sonication (vibra-cell Branson, 200 W with 40 cycles/sec) or mechanical agitation (ultra-turrax T25, IKA, Germany) at 18,000 rpm for 30 or 60 seconds. Then, the haemagglutinating activity of BmoLL was determined as described above.

The effect of organic solvent on the haemagglutinating activity of BmoLL was evaluated as following. 100 µl of BmoLL in water or citrate-phosphate buffered solution at pH 6.5 (41 µg/ml), 2 ml of organic solvent (methylene chloride or acetone), and 4 ml of distilled water were mixed together. The organic solvent was evaporated through stirring. Then, haemagglutinating activity of BmoLL was determined.

2.3 Preparation of BmoLL-loaded nanoparticles

A pre-formulation study was developed with different batches of unloaded nanoparticles prepared by using a modified multiple emulsion ($W_1/O/W_2$) method as previously described (Quelec *et al*., 1998). Sonication was used in the two-step emulsification process, in order to reduce the particle size of nanoparticles to a submicronic range. Varying type and concentration of constituents optimized the formulation of nanoparticles. The volume of the organic phase was 0.5 or 1 ml. The volume of the internal aqueous phase was varied from 0.025 to 0.5 ml. The external aqueous phase (1 or 2 ml) was constituted of 0.3% PVA or 0.6% SC. The final volume of the dispersant phase was 10, 25 or 50 ml containing 0.1% PVA or 0.1% SC.

According to the stability of nanoparticles previously obtained, PCL 2,000 Da, PLGA 50/50 and 75/25, and PLA 50 were chosen to prepare BmoLL-loaded nanoparticles, which are produced as follows. First, an emulsion was prepared with 0.5 ml of a polymer dissolved in methylene chloride (25 mg/ml) and 0.5 ml of BmoLL suspended in citrate-phosphate buffered solution was added under vigorous stirring by vortex during 1 min. Then, the emulsion was sonicated for 20 sec (CV 145 sonicator, Vibra cell France) at 40 W for 20 sec in an ice bath. A double emulsion W/O/W was obtained by adding 1 ml of sodium cholate solution (0.6% w/v) under vigorous stirring as previously described. The nanoparticle suspension was then sonicated for 30 sec and dispersed in 10 ml of sodium

cholate solution (0.1% w/v). The organic solvent was rapidly eliminated by evaporation under reduced pressure, and residues of SC were eliminated by dialysis against distilled water (membrane cut-off: 10,000 Da) during 12 h. Finally, the nanoparticles were collected by centrifugation (L-55 centrifuge, Beckman, USA) at 144,000 g for 30 min and washed twice with water before lyophilization.

2.4 Characterization of BmoLL-loaded nanoparticles

The mean diameter of particles and size distribution were determined in water at 20°C by photon correlation spectroscopy (PCS) using a Nanosizer N4-MD® (Coulter, France). The surface charge of nanoparticles diluted in 10 mM NaCl solution was evaluated by measuring the Zeta Potential using a Zetasizer (4® Malvern, UK). The morphological examination was performed using scanning electronic microscopy (SEM) after fixation and metallization of nanoparticles with colloidal gold. The amount of non-entrapped BmoLL was determined by Lowry-Peterson protein assay (Peterson, 1997) in the supernatant obtained after two ultracentrifugation and washing of nanoparticles. A calibration curve was prepared by using BSA as standard in a concentration range of 10 to 100 µg/ml. This procedure permits analysis of very dilute protein solutions with removal of most interfering substance (Quellec *et al*., 1998).

2.5 IN VITRO RELEASE KINETIC PROFILES OF BMOLL-LOADED NANOPARTICLES

Samples of 10ml of BmoLL-loaded nanoparticles [160µg/ml] were suspended in 10 ml of phosphate buffer saline (PBS, pH 7.4) containing 0.1% (w/v) sodium azide and incubated at 37°C under moderate magnetic stirring. At predetermined time intervals, one

sample was withdrawn and centrifuged at 144,000 g for 30 min. The supernatant was removed (1 ml) and the released BmoLL was measured as previously described.

2.6 In vitro adsorption kinetic profiles of BmoLL onto nanoparticles

The adsorption of BmoLL onto the surface of nanoparticles was evaluated. Lyophilized unloaded-nanoparticles (4 mg) were resuspended in 10 ml of 10 mM phosphate buffered saline (pH 7.4) containing 72 µg of lectin, and incubated under magnetic agitation at room temperature. At different time intervals, an aliquot of 1.2 ml was collected and submitted to centrifugation at 144,000 g for 30 min. The free BmoLL on the clear supernatant was determined by Peterson method as described above. The amount of adsorbed BmoLL onto the surface of nanoparticles was calculated from the values of the dosed BmoLL in the supernatant.

3. RESULTS AND DISCUSSION

3.1 Stability of BmoLL

Effective biopharmaceutical product development requires a good understanding of the degradation mechanism of the macromolecule of interest. As degradation may occur during the production, isolation, purification, formulation, storage and delivery of the macromolecule, it is required for the formulation scientist to work in a multidisciplinary team for developing adequate bioprocessing and analytical methodologies (Parkins and Lashmar, 2000). Many macromolecules have a relatively short shelf life in aqueous medium, which can lead to both poor bioavailability and poor stability in the final product. Factors such pH, ionic strength, buffer composition, other excipients and temperature may

all affect the mechanism of degradation, and should be evaluated (Parkins and Lashmar, 2000).

The influence of conditioning of BmoLL in citrate-phosphate buffered solution or in a lyophilized form on its specific-haemagglutinating activity (sHA) was evaluated as shown in Table 1. It can be observed that sHA remained at 320 ng/ml after submission of lyophilized BmoLL to stressful conditions such as ultrasounds, ultra-turrax agitation or solvents, except for a long exposition to ultrasounds during 60 sec (641 ng/ml). In contrast, a reduction of twice on sHA was observed for BmoLL in buffered solution, after exposition to 30 sec (641 ng/ml) of ultrasounds or ultra-turrax agitation. Similar results were found by Zambaux and co workers (1999) when investigating the influence of the sonication time on the activity of protein C loaded in PLA-nanoparticles, observing a 50% reduction on its anticoagulant activity.

It is well known that during the manufacture of nanoparticles by emulsification based on solvent evaporation method, proteins, which are generally surface active, tend to migrate to the interface between the aqueous and organic phases. At the interface, usually, the molecule of proteins may unfold provoking modifications on their structure or conformation (Zhou *et al*., 2001). In fact, many works showed protein modification due to the harsh manufacture conditions of micro and nanoparticles by emulsification process (Lu *et al*., 1995, Uchida *et al.*, 1996, Zambaux *et al*., 1999). Emulsification is a crucial step in the preparation process and it is obtained by sonication. In fact, sonication induces an increase in temperature, which is moderated by the use of an ice bath. Moreover, during sonication, cavitations occur and can induce conformational changes of proteins that can disturb their activity (Niven *et al*., 1995, Zambaux, *et al.,* 1999).

The BmoLL activity was not affected in the presence of the methylene cloride or the methylene cloride and acetone mixture. These results are contradictory to those ones

obtained by Zambaux and co workers (1999) when the presence of the solvent mixture improved the anticoagulant activity of protein C, which was two fold great than with methylene cloride. However, it was showed that proteins suffered denaturation in aqueous organic mixtures but not in pure organic solvents (Gribenow and Klibanov, 1996). The hypothesis that proteins remain folded and active in organic solvents is counterintuitive. It was related that proteins are stable in some organic solvents (Mattos and Ringe, 2001).

Therefore, results showed that the BmoLL conserved its activity under various experimental nanoparticles manufacturing conditions. Moreover, it seems that BmoLL should be lyophilized to preserve its characteristics during storage.

3.2 BmoLL-loaded nanoparticles

The nanoparticles production deals with several independent variables of formulation and manufacturing, such as the surfactant/stabilizer type, the molecular weight and the concentration of the polymer. Considering polymeric nanoparticles, the particle size and the encapsulation efficiency are two more important characteristics. Another extremely relevant parameter for understanding the nanoparticle stability is the zeta potential, which determines the surface charge of particles (Blanco *et al*., 1997; Song *et al*., 1997; Feng *et al*., 2001, Hans and Lowman, 2002).

Parameters involved in the preparation of the unloaded-nanoparticles (organic and aqueous phase ratio, type of polymers and concentration surfactants) were optimized in order to obtain a formulation presenting monodispersed particles with a diameter lower than 200 nm (Table 2). Certainly, the use of SC as surfactant in the external phase, contributed for the production of (200 nm)-sized nanoparticles. Similar results were previously reported by Zambaux and colleagues (1998, 1999).

The use of PVA promoted nanoparticles with a diameter of about 400 nm. These findings corroborated some data described by Lamprecht and collaborators (2000, 2001). It is well known that the manufacturing of nanoparticles by using emulsification techniques required the use of surfactants to stabilize simple or multiple emulsions formed during the process. Addition of surfactants in the continuous phase reduces the "caking" current problems, which take place during the solvent evaporation process. In fact, the gradual reduction of the volume of the dispersion phase promoted an increase on the medium viscosity, affecting the balance of the size of dispersed droplets. This phenomenon promotes the coalescence and the agglomeration of the droplets (Murakami *et al*., 1999). PVA is one of the most employed surfactants. However, it has been demonstrated that PVA residues remain bounded to the surface of particles and they are hard to be totally removed (Zambaux *et al.*, 1998 and 1999). On the contrary, sodium cholate (SC), which is effective to produce nanoparticles, has the advantage to be more easily removed by washing after preparation. However, the resuspension in water of the nanospheres prepared with SC seems to be difficult. Apparently, this problem can be solved by a dialysis process against water (8 hours) before the recovering of nanoparticles by centrifugation (Quellec *et al*., 1998).

BmoLL was efficiently loaded in nanoparticles of PLA 50, PCL or PLGA. Sodium cholate was used as surfactant in the continuous aqueous phase. The nanoparticles were prepared by the double emulsion method.

3.3 Characterization of BmoLL-loaded nanoparticles

BmoLL-loaded nanoparticles were obtained with a mean diameter varying from 143 ± 47 nm to 163 ± 47 nm for PLA 50, PCL or PLGA (Table 2). The highest rate of BmoLL encapsulation was obtained by using the copolymer PLGA 50/50 (69% ± 5%), whereas

the nanoparticles prepared with PCL 2,000 Da presented the lowest encapsulation rate (40% ± 8%). Thus, a loading about 1.5 μg of protein/mg of polymer were obtained. However, a lowest polydispersity index (0.09) was obtained with PLA 50 (42,000 Da) for 58% BmoLL encapsulation ratio.

It can be observed that the mean diameter of nanoparticles and the BmoLL encapsulation efficiency were both dependent on the polymer molecular weight (Table 2). In fact, PCL (2,000 Da) promoted the smallest BmoLL encapsulation rate (40 %) and the most charged surface (ξ = - 4.5 mV). This suggests that Bmoll was preferentially absorbed onto the surface of nanoparticles. In contrast, BmoLL encapsulation rate was increased 20% in PLA in comparison with PCL nanoparticles. Moreover, loaded PLA-nanoparticles presented a small zeta potential (ξ = - 8.9 mV), indicating that BmoLL was preferentially loaded in the polymeric core of nanoparticles. Although these nanoparticles presented a slight higher mean diameter size (151 ± 51 nm) than PCL (143 ± 47 nm), they displayed a smaller polydispersity index (0.09). Therefore, PLA50 promoted a monodisperse size distribution of particles. A higher BmoLL encapsulation efficiency was found with PLGA 50/50 (69 %) and a smaller size diameter (155 ± 51 nm) when compared to PLGA 75/25 nanoparticles, which promoted an encapsulation efficiency of 58 % and a mean diameter of 163 ± 47 nm. Corresponding zeta potential values were –6.8 mV and –5.6 mV, suggesting that BmoLL was more adsorbed at the surface of PLGA 75/25 than at the surface of PLGA 50/50.

The morphological aspects of the unloaded and BmoLL-loaded nanoparticles are displayed in Fig. 1. Nanoparticles containing BmoLL presented submicronic size, well-defined spherical shape, and a relative monodispersity of particle size distribution.

3.4 in vitro release kinetic profiles of bmoll-loaded nanoparticles

The BmoLL release characteristics of PLGA (75/25,50/50), PCL 2K e PLA50 nanoparticles were investigated in vitro. In vitro kinetic profile of BmoLL from nanoparticles revealed a releasing of 40% at 24h, independently of the used polymer (Fig. 2). A more or less pronounced burst release was observed during the first two hours and tends to a plateau after eight hours. The burst affect could be attributed to the immediately release of BmoLL molecules adsorbed at the surface of nanoparticles. After the initial burst, BmoLL release profile displayed a sustained behavior, especially from PLGA 50/50 nanoparticles, which presented the highest BmoLL entrapment in the polymeric core. The sustained release pattern can be attributed to the diffusion of BmoLL through the polymeric matrix as well as some polymer erosion. Such erosion was more pronounced for PLGA 75/25 and PCL nanoparticles. As noticed, drug release from polyesters, such PLA, PLGA and PCL, is generally controlled by both drug diffusion and polymer erosion. Hydrophilic drugs with molecular weight above 0.5 kg/mol, such as peptides and proteins, have especially shown in vivo plasma level/time curves with clear discontinuities. In an initial phase, release occurs predominantly by diffusion through an interconnecting network formed by the dissolving drug substance itself. The second release phase is governed by the polymer degradation (Zambaux *et al*., 1999, Breitenbach *et al*., 2000, Li *et al*., 2001).

3.5 IN VITRO ADSORPTION KINETIC PROFILES OF BMOLL-LOADED NANOPARTICLES

The kinetic patterns of BmoLL adsorption on the surface of nanoparticles is displayed in Fig. 3. It can be observed that 30-45% of BmoLL is adsorbed onto nanoparticles at the first two hours and it remained adsorbed for all the experiments (24 h). Apparently PLGA

75/25 and PCL promoted a higher "affinity" to BmoLL than PLA 50. These findings corroborate the analysis of surface change by the zeta potential values (Table 2). In fact, PCL and PLGA 75/25 nanoparticles presented higher ζ values (-4.5 and –5.9 mV, respectively) and the highest BmoLL adsorption (40 and 45%, respectively).

4. Conclusion

Results suggested that the lectin haemagglutinating activity was more dependent on the BmoLL storage conditions than to the manufacturing process of nanoparticles. Moreover, BmoLL was efficiently encapsulated into nanoparticles of size about 160 nm. The in vitro kinetic profile revealed that BmoLL-loaded nanoparticles could be used as controlled release systems. Currently studies are carrying out in our laboratory to evaluate the bioavailability of BmoLL-loaded nanoparticles using a Europium derivative as an alternative marker.

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Table 1. The evaluation of the effect of time exposure to ultrasounds, mechanical agitation and organic solvents on the specific haemagglutinating activity of BmoLL. Assessment of the influence of storage conditions (lyophilized or in 10 mM citrate-phosphate buffered on the stability of BmoLL.

		Storage of BmoLL Sample			
		Lyophilized		Buffered solution	
BmoLL exposition	**Time (sec)**	**sHA**	**[BmoLL] (ng/ml)**	**sHA**	**[BmoLL] (ng/ml)**
	0	3,129.58	320	3,129.58	320
ULTRASOUNDS	**30**	3,129.58	320	1,564.79	641
	60	1,564.79	641	1,564.79	641
	0	3,129.58	320	3,129.58	320
ULTRA-TURRAX	**30**	3,129.58	320	1,564.79	641
	60	3,129.58	320	1,564.79	641
Solvents	**Methylene chloride**	3,129.58	320	3,129.58	320
	Methylene chloride/acetone	3,129.58	320	1,564.79	641

BmoLL concentration at 40.9 µg/ml in 10 mM citrate phosphate buffered solution (pH 6.5) solution, pH 6.5).
sHA = specific Haemagglutining Activity
[BmoLL] = concentration of BmoLL corresponding to sHA

Table 2. The evaluation of the influence of polymer on the mean diameter, polydispersity index and BmoLL encapsulation efficiency into nanoparticles.

Polymer	**Mean Diameter (nm ± SD)**	**Polydispersity Index**	**BmoLL encapsulation Ratio (%)**	**Zeta Potential (mV)**
PLA 50 (42,000 Da)	153 ± 51	0.09	58 ± 5	- 4.5
PCL (2,000 Da)	143 ± 47	0.22	40 ± 8	- 8.9
PLGA 50/50	155 ± 49	0.19	69 ± 5	- 6.8
PLGA 75/25	163 ± 47	0.14	58 ± 8	- 5.9

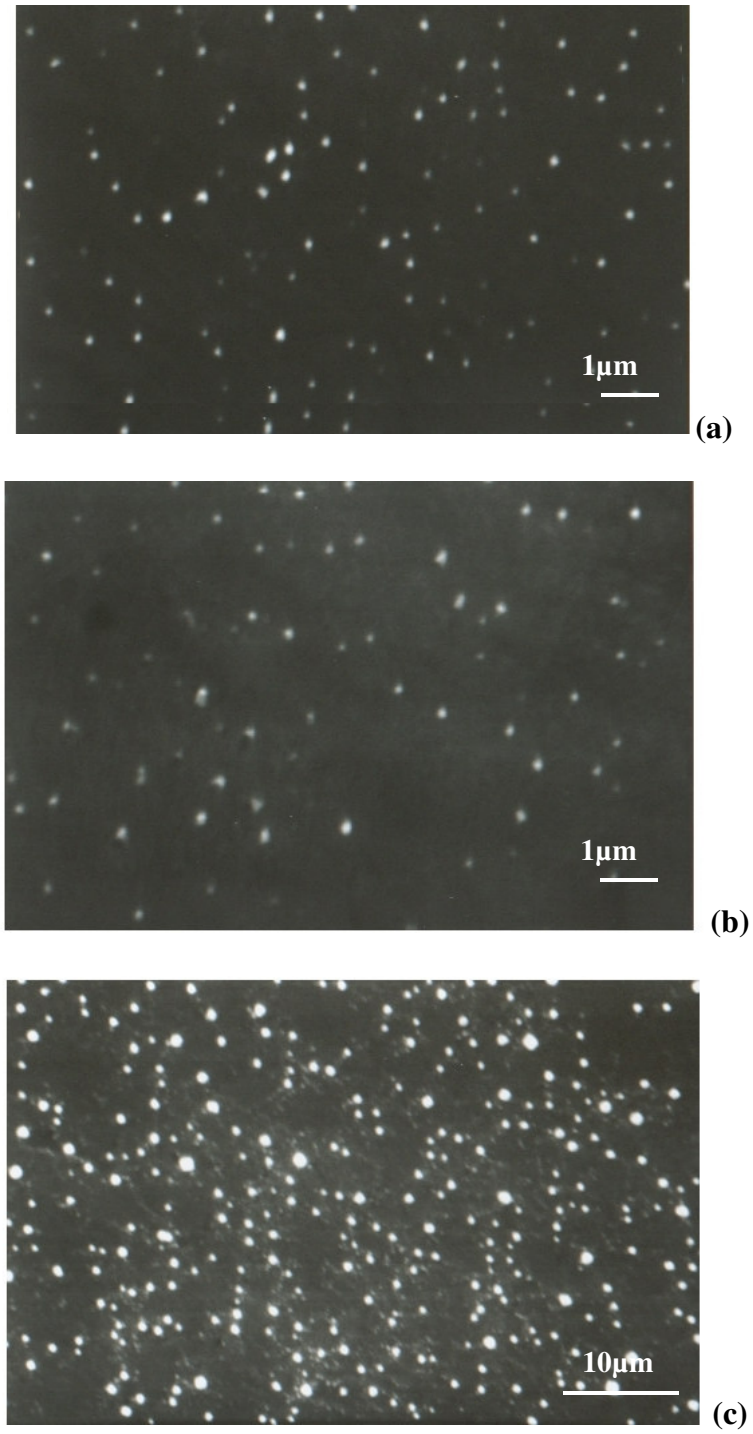
1.5µg of protein /mg of polymer

List of captions

Figure 1. Scanning electronic microscopic images of nanoparticles prepared by double emulsion method using sodium cholate as surfactant. PLGA 50/50 (**a**) PLGA 75/25 (13,000 ×) (**b**) unloaded nanoparticles and BmoLL-loaded PLGA 75/25 nanoparticles (2,500 ×) (**c**).

Figure 2. In vitro kinetic release profiles of BmoLL from PLGA 75/25 (■), PCL (●), PLGA 50/50 (∇) and PLA (◆) nanoparticles in phosphate buffered solution (pH 7.4) at 37°C. Data are shown as mean ± S.D of three assays.

Figure 3. In vitro adsorption of BmoLL onto the surface of PLGA 75/25 (■), PCL (●), PLGA (∇) and PLA 50 (◆) nanoparticles in phosphate buffered solution (pH 7.4) at 37°C. Data are shown as mean ± S.D. of three assays.



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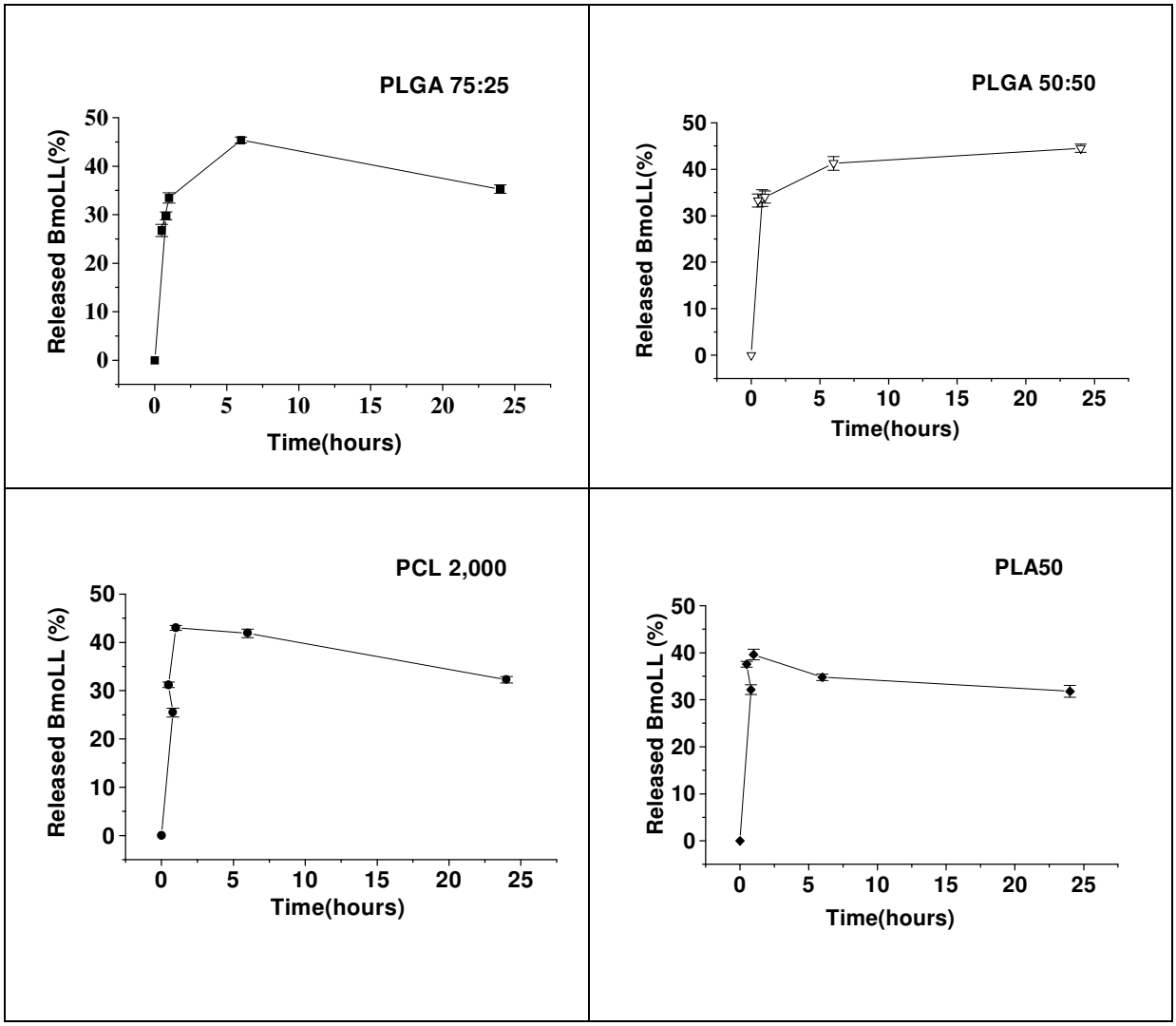

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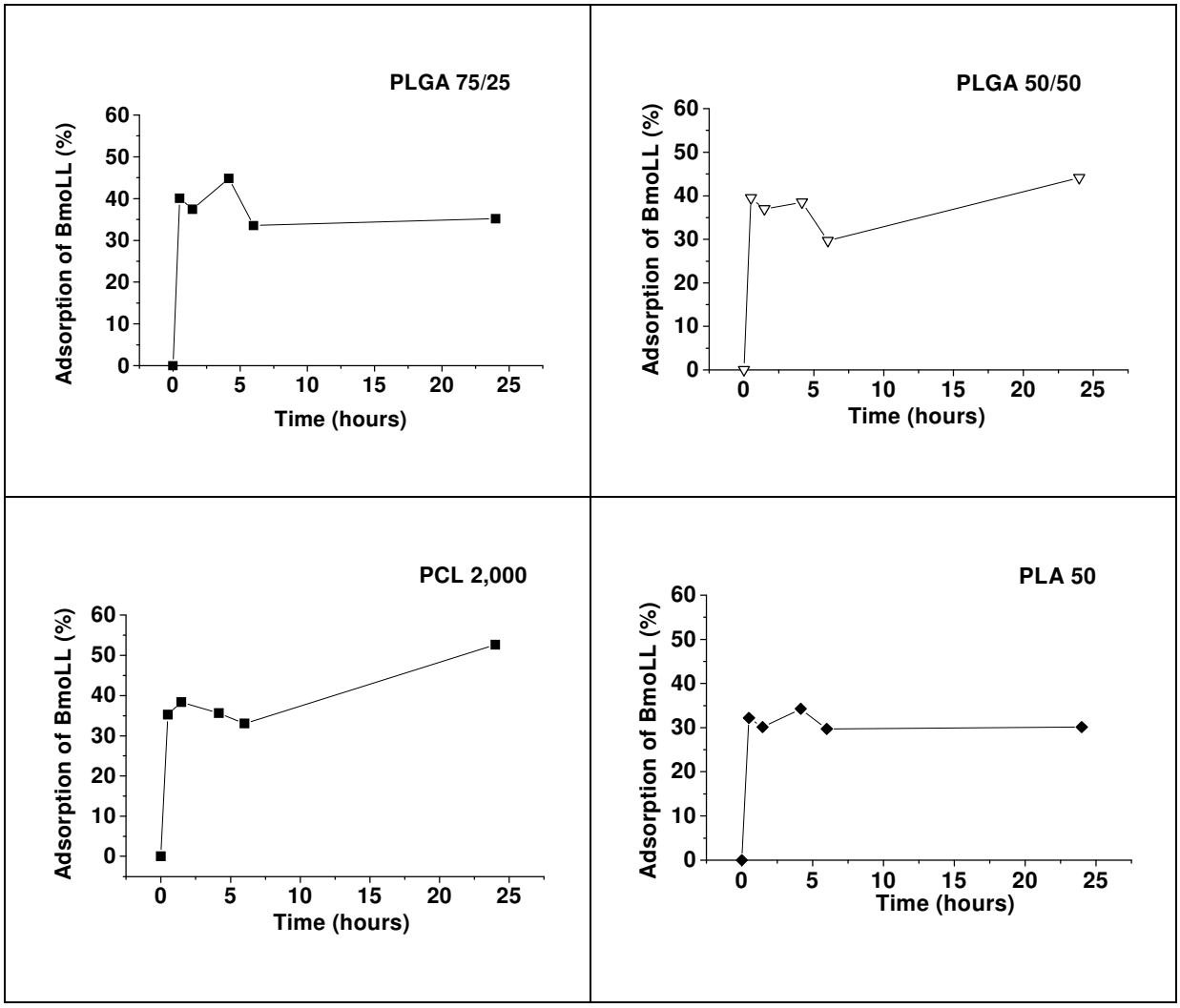

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POLISSACARÍDEOS ENXERTADOS COM POLIÉSTERS: NOVOS COPOLÍMEROS ANFIFÍLICOS PARA APLICAÇÃO BIOMÉDICA

Trabalho aceito no **Macromolecules**

Polysaccharides grafted with polyesters : novel amphiphilic copolymers for biomedical application

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Summary

New amphiphilic polysaccharides with controlled structure were synthesized by coupling between a carboxylic function of preformed polyesters chains and a hydroxyl group naturally present on polysaccharides. Firstly, the synthesis of poly(ε-caprolactone) monocarboxylic acid (R-PCL-COOH) was carried out by ring-opening uncatalyzed polymerization of monomer in the presence of a carboxylic acid (R-COOH). R-PCL-COOH was reacted with carbonyl diimidazol and the resulting activated intermediate (imidazoline) was further reacted with dextran (Dex) at different molar ratios to obtain amphiphilic copolymers with various hydrophilic-lipophilic balance. The coupling reaction was followed by GPC, indicating a total conversion. The copolymers were further characterized by GPC, 1H NMR and FTIR. Nanoparticles of less than 200 nm, with potential interest for controlled release of bioactive compounds, were successfully prepared by using these new materials.

Introduction

One remarkable feature in drug delivery technology is the central role that polymer plays in the control of drug administration, transport, and delivery at the desires site of drug action. Encapsulation of active compounds needs to conceive new biocompatible polymers for the design of particulate carriers (nanoparticles) with engineered surface properties for targeting purpose. The concept of nanoparticles surface modification to control the specific interaction with target cells as well as the non specific interaction with blood components and phagocytic cells raises a question about the optimal polymer/copolymer composition. Even if polyesters such as poly(lactic acid) (PLA), poly(lactic-co-glicolic acid) (PLGA) and poly(ε-caprolactone) (PCL) are now recognized as safe materials, none of the actual available polymers/copolymers are satisfactory. Indeed, either they are hydrophobic, thus activating the complement and leading to unwanted liver accumulation or, when coated with hydrophilic chains such as poly(ethylene glycol) (PEG)[1,2], they do not possess reactive groups at their surface allowing ligand coupling. To address the issue, polysaccharide based polymers represent an interesting alternative to PEG since poly and or/ oligosaccharides may have per se a lot of recognition functions allowing specific mucoadhesion or receptor recognition. For instance, fucosylated oligosaccharide ligands mediate cell-cell adhesion through binding to cell-surface selectins[3] and galactose containing oligosaccharides have affinity for asiologlycoprotein receptors in liver tumor cells[4]. Other polysaccharides like dextrans have molecular characteristics (hydrophobicity and mobility) able to prevent protein opsonization and complement, thus avoid liver recognition. Thus, biocompatible polyesters with the functionality of polysaccharides should allow the design of ideal material for drug targeting.

However, there are only few examples of covalently linked polysaccharides-polyester copolymers in the literature. One strategy involved a catalytic ring-opening polymerization of monomers (lactide or caprolactone) in the presence of polysaccharides[5-9]. The major inconvenient of this approach is the difficulty to obtain controlled structures since all the hydroxyl groups on the polysaccharides, except the ones sterically hindered, initiate the polymerization[7-9]. Alternatively, it was recently suggested[10-12] that hydrophobization of polysaccharides is possible by bulk polymerisation of monomers in the presence of partially protected (silanyzation) hydroxyl groups of polysaccharides, followed by deprotection. However, bulk polymerization lead to inhomogeneous substitution ration and high polydispersities[7-9]. The resulting large number of grafted chains with variable length may mask the polysaccharidic backbone and modify its physicochemical properties. This is a major disadvantage for biomedical; applications where interactions of the biological environment with polymeric surfaces are of polymeric surfaces are of significant importance for bioadhesion, steric repulsion of seric proteins or targeting.

In addition, ring opening polymerizations were carried out using stannous octanoate as catalyst [5-9]. Although stannous octanoate is the most popularly used catalyst for the synthesis of biodegradable polyesters, it is always chemically polluted by 2-ethylhexanoic acid so that uncontrolled secondary polymerization may occur [13]. It is worth mentioning that the degree of polymerization is often low, in particular when using stanous octanoate as catalyst [6]. Moreover, the rather high content in the residues cannot be removed efficiently[13], which makes that the presence of this toxic compound in the resulting polymers is another inconvenient for biomedical applications.

In this paper, we describe the synthesis of new comb-like materials composed of a polysaccharide backbone on which preformed polyester (PCL) chains were grafted by

means of ester bridges. These polysaccharide-polyester copolymers with controlled structures were obtained by coupling between a carboxylic function present on the polyesters chains and a hydroxyl group naturally existent on the polysaccharidic backbone. It was therefore necessary to first synthesize a PCL monocarboxylic acid (R-PCL-COOH) of predetermined molecular weight[14]. The resulting amphiphilic Dex-PCL_n were characterized by various techniques. Moreover, a method has been developed to prepare nanoparticles with a mean diameter lower than 200 nm using these new copolymers.

Experimental

Materials

ε-caprolactone (> 99 %), capric acid (> 99,9 %), Dext from *Leuconostoc mesenteroides* with molecular weights 5 and 40.10^3 g/mole(as determined by GPC), named Dex 5 K and Dex 40 K were obtained from Fluka. DMSO and THF (HPLC quality) and carbonyldiimidazole (CDI) were purchased from Sigma-Aldrich. Regenerated cellulose membranes with a cut off 6-8000 Da (Spectra/por, Spectrum, Breda, The Nederlands) were used for dialysis.

Synthesis of R-PCL-COOH ($R=C_9H_{19}$)

Synthesis of low molecular weight (2 to 4 10^3 g/mole) PCL monocarboxylic acid (R-PCL-COOH) was carried out by bulk polymerization of ε-caprolactone in the presence of capric acid at various molar ratios. Caprolactone was freshly distilled under CaH_2 in vacuum before polymerization and capric acid was dried by azeotropic distillation.

For example, 20 g ε-caprolactone and 1.88 mL capric acid (molar ratio 16) were weighted in a 50 ml round-bottomed flask which was fitted with a reflux condenser and connected

to the vacuum/Ar (Argon) line. The flask contents were thoroughly degassed under high pressure using three freeze-pump-thaw cycles. Polymerizations were carried out under Ar, at 235 °C in a silicone oil bath. After 7 h the reaction was stopped by dipping the flasks in ice. The obtained polymers were purified by four successive precipitations using THF as solvent and methanol as non-solvent. The polymers were finally dried under vacuum. Polymer recovery yield was 75 wt %.

Synthesis of Dex-PCL$_n$

Dex and R-PCL-COOH were anhydrized by azeotropic distillation befor the coupling reaction. To synthesize a polymer named Dex-PCL$_n$ where n is the number of grafted PCL chains per Dex macromolecule, a three step reaction was carried on.

For example, 3.5 g anhydrous R-PCL-COOH (Mn=2100 g/mol) and 290 mg CDI (5 %) molar excess with regard to the COOH functions) were dissolved in 4 mL of anhydrous THF in a 50 mL round-bottomed reaction flask equipped with a reflux condenser and connected to the Ar/vacuum line. The flask was heated at THF reflux under Ar, when CO_2 formation was observed. After 3h, when CO_2 was no longer observed, the reaction was stopped and THF was evaporated. The so-obtained unisolated activated polymer (imidazolide) was dissolved in a minimal amount (~ 2 mL) of anydrous DMSO and 1.05 g Dex 5 K (23 wt %) solubilized by heating in a minimal amount (~ 6 mL) of anhydrous DMSO were added. The coupling reaction was carried on for minimum 3 h at 130º C under Ar. The reation mixture was recovered and dialyzed against demineralized water. Precipitated polymer was isolated by centrifugation, the supernatant was discarded, and the polymer was washed again with water and THF to remove respectively traces of unreacted Dex and PCL. It was finally lyophilized and dried over phosphorous pentoxyde. The copolymer recovery yield was higher than 80 %.

To synthesize other Dex-PCL_n copolymers, the weight ratio of Dex in the reaction mixture was varied from to wt %.

Polymer Charatcterization

Determination of molecular weight of R-PCL-COOH by titration

An exact mass (m) of dried polymer (about 100 mg) was dissolved in 7 mL pure acetone and the COOH end groups were dosed with a 10^2 M solution of KOH in ethanol, in the presence of phenolphthalein. Average number molecular weights (M_n), expressed in g/mole, were determined by relationship:

$$M_n = \frac{m10^3}{cV}$$

where c (mol/L) is the title of the KOH solution (verified using a HCL solution of known title) and V (mL) is the added volume of KOH solution. Mn were averaged on at least three independent measurements. The precision of this analysis was 5%.

Gel permeation chromatography (GPC)

Dex-PCL_n copolymers were analyzed by GPC using a triple detection system (Viscotek, Houston, Texas, US). The GPC system is composed of a VE 7510 degasser (Viscotek), a VE 1121 pump (Viscotek), a waters 712 WISP injector, a Waters 410 differential mounted in parallel with a Viscotek T60Ab dual (viscosity and light scattering) detector, and a Waters TCM heating column system. Polystyrene standards (Polymer Laboratories, Shropshire, UK) were used to determine molecular weights from universal calibration.

For the analysis of R-PCL-COOH polymers, the eluent was HPLC-grade THF at a flow rate of 1 mL/min. A GMH-HR M (Viscotek) column heated at 40°C was used. The injected volumers were 100 µL and the polymer concentration was 5 mg/mL.

For the analysis of Dex and Dex-PCL_n samples, the mobile phase was N, N dimethylacetamida (DMAC) containing 0.4 % LiBr at a flow rate of 0.5 mL/min. The injected volumes were 100 µL. Sample concentrations ranged from 5 to 10 mg/mL. To achieve the best resolutions, two GMH-HR H columns were mounted in series for samples containing Dex 5 K and a GMH-HR N column was used for samples containing Dex 40 K. The columns were heated at 60 °C.

The number "n" of polyester chains grafted per Dex macromolecules was calculated by subtracting the determined M_w of Dex (5000 g/mole) from M_w of Dex-PCL_n copolymers and dividing by M_w of polyester grafts (2100 g/mole).

^{1}H NMR and FTIR analysis

^{1}H NMR spectra were recorded with a 200 MHz Bruker B-ACS 60 spectrometer. R-PCL-COOH samples were analyzed in D-chloroform (> 99.8 %, SDS, Peypin, France) and D_6-DMSO (99.9 atom % deuterium (D), Sigma Aldrich, Steinheim, Germany), whereas Dex-PCL_n samples were dissolved in D_6-DMSO. Infrared spectra of dried polymer powders were recorded using a Bruker Vector 22 spectrometer. 16 scan were averaged for each sample.

Nanoparticles preparation

5 mg Dex-PCl_n copolymer, 1 mL dichloromethane and 5 mL demineralized water containing 0.1 % (w/v) sodium cholate were stirred together for 5 min at room temperature. The emulsion thus formed was sonicated (60 s, 20 W, pulses of 1 sec each,

vibra cell, Sonics & Materials, Danbury, US). After solvent evaporation (rotary evaporator, 30 min, room temperature), the resulting nanoparticle volume diameter was determined using a particles size distribution analyzer (PL-PSDA, Polymer Laboratories, Shropshire, UK). The eluent was demineralized water containing 0.1 % (w/v) pluronic F68, at a flow rate of 2.1 ml/min. A "cartridge type 2" column (polymer Laboratories), with a separation domain 20 - 1500 nm, was used to separate the nanoparticles on the basis of their hydrodynamic radius. To detect the nanoparticles, absorbance was monitored at 254 nm.

Results and Discussion

PCL monocarboxylic acids (R-PCL-COOH) obtained by polymerization of (ε-caprolactone in the presence of monocarboxylic acids are key compounds in the synthesis of amphiphilic polysaccharide-polyester copolymers with controlled structures. The molecular weight of these polymers should be a compromise. Indeed, for coupling reaction with polysaccharides, short PCL chains are required to increase the reaction rate. On the contrary, long PCL chains are need to achieve the desired hydrophilic lipophilic balance (HLB) without increasing too much the number of grafted side chains. In the polymerization conditions used in this work, the molecular weights M_n of the isolated polymers was in the range of 2 to 3 10^3 g/mole(Fig. 1), suitable for grafting to Dex. The obtained M_n were practically independent on the ratio monomer/acid. Similar finding were reported in the case of ε-caprolactone polymerization in the presence of succinic acid[11]. According to the results in Fig. 1, a 7h reaction time was chose because it enable to obtain a satisfying polymer recovery yield (~70 wt%) and appropriate molecular weights (2100-2500 g/mole). A typical ^{1}H NMR spectrum and peaks assignment of the R-PCL-COOH

($R=C_9H_{19}$) polymers is shown in Fig. 2. The proton of the carboxylic function was not visible at ppm higher than 7 (very weak signal).

Average number and weight molecular weights (M_n, M_w) of these polymers were determined both by GPC and by dosage of the acidic end groups. After four successive purifications by precipitation, M_n were constant (< 5 % variation) indicating that the R-PCL-COOH polymers were exampled of traces of unreacted acid. Less than 10 % differences were found in between the M_n determined by dosage of carboxylic en groups and by GPC (results not shown). According to GPC data, polydispersity indexes were low (< 1.5).

The theorical molecular weights (M_t) (at total monomer conversion) are defined as:

$$Mt = \frac{n_{eCL}}{n_{acid}} 114 + 172$$

where n_{eCL} and n_{acid} are respectively the number of moles of monomers and capric acid in the reaction mixture, 114 and 172 are respectively the molecular weights of monomers and capric acid. A good agreement was found between M_t and M_n determined by titration after 7 h reaction time. For example, for a molar monomer : capric acid of 16, M_n was 2100 g/mole and M_t was 2000 g/mole.

It was found that water accelerates the polymerization of ε-caprolactone, but it might lead to the formation of undesired HO-PCL-COOH polymers. Studies concerning the influence on initiator structure were reported by Bixler *et al.*[16]. Because water is a comparative initiator of polymerization, drastic conditions (high purity reagents and anhydrous conditions) were necessary to obtain polyester carboxylic acids.

For the coupling reaction between R-PCL-COOH and Dex, different strategies were envisaged. For example, the carboxylic function of R-PCL-COOH was activated with N-

COOH in corresponding ester of NHSI in the presence of dicyclohexylcarbodiimide (DCC) was quantitative, but coupling with polysaccharide needed several days. Reaction yield did not exceed 50 %. DDC was used to couple polyesters and PEG[17]. However, in our case direct coupling using DCC needed prolonged reaction times and lead to low conversion yields.

The best results were obtained using CDI as coupling agent (short reaction times, good conversation yields). Selectivity, reactivity and efficiency of this acylating agent in various conditions was studied[18]. Examples of applications in polymer chemistry were reported particularly in the case of polysaccharides[19,20]. Coupling with CDI can be carried on in various usual solvents such as THF, DMF or DMSO[18]. In this work, DMSO was chosen because it is the best solvent for the used polysaccharides. The coupling reaction with CDI was carefully inspected and the reaction conditions were optimized using Dex 5 K and 40 K as starting materials. The different steps of the synthesis of Dex-PCL_n copolymers are schematized in Fig. 3. Firstly, CDI in excess was used for the conversion of carboxilic acid of R-PCL-COOH in corresponding imidazolide intermediate. In a second stage, Dex was added to the non isolated mixture containing imidazol and the reaction mixture was heated at 130 °C.

The optimal reaction time corresponding to more than 90 % conversion was determined by GPC. A typical GPC follow-up of the coupling reaction is shown in Fig. 4. The reactants were Dex 40 K (curve 1) and R-PCL-COOH 2100 g/mole (curve 2). The amount of Dex 40 K in the reaction mixture was 10 wt %. On the chromatogram of the initial reaction mixture (curve 3), both peaks of Dex and R-PCL-COOH can be observed. After one hour of reaction at 130 °C, the area of the peak corresponding to R-PCL-COOH (curve 4) was drastically reduced. It became negligible after 3h (curve 5), showing a

practically total conversion of R-PCL-COOH. Therefore, reaction times of 3 h were chosen for the coupling reactions.

After 1 and 3 h (curves 4 and 5), a new peak corresponding to higher molecular weights appears at shorter retention volumes. This, together with the disappearance of the peaks corresponding to Dex and R-PCL-COOH, clearly indicates that R-PCL-COOH could be detected by titrimetry in the isolated Dex-PCL_n copolymers, showing once more that the grafting was efficient.

The copolymers were analyzed by GPC, 1H NMR and FTIR. These techniques allowed to determine their average molecular weights, polydispersity index, gyration radius, viscosity, as well as their global composition. In particular, it was possible to determine the amount of Dex effectively incorporated into the Dex-PCL_n copolymers and the substitution degree.

Dex-PCL_n copolymers obtained using Dex 40 K and more than 15 wt % Dex in the reaction mixture were poorly soluble in DMAC and therefore their analysis by GPC was not possible. This was not the case with a family of Dex-PCL_n copolymers prepared using Dex with lower M_w (Dex 5 K), and even in amounts up to 33 wt % in the reaction mixture. All these isolated Dex-PCLn copolymers showed an unique peak in GPC. Table 1 gathers the GPC characteristics of Dex-PCLn copolymers obtained using 5, 20 and 33 wt % Dex 5 k in the reaction mixture and R-PCL-COOH 2100 g/mole. Average number and weight molecular weights (M_n and M_w), gyration radius and intrinsic viscosity increased with the wt % R-PCL-COOH in the reaction mixture, as a consequence of the increase of the number n of grafted PCL chains. Polydispersity of the Dex-PCL_n copolymers slightly increased with their M_w but remained low (<1.5).

Data in Table 1 allowed to calculate the number of grafted PCL chains per Dex backbone. It appears that approximately 3.5.5 and 7.1 polyester chains were grafted to Dex in Dex-

PCL_n copolymers synthesized with respectively 33 %, 20 % and 5 wt % Dex in the reaction mixture.

A representative 1H NMR spectrum of PCL, Dex and Dex-PCL_3 is shown in Fig. 5, together with the attribtion of the various peak. All and only the peaks corresponding to Dex and to PCL can be observed in the spectrum of Dex-PCL_3 copolymer. Due to the very low number of grafted PCL chains, about 3 in this case, no peak corresponding to the ester bridges between PCL and Dex could be observed.

In particular, it can be observed on Fig. 5 that the signal from the anomeric proton (f) of the glucopyranosyl ring (4.65 ppm) of Dex is well separated from the other proton signal (3.2 to 3.7 ppm) and the signal from the OH groups (4.4, 4.8 and 4.9 ppm). Therefore, the Dex wt % in the Dex-PCL_n copolymers can be calculated using the following relationship:

$$\text{Dex wt \%} = \frac{162A_{4.65}}{162A_{4.65} + 114/2\ A_4}$$

where 162 and 114 are respectively the molecular weight of α-D glucopyranose and caprolactone units. $A_{4.65}$ and A_4 are respectively the area of the peaks corresponding to the anomeric H of Dex and to the H of the CH_2 group linked to -C(O)-O in PCL chains (peak a, Fig. 5). For example, the so-calculated value of Dex in the copolymer, 31 wt %, was very close to 33 wt %, initially introduced in the reaction mixture. Differences could possibly be due to the elimination of the less substituted fraction by repetitive washing. In the case of the less substituted polymers, the differences between theorical and the calculated values of Dex in the copolymer were higher (~15 %).

Fig. 6 presents the superposition of FTIR spectra of Dex 5 K (curve 1), R-PCL-COOH (curve 5) and the Three Dex-PCL_n copolymers (n=3 to 7). Dextran presents a broad absorption band in the region 3200-3500 cm^{-1}, assigned to OH stretching vibrations. A

large peak at about 2900 cm^{-1} attributed to the CH/CH_2 vibrations is also observed. In the case of PCL chains, the peaks corresponding to these vibrations (2994 and 2865 cm^{-1}) are sharper. The characteristic C=O stretching vibration of PCL chains can be observed at 1722 cm^{-1}. The second main IR absorption band of Dex has a maximum at 999.3 cm^{-1}. This region is dominated by ring vibrations overlapped with stretching vibrations of (C-OH) side groups and the (C-O-C) glycosidic bond vibration [21]. The intensity of all these characteristic peaks of Dex increases in the Dex-PCL_n copolymers with the Dex wt % increase. Simultaneously, the intensity of the characteristic peaks of PCL decreases. Therefore, in a similar way as NMR spectroscopy, FTIR spectroscopy showed that the Dex, Dex-PCL_n and PCL samples were structurally related.

Moroever, the expected composition of the Dex-PCL_n copolymers could be calculated from the intensity of the carbonyl group band at 1722 cm^{-1}, characteristic of R-PCL-COOH (Table 2). Table 2 allows a comparison of the two different techniques used to determine the Dex wt% in Dex-PCL_n copolymers. FTIR spectroscopy reveals to be the most accurate one. 1H NMR was accurate too, but could not be applied to copolymers with low amounts of Dex, because of the very low intensity of the corresponding peaks. In the case of Dex-PCL_3 and Dex-$PCL_{5.5}$ (respectively 33 wt % and 20 wt % Dex in the reaction mixture), a good agreement was found between IR and NMR methods. Both these methods gave Dex wt% values lower than the ones in the reaction mixture. Possibly, during polymer purification by washing the faction of Dex-PCL lost in the aqueous phase was the less substituted one (with highest solubility in water and/or forming colloidal dispersions). As a consequence, the recovered polymers was impoverished in Dex.

As a conclusion, GPC reveal effective to determine the selectivity and the conversion ration of the coupling reaction, in particular to prove that Dex-PCL_n did not contain the starting material (R-PCL-COOH and Dex), and to calculate polymer characteristics such

as average molecular weights and polydispersity indexes (Table 1), IR and NMR spectroscopies used to the global compositions of the Dex-PCL_n copolymers, in particluar their wt % of Dex and PCL.

The HLB of the synthesized Dex-PCLn copolymers ranged from 1 to about 7. They revealed excellent oil-in-water emulsion stabilizing abilities, due to their amphiphilic nature. This property was further used to elaborated core-shell nanoparticles from these copolymers. The nanoparticles preparation methods generally involve the dissolution of the preformed polymer in an organic phase, but since the grafted Dex-PCLn copolymers here synthesized were insoluble in the most commonly used organic solvents for nanoparticles preparation (acetone, ethyl acetate, methylene chloride), an "interfacial migration/solvent evaporation" method has been developed. For this, amphiphilic copolymer or suspended in a mixture of water and an immiscible organic phase (ethyl acetate, methylene chlorid), were allowed to migrate at the oil/water interface, by stirring the two phases. The diameter of the oil droplets was reduced by sonication and the solvent was evaporated, leading to the formation of nanoparticles. A typical diameter distribution in the case of nanoparticles made of Dex-PCL 5.5 copolymers is shown in Fig. 7. Nanoparticles diameter ranged from 45 to 140 nm, with an average volume diameter of 70 nm. This size lower than 200 nm is particularly advantageous for intravenous administration. The investigation of the pharmaceutical applications of this new type of particles is underway.

Conclusions

The method describe in this paper allows to obtain a family of copolymers of the type Dex-PCL_n (n=3 to 7) with well defined structure. The number of grafted PCL chains is predeterminated from the mass ration Dex : R-PCL-COOH in the reaction mixture. With

the aim to further use these materials for biomedical applications, grafting was achieved through labile ester bridges to ensure a good biodegradability. The synthesis involves three steps.

First, a functionalized polyester, R-PCL-COOH, was obtained by uncatalyzed ring-opening polymerization of ε -caprolactona in the presence of capric acid. This polymer was activated with CDI. The reactive imididazole intermediate was effectively coupled to the Dex backbone. Although in general substitution reactions on polysaccharides are difficult, in this study it was shown that CDI was an excellent acylation agent for Dex. A follow-up of the coupling kinetics by GPC revealed that the reaction was fast (3h) and that conversion was practically complete (>90%). However the copolymer recovery yield was about 70 wt %, because of loss by extensive washing and colloidal particle formation. The solubility of the Dex-PCL_n copolymers depends upon their substitution yield. The HLB balance varied in a large domain (1-7). The new copolymers had excellent abilities to stabilize emulsions. This property was used to elaborate nanoparticles of less than 200 nm by using Dex-PCL_n copolymers. This low diameter is compatible with intravenous administration. Studies are to determine these nanoparticles and to explore their biomedical applications. By taking into consideration the physiochemical properties of a given drug it should be possible to chose the optimal Dex-PCL_n copolymer composition to achieve the best results in terms of entrapment and release.

Current research deals with coupling of mono or dicarboxylic polyesters to oligo-and polysaccharides such as cyclodextrins, amilose, chitosan or hyaluronic acid.

Aknowledgements
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Table 1 - GPC characteristics of dextran (Dex 5K) and three Dex-PCL_n copolymers with 3, 5.5 or 7.1 grafted polyester side chains: average weight (Mw) and number (Mn) molecular weights, polydispersity (Pd), average weight intrinsic viscosity (Iv_w), average weight gyration radius (Rg_w) and variation of the specific refraction index with concentration (dn/dc). For GPC conditions see experimental section.

Copolymer	**Dex**	**Dex-PCL_3**	**Dex-$PCL_{5.5}$**	**Dex-$PCL_{7.1}$**
M_w (g/mol)	5000	10900	16000	19100
M_n (g/mol)	4700	9900	11700	13500
Pd	1.06	1.10	1.37	1.41
Iv_w (dl/g)	0.087	0.12	0.12	0.98
Rg_w(nm)	2.47	3.57	4.07	3.92
dn/dc (ml/g)	0.147	0.088	0.084	0.052

Table 2 - Composition (wt% Dex) of a series of Dex-PCLn copolymers with na average of 3, 5.5 and 7.1 grafted PCL chains, as determined by FTIR and 1H NMR spectroscopies, compared to the Dex wt% in the reaction mixture (r.m).

*not determined (n.d.), because of the too low intensity of the Dex signals.

Copolymer	**Dex-PCL$_3$**	**Dex-PCL$_{5.5}$**	**Dex-PCL $_{7.1}$**
wt% Dex (r.m)	5%	20%	33%
wt% Dex (FTIR)	9 ± 4	16 ± 3	26 ± 3
wt% Dex (^{1}H NMR)	n.d.*	17 ± 2	30.7 ± 2.3

List of Captions

Figure 1 - Yield (■) of R-PCL-COOH (R=C_9H_{19}) recovery and average number molecular weight (♦) of R-PCL-COOH polymers as a function of reaction time. The molar ratio caprolactone : capric acid in the reaction mixture was 16:1.

Figure 2 - ^{1}H NMR (200MHz) of R-PCL-COOH (R=C_9H_{19}, M_n 2100 g/mole) in D-chloroform.

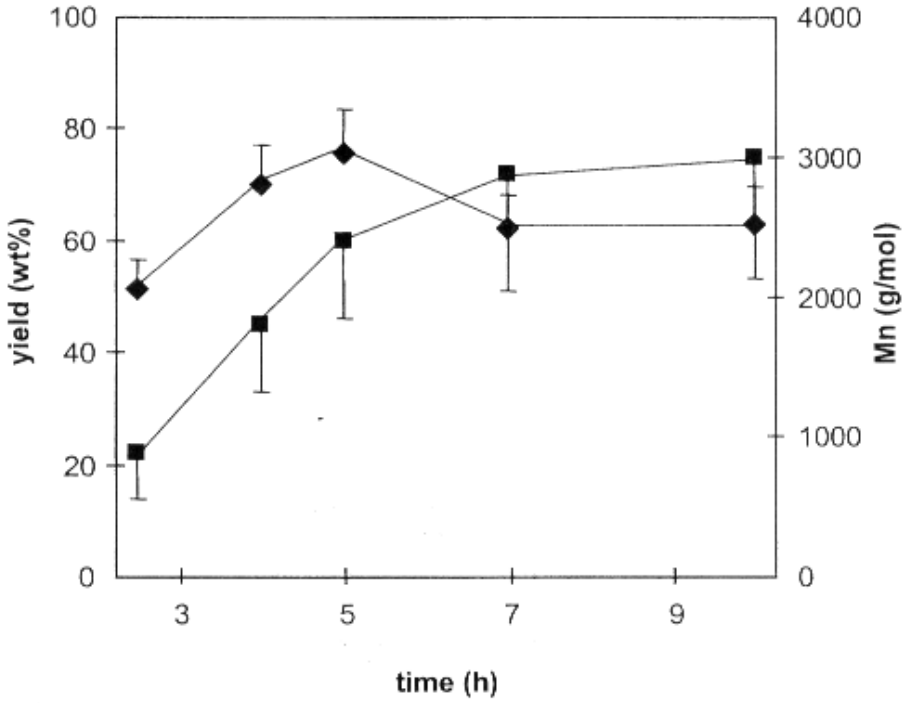
Figure 3 - Chemical reactions involved in the synthesis of Dex-PCL_n copolymers

Figure 4 - Coupling reaction time course as followed by GPC : Dex (4000 g/mole) (1), R-PCL-COOH (2100 g/mole) (2), starting mixture (Dex and R-PCL-COOH) (3), reaction mixture after one hour (4) and 3 hours (5).

Figure 5 - ^{1}H NMR (200MHz) in DMSO-D_6 : R-PCL-COOH (R=C_9H_{19}, 2100 g/mole) (**A**), Dex 5K (**B**) and Dex-PCL_3 copolymer (**C**).

Figure 6 - FTIR spectra of Dex 5K (1), Dex-PCL_3 (2), Dex-$PCL_{5.5}$ (3), Dex-$PCL_{7.1}$ (4) and R-PCL-COOH (R=C_9H_{19}, 2100 g/mole) (5) copolymers.

Figure 7 - Volumic diameter distribution of nanospheres prepared using Dex-PCL5.5 copolymer and following an "interfacial migration/solvent evaporation" technique.



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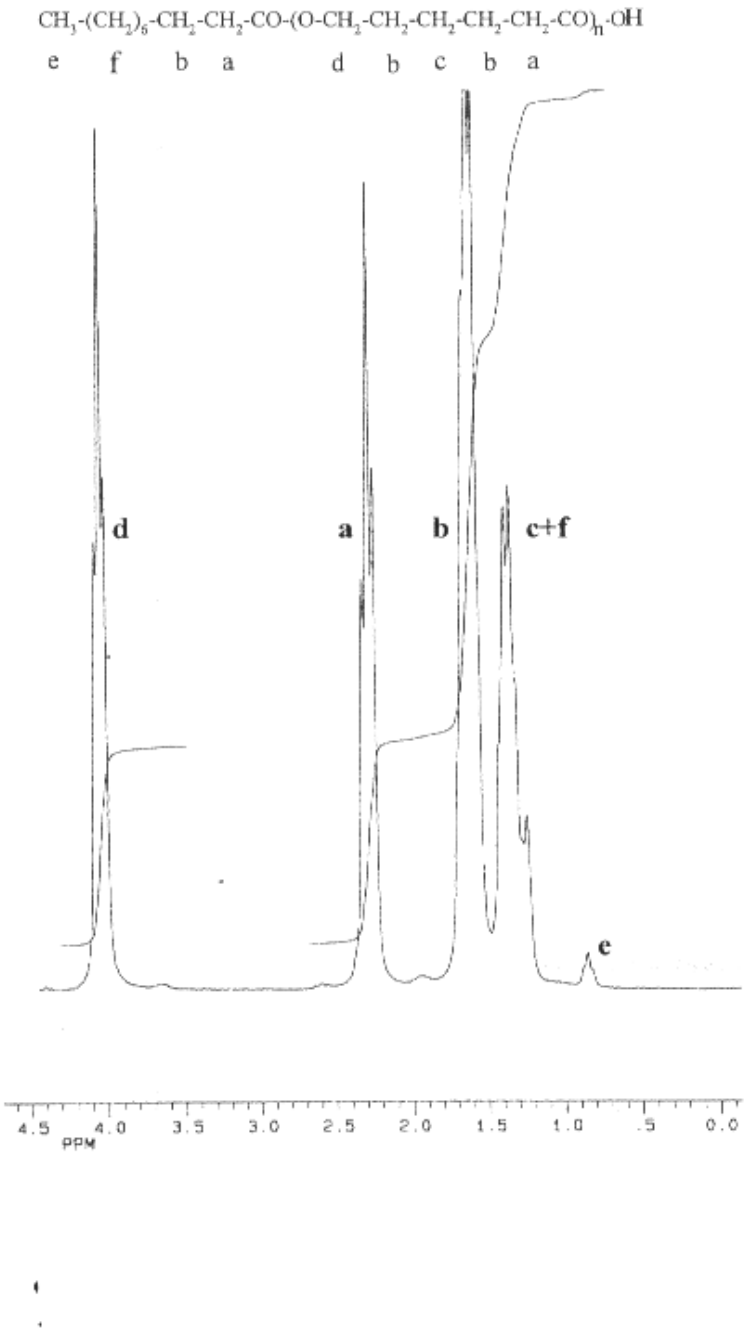

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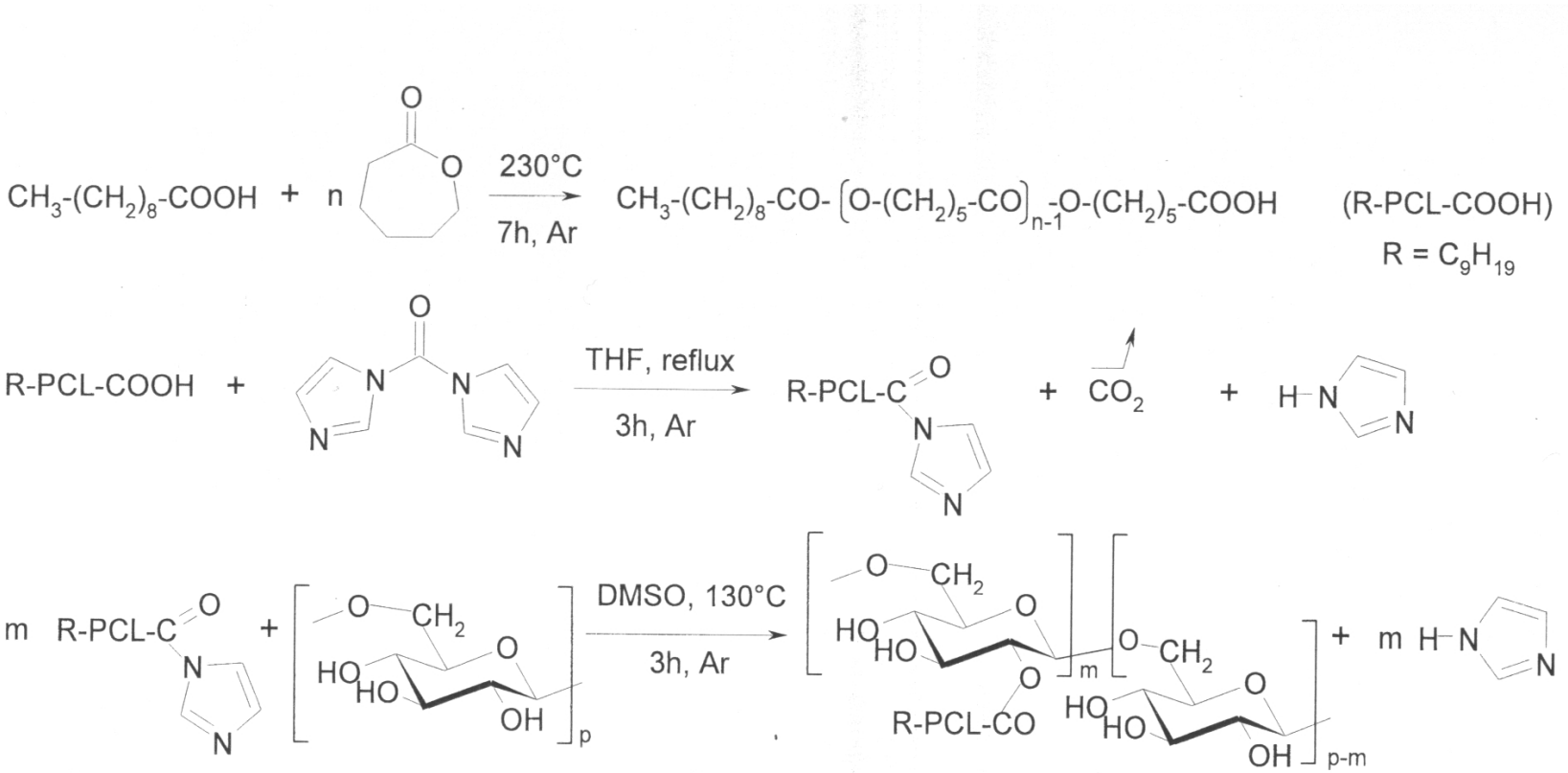

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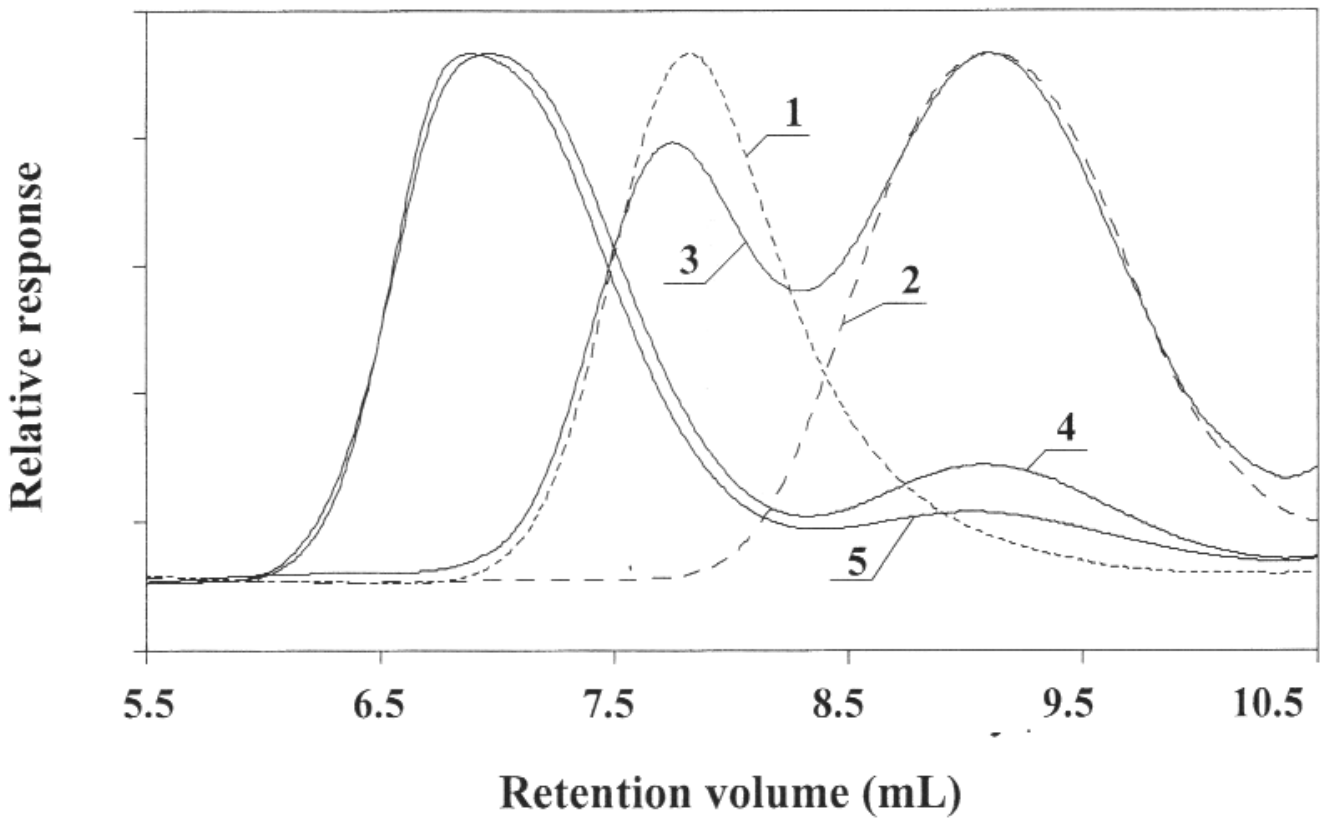

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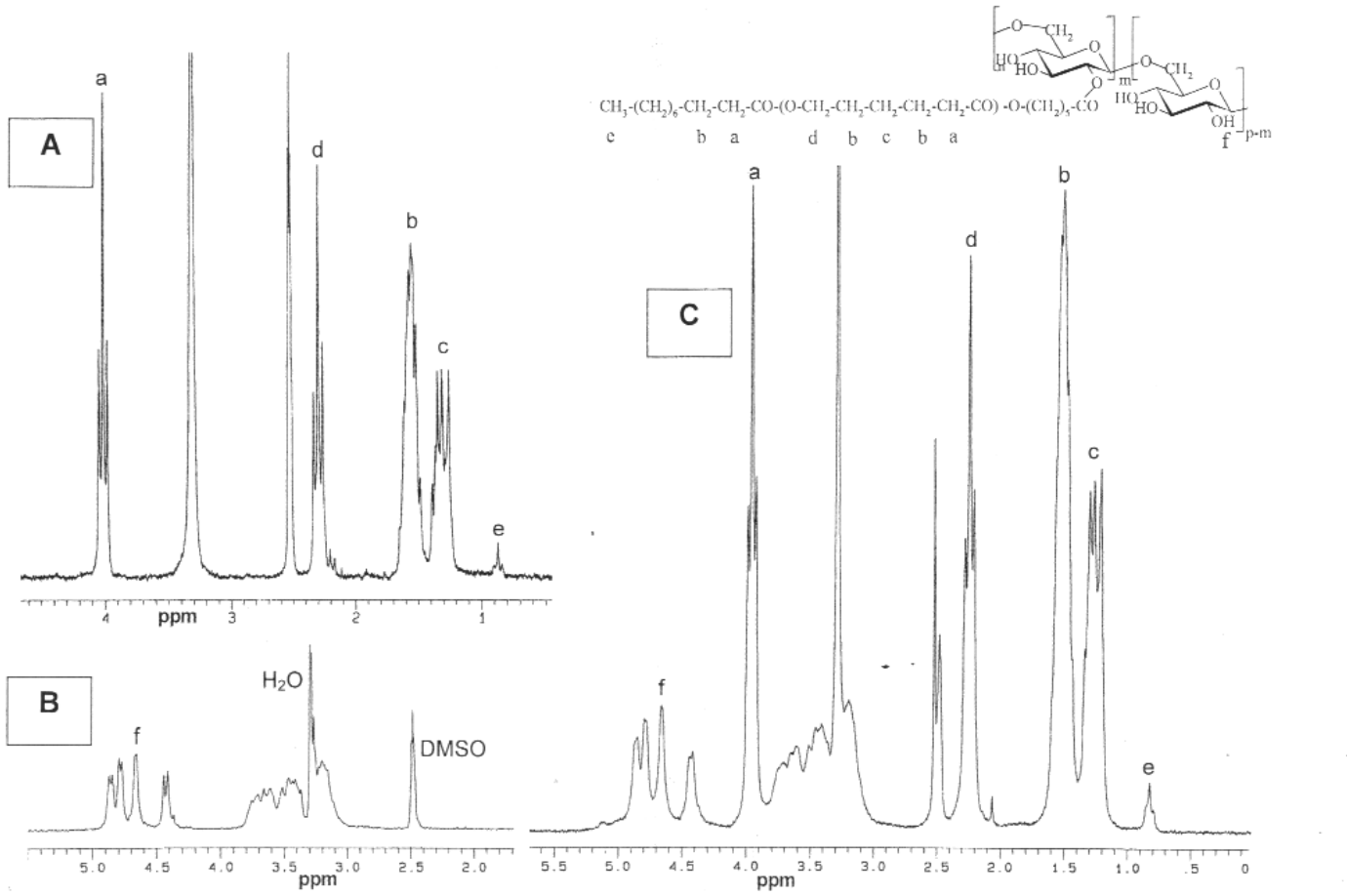

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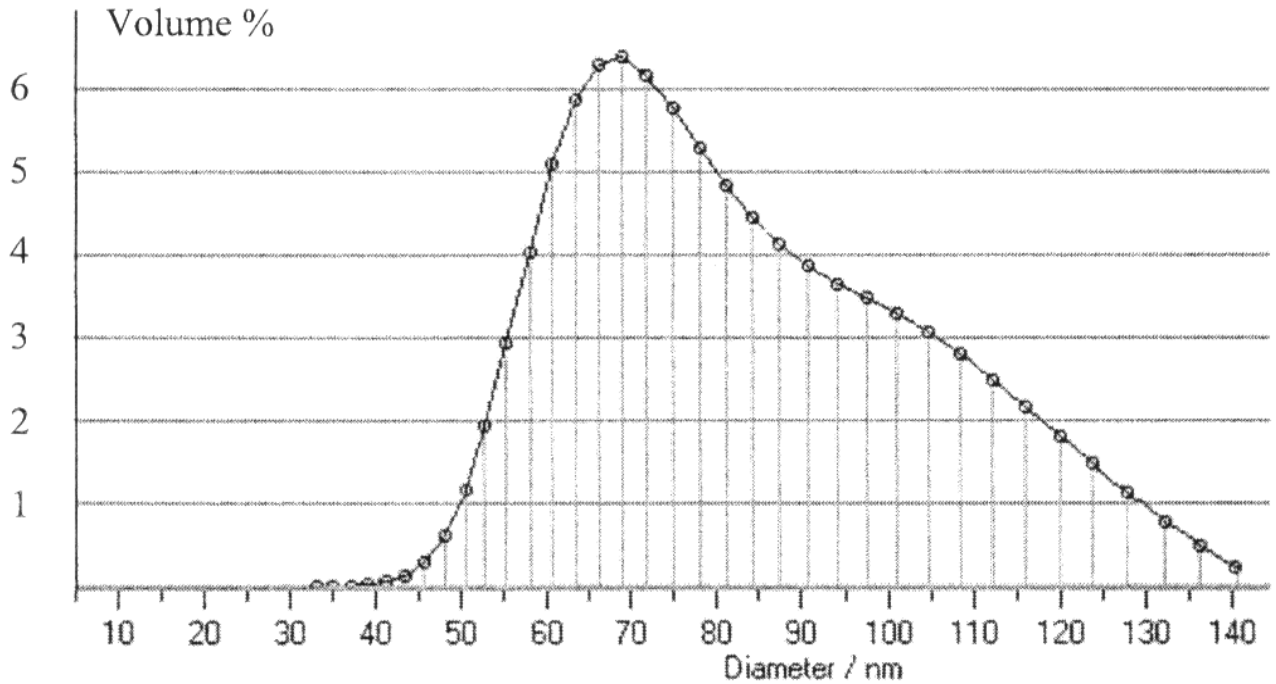

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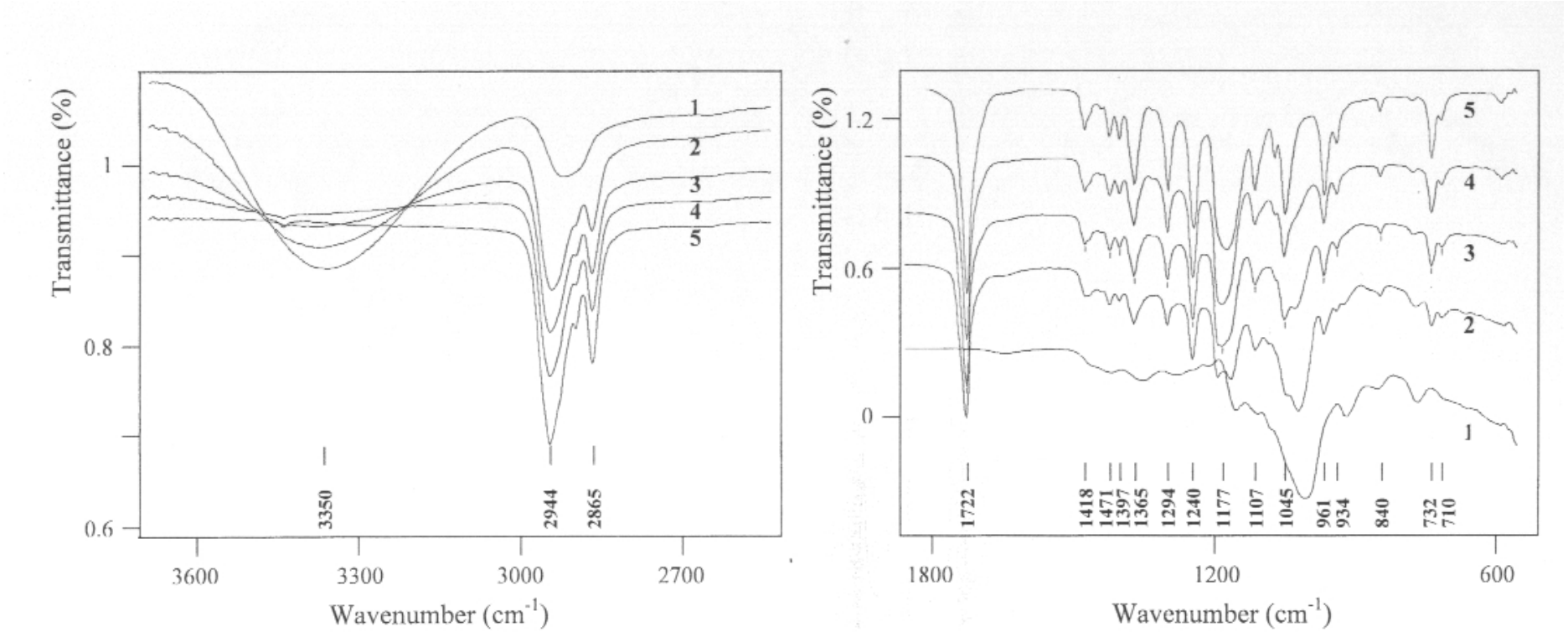

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Nanopartículas com superfície modificada do tipo núcleo-coroa constituídas de novos copolímeros de dextrana e policaprolactona

Trabalho a ser submetido ao **Journal of Controlled Release**

New modified surface nanoparticles prepared with dextran-polycaprolactone block copolymers (Dex-PCL_n)

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Abstract

The purpose of this work was to develop and characterize new biodegradable nanoparticles presenting a hydrophobic polycaprolactone core and a dextran hydrophilic corona. Recent synthesis of copolymers of dextran and caprolactone (PCL_n) instigates a successful protein entrapment into nanoparticles. The ability of new Dex-PCL_n copolymers to form nanoparticles was evaluated in comparison with some conventional caprolactone polymers (PCL 2K, 10K and 40K). Two different preparation methods were tested for evaluating the encapsulation efficiency of BSA, BmoLL and *Lens culinaris* lectins into Dex-PCL_n nanoparticles. The transepithelial resistance (TEER) of Caco-2 cells monolayers was measured on Transwell® filters and the bioadhesive potential of the nanoparticles was determined by incubating radiolabelled nanoparticles in the presence of Caco-2 cells. A lectin from leaves of *Bahunia monandra* (BmoLL) was successful loaded in Dex-PCLn nanoparticles. Furthermore, bovine serum albumin, BmoLL and lectin from *Lens culinaris* (LC) were adsorbed into the surface of Dex-PCLn nanoparticles. Results of bioadhesion evaluation showed that the non-specific interactions of the particles with the Caco-2 cells membranes was favored in the case of the Dex-PCL particles, compared to the plain PCL particles. In conclusion, a core/corona system based on amphiphilic copolymers has been prepared and characterized. Reasonable encapsulation levels can be obtained for proteins and surface modifications offer the possibility of preparing ligands conjugated systems of interest for targeting applications.

1. Introduction

The development of appropriate delivery systems for new macromolecules coming out of the biotech industry is a meaningful challenge for pharmaceutical scientists. Peptides, proteins, oligonucleotides, and genes are very unstable compounds that need to be protected from degradation in the biological environment. Moreover, their efficacy is highly limited by their ability to cross biological barriers and reach the target site. As such, the future of these molecules as therapeutic agents clearly depends on the design of appropriate carriers for their delivery into the body [1].

Lectins are carbohydrate-binding proteins of non-immune origin from plants, microorganisms or animals. They are multivalent molecules, which possess two or more sugar-binding sites for agglutinating plant and animal cells, and for precipitating polysaccharides, glycoproteins, peptidoglycans, teichoic acid, glycolipids, etc. The specificity of lectin is essentially driven by monossacharides or oligossacharides that inhibit lectin-induced agglutination or precipitation reactions [2]. Such a specificity, multivalent featured, as well as their non-immunogenic properties render lectins appealing candidates for developing a third-generation of site-specific coated nanosystems as carriers for drug delivery [3,4].

Carrier systems consisting of polymers and proteins of low immunogenicity are advantageous in pharmaceutical applications, e.g., for slow or stimulated release of anticancer agents, radical scavengers, etc. Two approaches have been applied to introduce proteins in therapy. In the first case, the protein itself presents biological activity and it can be encapsulated or incorporated into nanosystems such as liposomes or nanoparticles [5-10]. In most conventional systems, the control of release is based on encapsulation and/or non-specific reversible interactions between the carrier and the active agent. On the other

hand, proteins, much specifically lectins, could also bind at the surface of nanodispositives for site-specific drug targeting. One of the crucial and pervasive troubles in the human therapy is to achieve a satisfactory balance between the toxicity and the therapeutic effect of drugs. Site-specific delivery could prevent possible side effects at non-target sites and increase the efficacy of the therapeutic agent.

In this framework, the development of drug targeting systems could be based on the design of strategies for producing lectin-conjugated nanoparticles, nanoparticles with neoglycoconjugates, and monoclonal antibody-nanoparticles. In this light, nanoparticles have already been proposed as drug delivery carriers for controlled release of proteins and peptides [11-15].

It is well known that lectins are involved in the molecular interactions between cells recognition mechanisms. Biological information and transfer by the sugar code encompasses a complementary recognition step on the level the glycoligand (code word/message) and the lectin (receiver/translator) as effectors mechanisms. This background information is the base to devote efforts for designing new strategies of lectin-mediated drug targeting. Endogenous lectins harbor the potential to be pharmaceuticals in their own right, e.g., in anti-adhesion approaches, immunomodulation or growth controls [4]. A series of works reported in literature [3,8,10,11, 12, 13] showed the use of lectins in the field of Pharmaceutical Nanotechnology as a strategy for lectin-mediated drug targeting. The development of liposome or lectin-nanoparticle conjugated is based on the background of understanding molecular level interactions between glicoligands (code word/message) and lectin (receiver/translator), which can trigger a variety of post-binding signaling mechanisms [5]. The concept of lectin-mediated drug targeting is anchored in chemical properties of the spacer and the ligand-presenting scarf-fold as well as with the ligand structure, density and the topology of targeting device [4].

Despite that the field of lectin-mediated drug delivery systems is up till now in its early life, a variety of studies have corroborate the appeal of such an approach. Future works will cope with the challenges of enhancing *in vivo* stability until the target is reached, improving selectivity of the triggering by better linker design and enhancing the entrée of the drug to the (specific) site of action. Numerous *modus operandi* have been developed for attaching ligands to liposome surface [6,13]. In contrast, a few technical difficulties remained in technology of biodegradable nanoparticles lectin-conjugated. Specifically, novel biodegradable polymers should be conceived in order to get better covalent linkage of lectins at the surface of nanoparticles.

The convenience of lectins as oral drug cancer therapy is to link peptide or protein directly through a spacer arm to the lectin, which release the peptide or protein into concurrence with epithelial cells. Due to their ability for endocytic uptake, lectins have been the focus of special interest in drug delivery because they accept neoglyco-comjugtes with their payload like physiological ligands such as the asiologlycoprotein or mannose receptors of liver cells and macrophages. Lectins have also been offered so as to block the adhesion of bacteria such as *Helicobacter pylori* to the stomach epithelium, which is dependent on glycans containing both N-acetyl-neuramic acid and fucose in terminal positions [16]. A hemagglutinating test showed that gliadin nanoparticles conjugated with *Dolichos biflorus* lectrin (DBA) exhibit biological activity. The adhesive capacity of gliadin DBA conjugated nanoparticles was evaluated in Payer´s patches. The conjugates displayed a high specificity for intestinal mucosa [17, 18].

The main goal of this work was to develop and characterize new biodegradable nanoparticles presenting a hydrophobic core of polycaprolactone and a dextran hydrophilic corona for oral protein delivery. The syntheses of dextran and caprolactone (PCLn) copolymers make possible a successful protein entrapment in nanoparticles. The

ability of new Dex-PCL_n copolymers to form nanoparticles was evaluated in comparison with a number of conventional caprolactone polymers (PCL 2K, 10K and 40K). Two different methods of preparation were tested. The encapsulation efficiency of bovine serum albumin, lectin from leaves of *Bahunia monandra* (BmoLL) and *Lens culinaris* lectin (LC) into Dex-PCL_n nanoparticles was evaluated for these methods. Furthermore, the surface adsorption of both BmoLL and LC lectins onto Dex-PCLn nanoparticles was investigated.

2. Materials and methods

2.1 Materials

Block copolymers of dextrana-caprolactone (Dex-$PCL_{7.1}$, Dex-$PCL_{5.5}$ e Dex-PCL_3) were synthesized according to Gref and co-workers [19]. Caprolactone polymers (MW 2,000 Da, 10,000 Da and 40,000 Da) namely PCL 2K, PCL 10K and PCL 40K were furnished by Aldrich (USA). Serum bovine albumin (BSA, fraction V), sodium cholate (SC, MW 430.6 Da), and lectin from *Lens Culinaris* (LC) were purchased from Sigma (USA). Lectin from leaves of *Bauhinia monandra* (BmoLL) was purified and characterized by Coelho and Silva [20]. All the other chemical reagents were obtained from Merck (Darmstadt, Germany) of analytical grade.

2.2 Preparation of protein loaded-nanoparticles

Nanoparticles derived from Dex-PCL_n copolymers were manufactured. Two different methods of preparation based on emulsification-solvent evaporation were tested and the encapsulation efficiency of BSA, BmoLL and *Lens culinaris* lectin into Dex-PCL_n nanoparticles was assessed.

Initially, nanoparticles were prepared by a modified double emulsion ($W_1/O/W_2$) technique [21, 22]. It was prepared a W/O emulsion by adding 200 µL of water to 1.0 ml of Dex-PCL_n in diclorometh ane (4 mg/ml) under vigorous stirring during 1 min. The broad W/O emulsion was sonicated (CV 145 sonicator, Vibra Cell, France) at 40 W during 20 sec in an ice bath, and 4 ml of a sodium cholate solution (0.1%, w/v) was added under stirring by vortex for 3 min. The resulting double $W_1/O/W_2$ emulsion was sonicated as described above for 30 sec. The solvent was eliminated by evaporation under reduced pressure and the nanoparticles were recovered by centrifugation (Beckman L7-55 centrifuge, USA) at 144,000 g for 30 min and washed twice with water. SC was eliminated through dialysis against water during 6 h. Nanoparticles were diluted with 2 ml of 5% glucose, lyophilized and storaged at 4◦C. The efficiency of protein entrapment in Dex-$PCL_{5.5}$ nanoparticles was evaluated for BSA, BmoLL and *Lens culinaris* lectin. The proteins were diluted in the internal aqueous phase at different concentrations (0.3, 1.2 and 2.4 mg/ml).

Afterwards, a simple emulsion method [23] was used as a comparative to the modified double emulsion method in order to verify the efficiency on the preparation of Dex-PCL_n nanoparticles. Briefly, 1.0 ml of a polymer solution (4 mg/ml) in diclorometh ane was added to 200 µL of water and 4.0ml of a sodium cholate (SC) solution (0.1%, w/v) under vigorous stirring by vortex (3 min). The broad o/w emulsion was sonicated (CV 145 sonicator, Vibra Cell, France) at 40 W during 20 sec in an ice bath. The solvent was eliminated by evaporation under reduced pressure. The nanoparticles were recovered by centrifugation and treated at the some conditions as previously described in the double emulsion method.

2.3 Characterization of nanoparticles

After the fabrication, nanoparticles were analyzed according to morphology examination, particle size distribution, and density and zeta potential measurements.

The size distribution of nanoparticles was determined in water at 20°C by photon correlation spectroscopy (PCS) using a Nanosizer N4-MD® (Coulter, France). The Zeta Potential (ζ) was measured using a Zetasizer 4® (Malvern, UK). Samples of lyophilized nanoparticles were diluted in 10 mM NaCl solution and analyzed. The density of nanoparticles was estimated according the sucrose gradient method [24]. Sample of nanoparticles (0.5 ml) were put at the maximum of sucrose gradient (10 to 70%) and then submitted to centrifugation (L-55 centrifuge, Beckaman, USA) at 122,000 g (20°C, 15 min). After that, an aliquot was withdrawn and the density was determined in a densimeter (DMA 45, Instrulab France). The morphological examination of nanopartcicles was performed by using scanning electronic microscopy (SEM). A lyophilized sample of nanoparticles was resuspended in water and placed in a glass surface, which was fixed on metallic support with carbon-glue. After drying, the sample was directly coated with colloidal gold using a gold sputter module in a high-vacuum evaporator (JFC-1100, JOEL, Japan). Samples were observed with a scanning microscope (JOEL, Japan) at 20 KV.

2.4 Determination of protein entrapment efficiency

The non-entrapped protein content was determined by the Lowry-Peterson protein assay [25]. The supernatant was obtained after two centrifugation steps (L7-55 centrifuge, Beckman, USA) at 87,000 g for 15 min and 144,000 g for 30 min intercalated by nanoparticles washing. The amount of entrapped BSA within nanoparticles was calculated by the difference between the initial amount of BSA used to prepare nanoparticles and the content of BSA present in supernatant after centrifugation. Each sample was assayed in triplicate.

2.5 In vitro Kinetic release of BSA from Dex-PCL$_n$ nanoparticles

Samples of 10 mL of BSA -loaded nanoparticles were suspended in 100 mL of phosphate buffer saline (PBS, pH 7.4) containing 0.1% (w/v) sodium azide and incubated at 37°C under moderate magnetic stirring. At predetermined time intervals, one sample was withdrawn and centrifuged at 144,000 g for 30 min. 1.2 mL of the supernatant was removed and the released BSA was determined as described above. The same volume of PBS replaced the removed aliquots of the kinetic milieu.

2.6 Adsorption of BmoLL onto Dex-PCL$_n$ nanoparticles

The adsorption of BmoLL and LC lectins onto the surface of Dex-PCL$_{5.5}$ nanoparticles was evaluated. An amount of 4 mg of lyophilized unloaded Dex-PCL$_{5.5}$ nanoparticles was diluted in 1 ml of 10 mM phosphate buffered saline (pH 7.4) containing 30 µg of lectin, and incubated overnight under magnetic agitation at room temperature. Then, the conjugates were centrifuged (Beckman L7-55 centrifuge, USA) at 144,000 g for

30 min and washed twice with water for removing free lectin. The amount of adsorbed lectin was calculated from the values of the dosed lectin in the supernatant.

The adsorption of BmoLL on the surface of Dex-$PCL_{5.5}$ nanoparticles was verified by the haemagglutinating activity and specificity for D (+)-galactose assays. The pellet of BmoLL-adsorbed nanoparticles was resuspended in 500 µl of 10 mM PBS and serially diluted for determination of haemagglutinating activity [26]. The specificity of BmoLL was assessed by determining the haemagglutinating activity in the presence of 3 mM D (+)-galactose.

2.7Evaluationofthecytotoxicityof Dex-PCL_n nanoparticles

The cytotoxic effect of Dex-PCL_n nanoparticles and their constituents, expressed as cell viability, was evaluated on cells of human colon carcinoma (Caco-2) by using the colorimetric technique with 3-(4,5-dimethyltiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Row material and nanoparticles were placed in contact with Caco-2 cells at different concentrations. The cells were grown as monolayers in Dubelcco's modified Eagle's MEM medium (DMEM) containing 25 mM glucose (Eurobio, France), supplemented with 10% heat-inactivated fetal calf serum (Boehreinger, Germany) and 1% nonessential amino acids. For viability experiments, cells were grown in flasks until 80-100% confluence during 15 days. Then, cells were seeded at a density of 7×10^4 per well and cultured in 96-well plates (Corning Glass Works, USA). The cultures were exposed to the Dex-PCL_n nanoparticles were firstly diluted in PBS at concentrations ranging from 50 to 750 µg/ml for 72 h. The viability of Caco-2 cells was also evaluated after treatment with sodium cholate solutions at 0.1 to 7.5 µg/ml concentration range, and BmoLL in phosphate buffered saline (pH 7.4) at concentration ranging from 30 to 500 µg/ml. After

72 h incubation, a solution of 0.05% MTT /well was added and plates were incubated for 4 hours. The supernatant was removed, DMSO (50 µl/well) was added and read at 590 mn. The maintenance of cells and all experiments were carried out at 37°C in a 5% CO_2 atmosphere. Different concentrations of nanoparticles were tested in quadruplicate and repeated three times in separated experiments. Results are expressed as the mean ± SD of viable cells.

Alternatively, Caco-2 cells monolayers were grown up on Transwell filters inserts (Dulbecco, France) ® (Beaver: porosity of 0.4 µm; diameter 12mm; 3 x 10^4 cells per cm2). The integrity of these monolayers has been assessed by measuring the transepithelial resistance (TEER) of these membranes. 0.5 ml of the cellular suspension was added in the superior part of the Transwell®, and 1.5ml of complete medium in the basolateral chamber. TEER was measured using a volt-ohmmeter (Millicel ERS, Millipor®), and was calculated in Ω.cm2 multiplying the electric resistance for the area of the surface of filtro. Briefly, the TEER was determined at the initial time of the experiment and further determined after increasing incubation times of the different preparations with the cells monolayers.

2.8 *Evaluation of the bioadhesive potential of the nanoparticles*

The bioadhesive potential of the nanoparticles has been determined by incubating radiolabelled nanoparticles in the presence of Caco-2 cells. Briefly, Caco-2 cells were placed in the wells of a cell culture plate as further described. Radiolabelled particles were prepared accordingly to the preparation procedure described in paragraph 2.2. The radiolabelling procedure was achieved by introducing ^3H PLA (obtained from Prof. M. Vert, Laboratoire des Biopolymeres, Montpellier, France). Due to the hydrophobic nature

of the ^{3}H PLA, this polymer could be easily inserted in the PCL core structure of the particles, resulting in a very stable labeling of the PCL modified particles. Aliquots of the prepared suspensions (100 µl, 27 and 10 µCi/g of particles for PCL and Dex-PCL particles, respectively) were introduced at the concentration of 83,25 µg in the wells and incubated for increasing periods of time. Further, the content of each well was withdrawn and added with two successive washings of the cells with PBS. Separately, the adherent cells were treated by Triton X100, in order to ensure a complete lyse of the cells in order to determine the amounts of the particles adhering to the cells and/or the amount of phagocyted cells. 4 mL of a radioluminescent cocktail (Ultima Gold, Packard, France) was added to the samples which were treated according to a routine procedure.

3. Results and Discussion

3.1 Protein-loaded Dex-PCL$_n$ nanoparticles

The capability of new copolymers Dex-PCL$_n$ to form core-corona nanoparticles with a caprolactone matrix coated by dextran molecular chains was accomplished by using both simple and double emulsion methods. Nanoparticles made of preformed (co)polymers are generally prepared following methods such as emulsification-solvent evaporation, nanoprecipitation or salting-out. All these techniques require the previous dissolution of the copolymers in an organic solvent. However, all Dex-PCL$_n$ copolymers were insoluble in most of the organic solvents commonly used for the preparation of nanoparticles (acetone, THF, ethyl acetate, methylene chloride, chloroform) at the opposite of the homopolymer PCL, soluble in all of them. At our knowledge, no method has been yet developed to prepare nanoparticles using copolymers which are insoluble in water and in the organic solvents compatible with biomedical applications. It was therefore the aim to develop an original "interfacial migration - solvent evaporation"

method leading to nanoparticle formation by using the newly synthesized family of insoluble Dex-PCL_n copolymers.

In this technique, the copolymers were first allowed to migrate to an o/w interface to form a stabilizing layer around the solvent droplets (Fig. 1). The droplet size was reduced by sonication and then the organic solvent was evaporated, leading to the formation of a fine aqueous suspension of nanoparticles. The size of the Dex-PCL_n nanoparticles could be reduced to less than 100 nm by using sodium cholate as a surfactant. The "interfacial migration - solvent evaporation" process could only be applied to Dex-PCL_n copolymers. Indeed, PCL or mixtures of PCL and Dex failed to produce nanoparticles in the absence of additional surfactants.

The size distribution of Dex-PCL_n nanoparticles was quite narrow, bellow to 200 nm. The entrapment of BSA, BmoLL and LC into Dex-PCL_n nanoparticles was successfully achieved. Furthermore, these nanoparticles improved surface protein adsorption, suggesting their application as oral drug delivery systems.

The influence of the type of polymer on the size diameter and the zeta potential of nanoparticles was evaluated (Fig. 2). The diameter and the polydispersity index of nanoparticles were about 200 nm (Fig. 2a) and inferior to 0.17, respectively, independently of the used polymer. As defined, the polysispersity index, which represents a distribution width of a sample, should be valued less than 0.1 for a sample to be considered as a monodispersive system without any larger aggregates [27]. Therefore, these results are an indicative of the narrow size distribution feature of nanoparticles.

The dextran corona outer the polycaprolactone core modulated the surface charge of nanoparticles, which was evaluated by zeta potential measurements (Fig. 2b). Zeta potential results corroborate the hypothesis of core-corona type for Dex-PCL_n nanoparticles (Fig. 1). The increase of graft PCL in copolymers promoted the formation of

nanoparticle sterically stabilizes by dextran chains arranged at their surface. The dextran corona induced to an increase on the surface charge of the nanoparticles. In fact, it can be seen that PCL 2K nanoparticles presented -38 mV zeta potential while Dex-PCL_3 (3 PCL grafted chains) had -18 mV zeta potential. As a result the zeta potential of nanoparticles was decreased with the increase of dextran content (lengh and density) of graft copolymers. This fact could be explained by the shielding effect of dextran chains, which probably occupy the outer coating of nanoparticles [28, 29].

Although Dex-PCL_n nanoparticles prepared by simple or double emulsion method presented almost the same mean diameter and particle size distributions (Fig. 3), a better reproductibility and stability was observed for nanoparticles obtained by double emulsion technique. Moreover, no significant difference was detected on the zeta potential values of Dex-PCL nanoparticles obtained by simple or double emulsion methods. The density of unloaded Dex-PCL_n nanoparticles was about 1.1 g/cm^3 whatever the Dextran-caprolactone copolymer ratio is.

The morphology of unloaded Dex-PCL_n nanoparticles was visualized by SEM, one day and fifteen days after fabrication (Fig. 4). Nanoparticles were spherically shaped and almost monodispersed, with a mean diameter of 200 nm. The surfaces of nanoparticles were found to be smooth showing some irregularities. Regarding the stability of Dex-PCL_n nanoparticles, it can be observed a clear evolution on the size and shape after 15 days of fabrication. The Dex-$PCL_{7.1}$ conducted to a great number of particles in the formulation, but a rapid size evolution provoking the formation of aggregates was observed. Dex-PCL_3 promoted smallest and monodispersed nanoparticles. Moreover, an inferior quantity of nanoparticles in the formulation was observed. Thus, Dex-$PCL_{5.5}$

seems to promote the slowest evolution on the particle size and, consequently a best stability of nanoparticles.

The use of Dex-$PCL_{5.5}$ copolymer promoted a significant improvement in BSA entrapment into nanoparticles compared with both nanoparticles prepared with PCL 2K (30%) and PCL 10K (23%). An increasing on the potential zeta of BSA loaded-nanoparticles was observed as compared to unloaded nanoparticles (Fig. 2). This suggests the presence of BSA also on the surface of nanoparticles, which increased the surface charges of nanoparticles. This behaviour was verified for nanoparticles prepared with PCL polymers or Dex-PCL_n copolymers.

Concerning the application of BmoLL, a previously study was performed to investigate the encapsulation of *Bauhinia monandra* lectin (BmoLL) in new nanoparticles type PCL-core and dextran-corone. The influence of the manufacturing conditions of nanoparticles on the hemagglutinating activity (HA) of BmoLL was evaluated [30].

In this work the influence of the preparation method on the BmoLL and LC lectins encapsulation rate in the Dex-$PCL_{5.5}$ nanoparticles was evaluated (Table 2). The entrapment efficiency of BmoLL and LC lectins was major affected by the manufacturing process. Significant improvements on the entrapment of LC (56%) and (26%) BmoLL were achieved by using double emulsion technique for the preparation of nanoparticles.Therefore, it can be deduced that the BSA loading had affected the surface charge of nanoparticles as verified by the zeta potential values. Presumably, BSA was also adsorbed into dextran corona provoking a change on the surface zeta potential of nanoparticles. ζ values were obviously proportional to the amount of PCL in the copolymer chains, indicating that a fine modulation of the surface charge of nanoparticles can be achieved by the controlled design of dextran and PCL quantities in copolymer molecules.

3.2 In vitro Kinetic release of BSA from Dex-PCL$_{5.5}$ nanoparticles

The release behavior of BSA from loaded-Dex-PCL$_{5.5}$ nanoparticles produced by simple and double emulsion methods was evaluated in vitro using phosphate buffered saline at pH 7.4 (Fig. 5). As can be seen, the BSA kinetic behavior from Dex-PCL$_{5.5}$ nanoparticles presented three different phases (burst, increasing the sustained release). An initial burst effect is produced at the first 24 h, followed by an increase on the release of BSA to attain 65% at 72h, and a sustained release for over 120 h. A significant difference on the BSA pattern release between nanoparticles prepared by simple or double emulsion technique was verified. Initially, a high burst effect on BSA release rate (35% of the entrapped BSA) was observed at the first 24 h for BSA-loaded nanoparticles prepared by simple emulsion method. In contrast, the BSA release from loaded-Dex-PCL$_{5.5}$ nanoparticles prepared by double emulsion method was retarded in 20% at the some time. This burst effect could be attributed to the immediately release of BmoLL molecules adsorbed at the surface of nanoparticles followed by the slowly diffusion of BSA from the polymeric caprolactone core. Another issue to be mentioned is that BSA affinity for the polycaprolactone is probably not enough to entrap a huge amount of molecules during the fast process of nanoparticles manufacturing by simple emulsion procedure. Therefore more BSA molecules should be retained on surface of nanoparticles. The sustained release behavior of BSA from Dex-PCL$_{5.5}$ nanoparticles over 120 h supports the hypothesis that the release mechanism is governed by the diffusion of the protein more than by the erosion of the polymer. Similar results were obtained by Kim and collaborators [31] studying the indomethacin kinetics from nanospheres prepared with pluronic-PCL copolymers.

Moreover it is well known that polycaprolactone and its copolymers have a slower permeability to proteins and peptides [32].

The ability of Dex-$PCL_{5.5}$ nanoparticles to provide a controlled release of BSA was confirmed, and it can be concluded that the effect of binding affinity of BSA in Dex-$PCL_{5.5}$ nanoparticles plays an important role in the in vitro release kinetic pattern of BSA.

3.3 Adsorption of BmoLL onto Dex-PCL nanoparticles

The adsorption of lectins onto Dex-$PCL_{5.5}$ nanoparticles prepared by using the double emulsion method was confirmed by the heamagglutinating activity of nanoparticles. The BmoLL adsorption was 25% (± 0.34) while LC was 40.4% (± 1.5) for incubation concentrations of 30 µg/ml. Free BmoLL presented a HA at 43 ng/ml while BmoLL adsorbed onto nanoparticles promoted HA only at 1.4 µg/ml. BmoLL galactose specificity was expressed by the inhibition of HA when BmoLL-adsorbed nanoparticles were placed in contact with galactose at 6.23 mM. These results corroborate the adsorption of lectins at the surface of Dex-PCL_n nanoparticles.

3.4 Evaluation of the cytotoxicity of Dex-PCL_n nanoparticles

The biocompatibility of Dex-PCL_n nanoparticles, BmoLL and sodium cholate was evaluated in vitro by the cytotoxicity test using Caco-2 cells. It can be seen that Dex-PCL_n nanoparticles had no toxic effect on Caco-2 cells even at 700 µg/ml (Fig. 6). The treatment of Caco-2 cells with Dex-PCL_n nanoparticles (41 µg/ml) prepared by simple emulsion method provoked an initial reduction of 30% on the cellular viability. However cellular viability was maintained at 70% even at high concentrations of nanoparticles (660 µg/ml). On the contrary, the treatment with Dex-PCL_n nanoparticles prepared by double emulsion method produced only a 15% reduction on cell viability. This fact can be attributed to a probably instability of nanoparticles prepared by simple emulsion method followed by realinsing of insoluble polymers or by the presence of sodium cholate residues.

BmoLL had no toxic effect on Caco-2 cells at the concentrations from 15 to 500 µg/ml. The cellular viability remained almost 90% from the initial cellular monolayer. As a result BmoLL was considered to be non cytotoxic.

The sodium cholate presented a pronounced toxic effect on Caco-2 cells at the concentrations above 0.5 µg/ml.

Therefore results showed that BmoLL, sodium cholate at 0.1% and nanoparticles prepared with the new Dex-PCL_n copolymers have no cytotoxicity and are apparently save for biomedical applications. Moreover these findings can provide tools to determine the conditions for studying in vitro the adhesion and the specificity of BmoLL and Dex-PCL_n nanoparticles for intestinal cells.

The measurement of the transepithelial resistance was an other mean to evaluate the cytotoxicity of the particles, using experimental conditions closer to oral delivery situations. Fig. 7a shows that the TEER progressively dropped following the contact of the unloaded particles with cells. Cells monolayers are very sensitive to changes in the mediums as shown by the controls. As can be seen the addition of PBS in the incubation medium is likely to produce considerable changes a decrease in the TEER. Unloaded nanoparticles had an additional effect on the TEER, compared to the control, which could be attributed either to the presence of traces of various substances used in the preparation of the nanoparticles, or to a direct contact of the particles with the cells [33]. Additionally, it can be seen that the toxicity of the sodium cholate used in the preparation as a stabilizer was attenuated in the colloidal preparations, compared to a simple sodium cholate solution at the same concentration. This could be probably attributed to at least a partial adsorption of the cholate to the particles, thus decreasing the potential of the sodium cholate to interact with the cells membranes. Similar trends can be seen with *Lens culinaris* lectins loaded particles (figure 7b). However, the TEER was more deeply affected than in the

case of the blank particles. This could be due to the presence of the lectin at the surface of the particles, which was very likely the result of increased interactions with the cells membrane. In fact, as early demonstrated [34] and as further demonstrated, the lectins are able to increase the bioadhesive interactions with cells surfaces bearing glycoproteins containing specific sugars in their glycoside moieties. In turn, these interactions are likely to modify the permeability of these membranes to ions, thus resulting in increased ions fluxes and increased electrical currents, resulting in a TEER decrease. Despite the rather pronounced effect of the particles on the TEER, the in vivo significance of these results is still unknown and transposition of these results to in vivo situation would be very hazardous.

3.5 Bioadhesive interactions of the lectins conjugated particles to Caco-2 cells

Fig. 8 shows the percentage of the particles which could interact strongly with Caco-2 cells through bioadhesive interactions and possibly further phagocytosis by the cells. Despite the preliminary nature of these results, examination of the data shows that the presence of the lectins in the surface of the particles resulted in striking adhesion to the Caco-2 cells. Caco-2 cells express various glycoproteins bearing a variety of sugars and sugars sequences which are likely to be recognized by lectins particles conjugates. In the present case, *Lens culinaris* is specific from D mannose, which is expressed at the surface of Caco-2 cells [34]. As can be seen, in the absence of the lectins, the binding of the conjugates to the cells is close to zero or very low (typically less than 0,1 %). On the contrary, the presence of the lectin resulted in interaction percentages which could be as high as 5%, depending on the incubation time, which represents tremendous increases in the interaction potential of the particles. As can be seen, the highest interactions were obtained for the PCL-dextran particles, compared to the PCL nanoparticles. In fact, it is well known that the development of specific interactions is a two step phenomenon which

necessitates at first a non-specific interaction of the particle with the substrate, followed by the development of the specific interaction. Accordingly, it was likely that the non-specific interactions of the particles with the Caco-2 cells membranes was favored in the case of the Dex-PCL particles, compared to the plain PCL particles, due to the differences in their zeta potential. The zeta potential of the protein charged Dex-PCL particles was probably close to the neutrality (as shown in the case of BSA) while on the contrary the one of the plain PCL particles was highly negative. As a consequence, the PCL particles could be efficiently repulsed by the electronegatively charged surface of the Caco-2 cells, resulting in a lowering of the number of particles able to develop further specific interactions through the lectin-sugar system. In view of this mechanism, it cannot be excluded that additional factors which were not studied here, be involved in the regulation of the bioadhesion process, including factors such as the lectin surface density, the modification of the polarity of the membrane due to the proteic nature of the lectins at the surface of the particles. Finally, it can be seen that the level of interaction increased when the duration of the incubation was increased. This could be the result of a time dependent phagocytosis of the particles by the Caco-2 cells, following their adhesion to the cytoplasmic membrane of the cells.

4. Conclusion

In conclusion, results confirmed the ability of new Dex-PCL_n copolymers to form nanoparticles with a narrow size diameter by using both simple and double emulsion methods. Results of zeta potential measurements of Dex-PCLn strongly suggested that the formed nanoparticles were core-corona type with dextran chains placed preferentially outer the surface of caprolactone matrix. A lectin from leaves of *Bahunia monandra* (BmoLL) was successful loaded in Dex-PCL_n nanoparticles as compared to bovine serum albumin used as a reference. Furthermore, BSA, BmoLL and lectin from *Lens culinaris* (LC) were adsorbed into the surface of Dex-PCLn nanoparticles, strongly suggesting their application as drug or protein carriers with modulated biodistribution and specificity for oral delivery. Moreover results indicated that the outer layer of dextran increase the adsorption capacity of Dex-PCL nanoparticles for proteins. A hypothesis that BSA or lectins was also loaded in dextran-layer was strongly supported by zeta potential measurements, kinetics and the in vitro adsorption studies. Therefore the present type core-corona Dex-PCL_n nanoparticles would be useful as both carriers for hydrophobic drugs and proteins as well as site-specific drug delivery since its possible to conjugate lectins on their surface leading to an efficient capture of the particles by the cells.

Acknowledgements

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Table 1.

Influence of the type of polymer in the mean diameter, the encapsulation of the BSA and the zeta potential of Dex-PCL $_{5.5}$, PCL 2,000 g/mole and PCL 10,000 g/mole nanoparticles. The final polymer concentration was 1mg/ ml. The nanoparticles were produced by the double emulsion method.

Polymers	**BSA (%)**	**Mean Diameter ± SD (nm)**	**Polydispersity Index**	**Encapsulation Rate (%)**	**Zeta Potential (mV)**
Dex-PCL$_{5.5}$	4.76	188±58	0.172	32.08	-5.2
	38.5	172±41	0.090	33.33	-2.4
	51.00	211±64	0.154	69.01	-1.3
PCL 2K	38.50	189±44	0.088	31	-8.9
	51.00	189±48	0.122	30.71	-6.4
PCL 10K	38.50	177±53	0.157	21.06	-4.9
	51.00	185±65	0.055	23.15	-3.5

Table 2.

The evaluation of the influence of the preparation method on the particle size diameter and lectin entrapment efficiency in Dex-$PCL_{5.5}$ nanoparticles. The polymer concentration was 1 mg/ml, and the lectin-polymer ratio was 5%.

Lectin	Preparation Method	Mean Diameter ± SD (nm)	Encapsulation rate (%)
***Lens Culinaris* Lectin**	**Simple Emulsion**	114 ± 40	58.91
	Double Emulsion	128 ± 51	79.84
BmoLL	**Simple Emulsion**	97 ± 34	22.64
	Double Emulsion	103 ± 39	52.41

The initial concentration of BmoLL and *Lens culinaris* lectin was 50 µg/ml.

List of captions

Figure 1. Schematic illustration of the mechanism of core–corona Dex-PCL_n nanoparticles formation.

Figure 2. Evaluation of the mean diameter (**a**) and surface charge (**b**) of unloaded nanoparticles prepared by double emulsion method, for new Dex-PCLn nanoparticles compared with PCL_n: (1) Dex-$PCL_{7.1}$, (2) Dex-PCL_3, (3) Dex-$PCL_{5.5}$, (4) PCL 2K, (5) PCL 10K, (6) PCL 40K.

Figure 3. Stability of unloaded Dex-PCL_n (n = 7.1, 5.5 and 3) nanoparticles produced by using simple emulsion (a) and double emulsion (b) methods. The nanoparticles were storaged at 4°C.

Figure 4. Evaluation of the morphology of unloaded Dex-PCLn nanoparticles by scanning electronic microscopy: Dex-$PCL_{7.1}$ (**a** and **b**), Dex-$PCL_{5.5}$ (**c** and **d**), and Dex-PCL_3 (**e** and **f**). Macrographs were taken one day after (**a, c** and **e)** and fifteen days **(b**, **d** and **f)** after fabrication of nanoparticles.

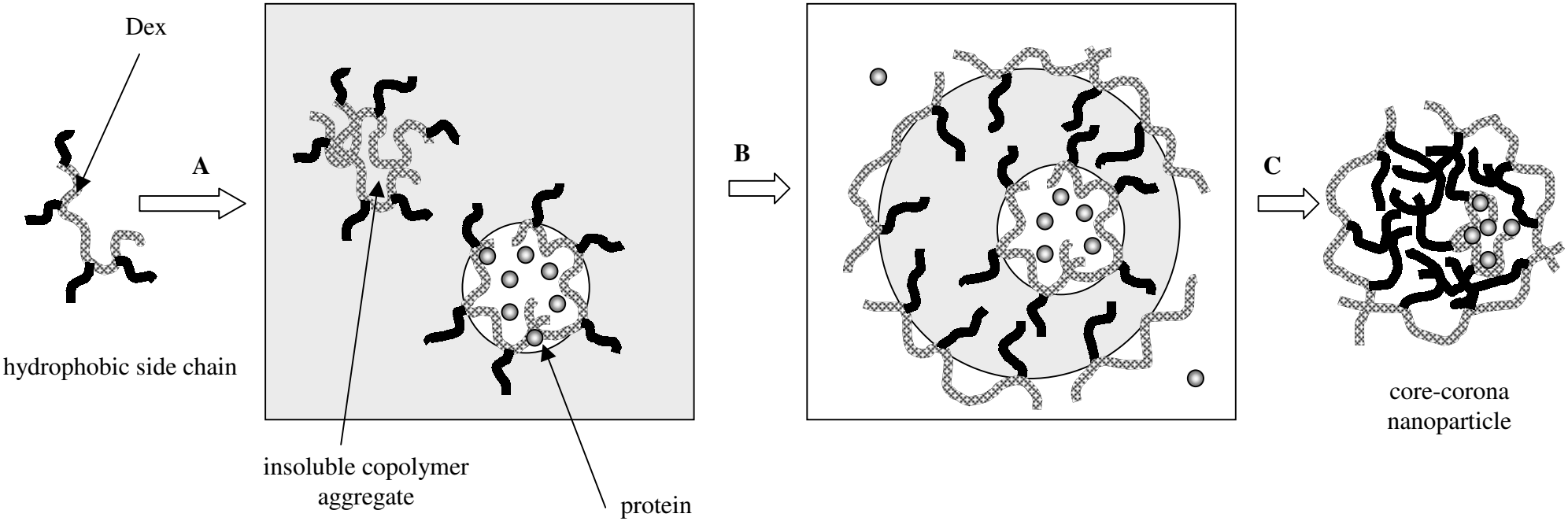
Figure 5. In vitro kinetic release profiles of BSA from Dex-$PCL_{5.5}$ nanoparticles prepared by simple (□) and double emulsion (■) methods. The concentration of BSA was 642 µg/ml of nanoparticles suspension.

Figure 6. The evaluation of the cytotoxicity: (**a**) unloaded Dex-$PCL_{5.5}$ nanoparticles and produced by double emulsion (■) and simple emulsion (□) methods, BmoLL (●), and (**b)** sodium cholate solution on the carcinoma of colon human cell (Caco-2) after 72 h of incubation. Cellular viability (%)=(N_t/N_c) x 100, where N_t and N_c are the number of surviving cells in the treated group and in the untreated group, respectively. Lyophilized nanoparticles were resuspended in 200 µl of phosphate buffered saline (pH 7.4). Serial

dilutions from 10.31 to 660 µg of particle/ml were added in each well. The cell viability end points were determined by MTT reduction.

Figure 7. Evaluation of transepithelial resistance (TEER) of Caco-2 monolayer cells in the presence of unloaded (**a**) PCL and Dex-$PCL_{5.5}$ nanoparticles; LC-loaded (**b**) PCL and Dex-$PCL_{5.5}$ nanoparticles: (♦, ◊) control, (■, □)PCL,(▲, Δ) Dex- $PCL_{5.5}$ nanoparticles and (●, ❍) sodium cholate. The concentration of samples were 83.25 µg/well.

Figure 8. Association of the nanoparticles, estimated as a percentage of the radioactivity recovered in the Caco2 cells, following incubation of nanoparticles suspensions for increasing periods of time. unloaded PCL (◆) and Dex-$PCL_{5.5}$ nanoparticles (●); LC-loaded PCL (■) and Dex-$PCL_{5.5}$ nanoparticles (▲). The concentration of samples were 83.25 µg/well.



(**A**) sonication of insoluble Dex-PCLn copolymers in the presence of water and methylene chloride, leading to the formation of a w/o emulsion. (**B**) sonication of the w/o emulsion in the presence of an aqueous phase, leading to w/o/w emulsion formation. (**C**) solvent evaporation, leading to nanoparticle formation.

Figure 1. Schematic illustration of the mechanism of core–corona Dex-PCL_n nanoparticles formation.

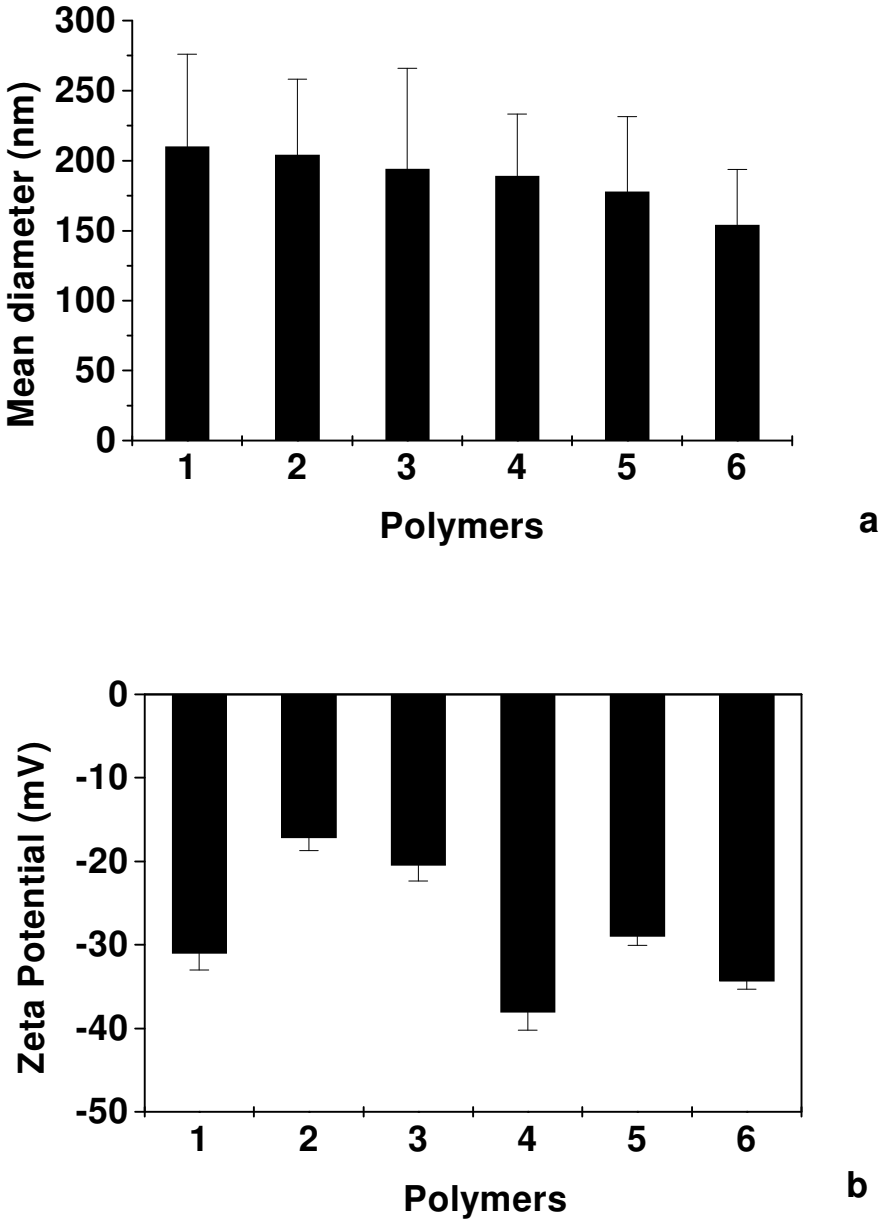


Figure 2. Evaluation of the mean diameter (**a**) and surface charge (**b**) of unloaded nanoparticles prepared by double emulsion method, for new Dex-PCLn nanoparticles compared with PCL_n: (1) Dex-$PCL_{7.1}$, (2) Dex-PCL_3, (3) Dex-$PCL_{5.5}$, (4) PCL 2K, (5) PCL 10K, (6) PCL 40K.

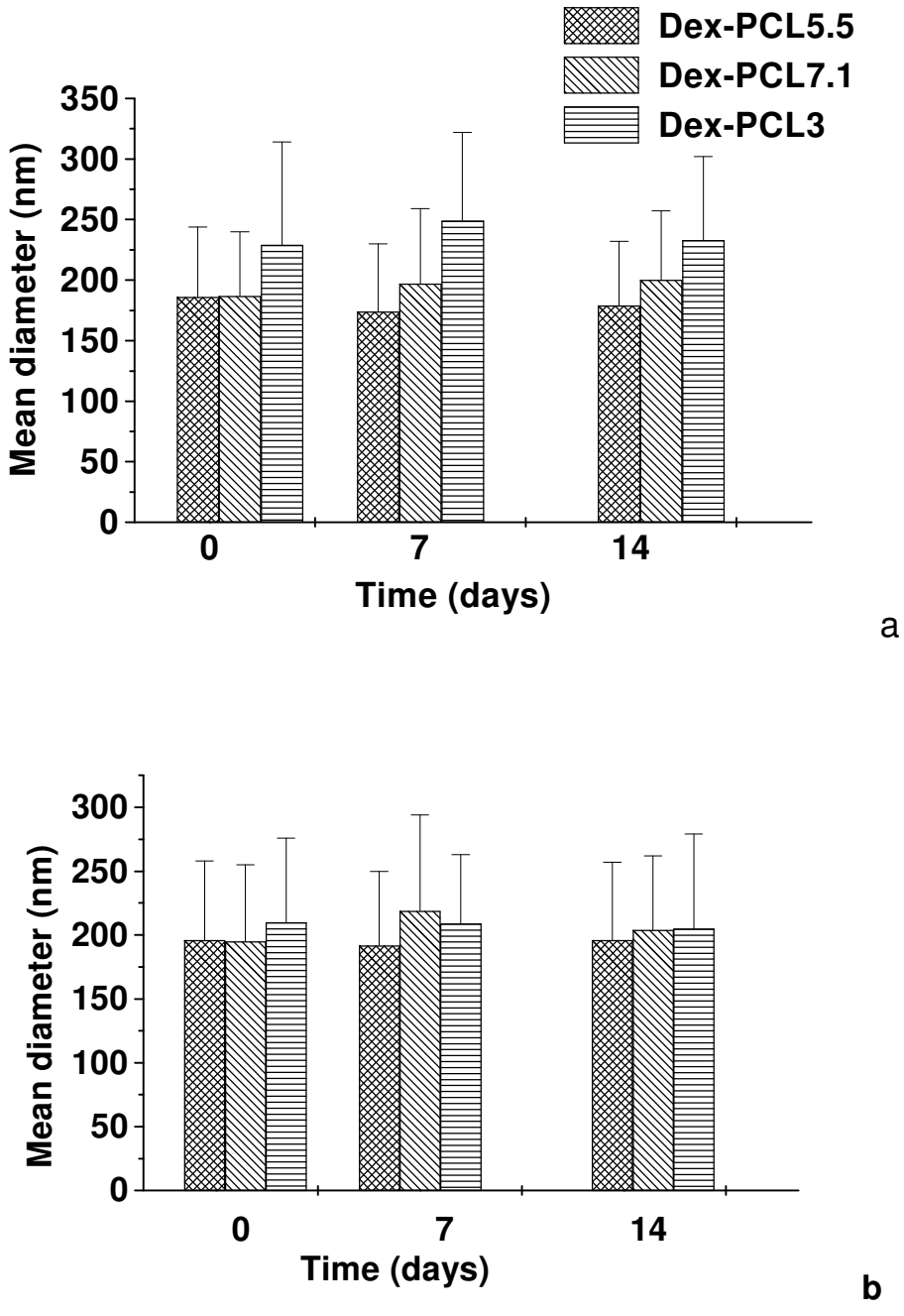


Figure 3. Stability of unloaded Dex-PCL_n (n = 7.1, 5.5 and 3) nanoparticles produced by using simple emulsion (a) and double emulsion (b) methods. The nanoparticles were storaged at 4°C.

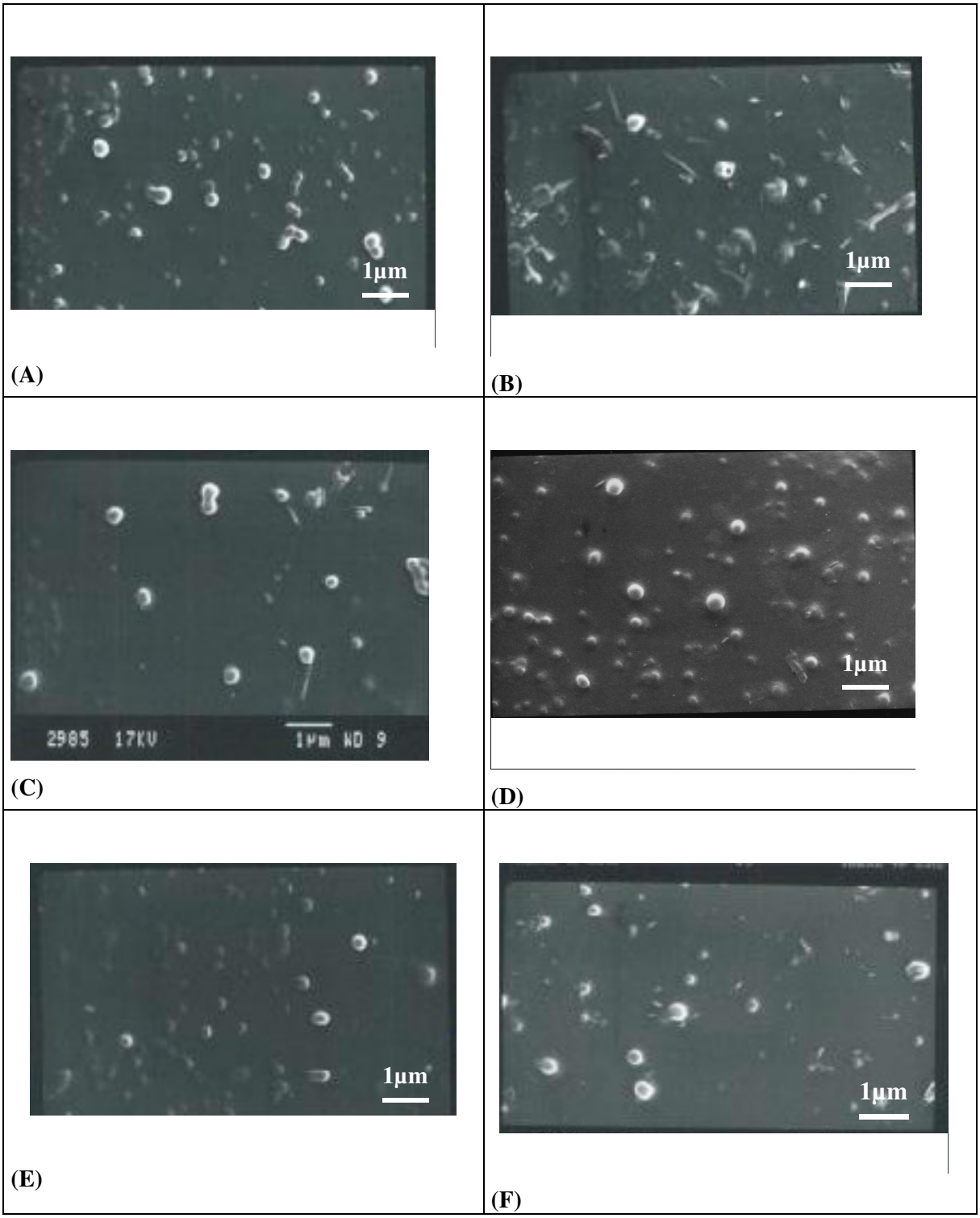


Figure 4. Evaluation of the morphology of unloaded Dex-PCLn nanoparticles by scanning electronic microscopy: Dex-$PCL_{7.1}$ (**a** and **b**), Dex-$PCL_{5.5}$ (**c** and **d**), and Dex-PCL_{3} (**e** and

f). Macrographs were taken one day after (**a, c** and **e)** and fifteen days **(b**, **d** and **f)** after fabrication of nanoparticles.

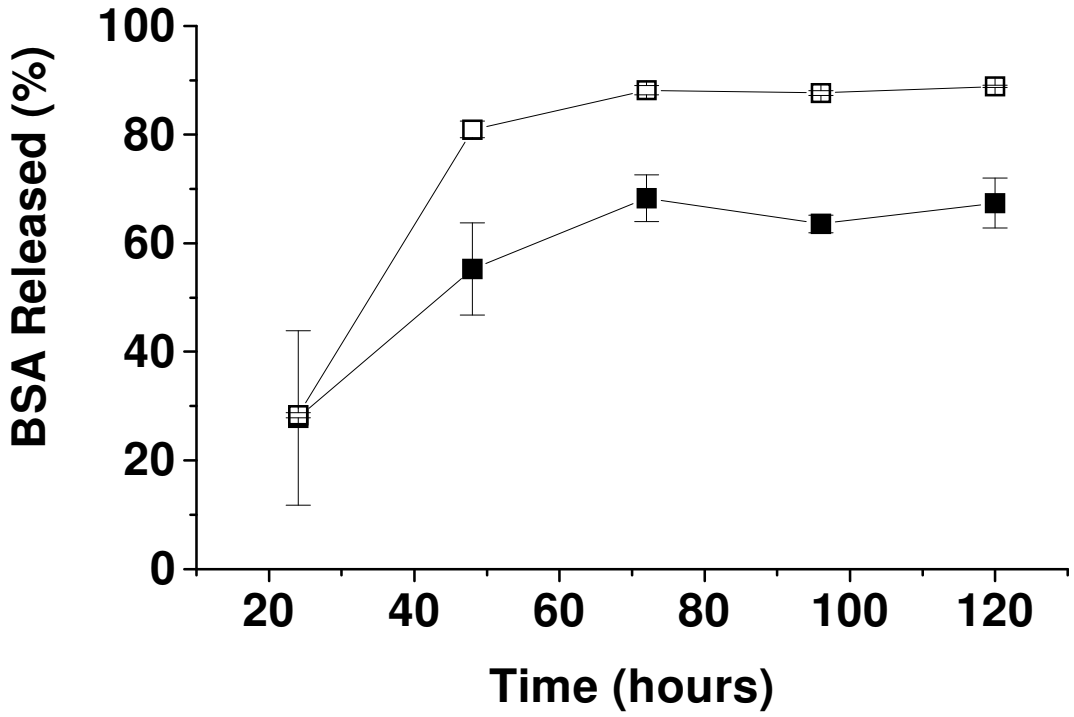
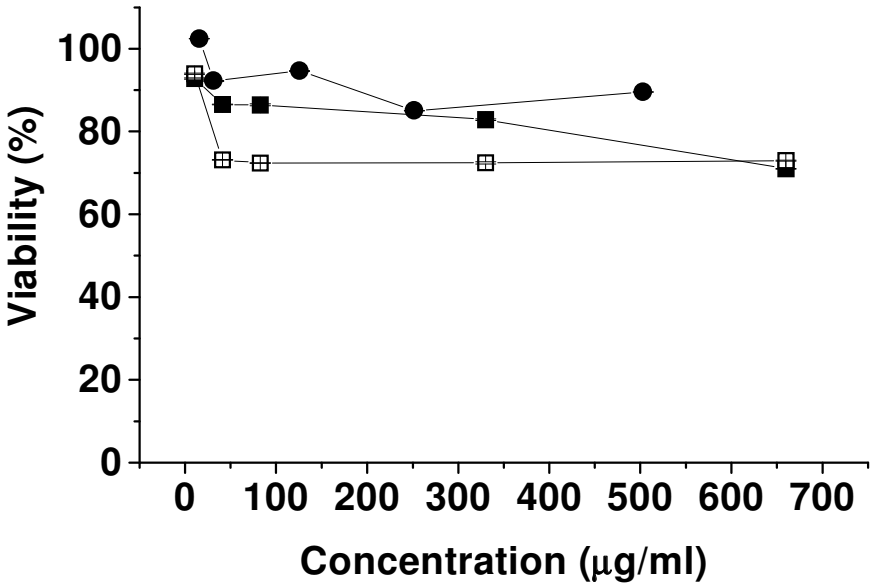


Figure 5. In vitro kinetic release profiles of BSA from Dex-PCL $_{5.5}$ nanoparticles prepared by simple (□) and double emulsion (■) methods. The concentration of BSA was 642 µg/ml of nanoparticles suspension.



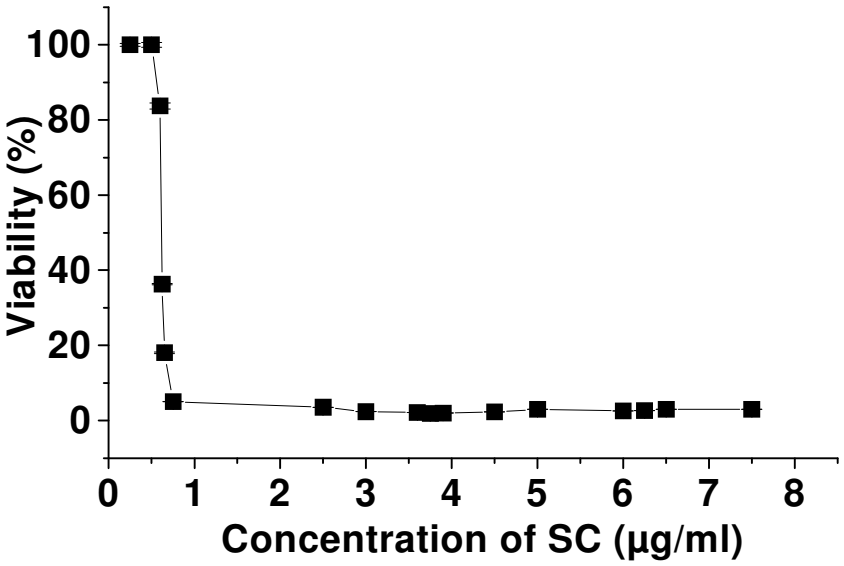
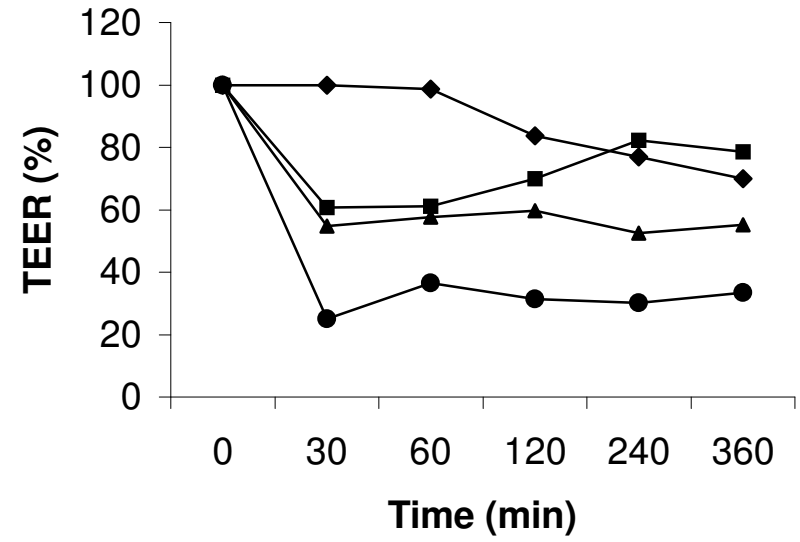
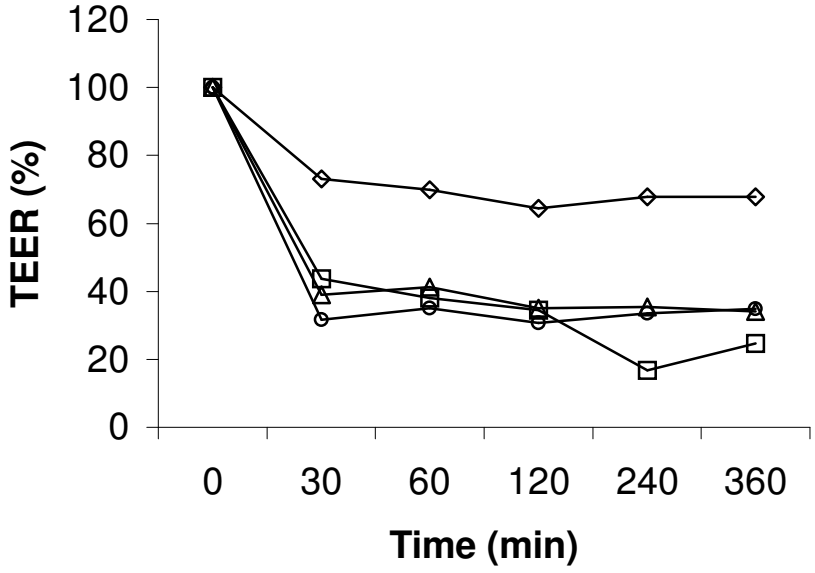


Figure 6. Association of the nanoparticles, estimated as a percentage of the radioactivity recovered in the Caco2 cells, following incubation of nanoparticles suspensions for increasing periods of time. unloaded PCL (◆) and Dex-$PCL_{5.5}$ nanoparticles (●); LC-

loaded PCL (■) and Dex-$PCL_{5.5}$ nanoparticles (▲). The concentration of samples were 83.25 μg/well.



(**a**)



(b)

Figure 7. Evaluation of transepithelial resistance (TEER) of Caco-2 monolayer cells in the presence of unloaded (**a**) PCL and Dex-$PCL_{5.5}$ nanoparticles; LC-loaded (**b**) PCL and Dex-$PCL_{5.5}$ nanoparticles: (♦, ◊) control, (■, □)PCL,(▲, Δ) Dex- $PCL_{5.5}$ nanoparticles and (●, ❍) sodium cholate. The concentration of samples were 83.25 μg/well.

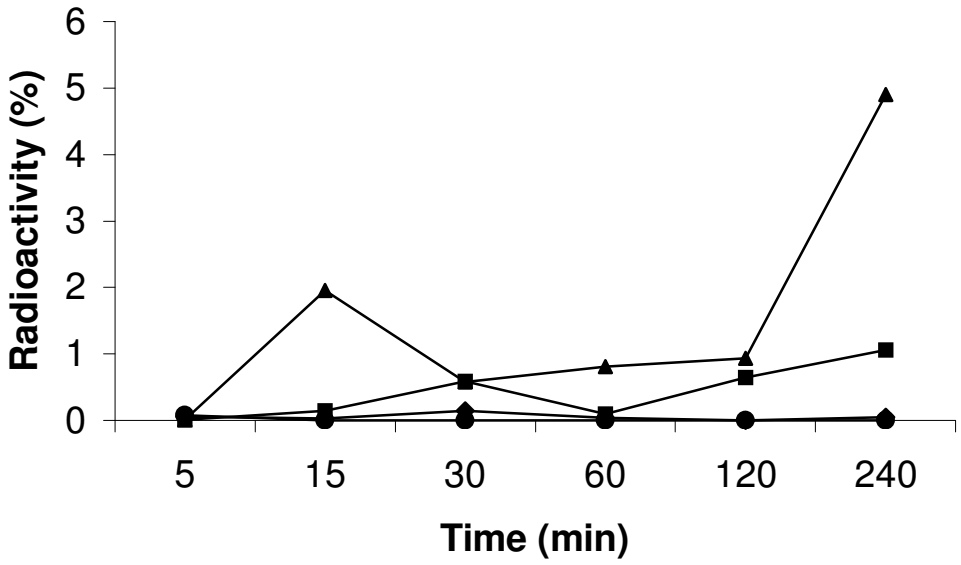


Figure 8. Association of the nanoparticles, estimated as a percentage of the radioactivity recovered in the Caco2 cells, following incubation of nanoparticles suspensions for increasing periods of time. unloaded PCL (◆) and Dex-$PCL_{5.5}$ nanoparticles (●); LC-loaded PCL (■) and Dex-$PCL_{5.5}$ nanoparticles (▲). The concentration of samples were 83.25 μg/well.

4. CONCLUSÕES

A análise dos resultados obtidos no desenvolvimento da presente tese permite a elaboração das seguintes conclusões:

- A atividade hemaglutinante da lectina de folha de *Bauhinia monandra* (BmoLL) está mais relacionada às condições de armazenamento (liofilização ou em solução tamponada) do que ao processo de produção das nanopartículas (ação de ultra-som, agitação mecânica do tipo ultra-turrax e presença de solventes). Os resultados sugerem que a BmoLL apresenta maior atividade quando armazenada na forma liofilizada e que a ação de ultra-som por um período curto de tempo de 30 segundos, de agitação mecânica e de solventes não parece ser deletéria sobre a atividade hemaglutinante;

- Nanopartículas de polímeros biodegradáveis convencionais (PCL, PLA e PLGA) contendo BmoLL encapsulada foram produzidas pelo método de emulsão múltipla, utilizando desoxicolato de sódio como tensoativo;

- As nanopartículas apresentaram formas esféricas bem definidas, com diâmetro médio inferior a 200 nm e distribuição de partículas relativamente monodispersa, independente da natureza ou do peso molecular do polímero utilizado;

- A taxa de encapsulação de BmoLL foi 30% superior para nanopartículas de PLGA 50/50 com relação às nanopartículas de PCL. No entanto, a avaliação da carga de superfície das partículas pela medida do potencial zeta demonstrou uma maior afinidade de BmoLL pela matriz polimérica de PCL;

- A liberação da BmoLL a partir das nanopartículas foi inferior 50% durante 24 h;

- BmoLL foi fortemente adsorvida na superfície de nanopartículas com maior afinidade para o PLGA 75/25;

- Novos copolímeros de dextrana e caprolactona (Dex-PCL_n) foram sintetizados em três etapas: (i) polimerização de ε-caprolactona derivada monocarboxílica, (ii) ativação de grupos carboxílicos com carbonildiimidazol e (iii) ligação com a dextrana com diferentes quantidades de cadeias de PCL ligadas a uma cadeia de dextrana através de ligações ésteres (n = 7,1; 5,5 e 3);

- Os novos copolímeros de Dex-PCL_n foram capazes de formar nanopartículas do tipo núcleo-coroa (matriz de PCL e coroa de dextrana), comprovada pela carga de superfície elevada das nanopartículas;

- BmoLL e lectina de *Lens culinaris* foram eficientemente encapsuladas em nanopartículas de Dex-$PCL_{5,5}$ quando comparada à proteína de referência BSA;

- A camada externa de dextrana aumentou a capacidade de adsorção de proteínas na superfície das nanopartículas de Dex-$PCL_{5,5}$;

- BmoLL, desoxicolato de sódio e nanopartículas de Dex-PCL_n não apresentaram citotoxicidade *in vitro* contra células de carcinoma humano de cólon (caco-2);

- Nanopartículas de Dex-PCL_n podem ser utilizadas como carreadores de fármacos hidrofóbicos ou de proteínas, bem como apresentam potencial para conjugação com lectinas para liberação sítio-específica de fármacos;

- A presença de lectina diminui a resistência transepitelia (TEER) da monocamada de células Caco-2 facilitando a adesão das nanopartículas Dex-PCL;

- A interação bioadesiva das nanopartículas Dex-PCL radiomarcadas foi superior àquela de nanopartículas de PCL.

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[5] N.F. Cardarelli, K.E. Walker, G. Zweig, Development of registration criteria for controlled release pesticide formulations, U.S. Environmental Protection Agency, Washington, DC 20460, EPA-504/9077-916, January 1978.

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RODRIGUES, Jaqueline Silva, GREF, Ruxandra, PONCHEL, Gilles, COELHO, Luana C. B., SANTOS-MAGALHÃES, Nereide Stella. Characterization of Dextran-Policaprolactone nanoparticles. In: XXXI REUNIÃO ANUAL DA SOCIEDADE BRASILEIRA DE BIOQUÍMICA E BIOLOGIA MOLECULAR - SBBQ, 2002, Caxambu. Anais da XXXI Reunião Anual da SBBq, 2002, p.H60.

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