



UNIVERSIDADE FEDERAL DE PERNAMBUCO

CENTRO DE CIÊNCIAS BIOLÓGICAS

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

**ESTUDO DA UTILIZAÇÃO DE FUCANA COMO AGENTE
DE DIRECIONAMENTO DE NANOPARTÍCULAS
PARA MACRÓFAGOS**

MARIANE CAJUBÁ DE BRITTO LIRA

RECIFE, 2009



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ESTUDO DA UTILIZAÇÃO DE FUCANA COMO AGENTE DE DIRECIONAMENTO DE
NANOPARTÍCULAS PARA MACRÓFAGOS

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*"C'est la curiosité, l'obsession et la simple persévérance
qui m'ont conduit à mes idées" Albert Einstein*

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LISTA DE ABREVIACÕES

AEP – Polimerização por emulsificação aniônica
CV – Coeficiente de variação
SFB – Soro fetal bovino
DMEM – “Dulbecco's Modified Eagle *Medium*”
DMSO – Dimetilsufóxido
DSC – Calorimetria exploratória diferencial
IC₅₀ – Concentração
GIT – Trato gastrointestinal
IBCA – cianoacrilato de isobutila
IR - Infravermelho
LUV – Vesícula unilamelar grande
MLV – Vesícula multilamelar
MPS – Sistema fagocítico mononuclear
MSR – “Macrophage Scavenger Receptors”
MWCO – Molecular Weight Cut Off
MW – Massa molecular
NMR H¹ – Ressonância magnética nuclear de hidrogênio
PACA – Poli(cianoacrilato de alquila)
PEG – Polietilenoglicol
PI – Índice de polidispersão
PIBCA – Poli(cianoacrilato de isobutila)
RPMI – Instituto memorial Roswell Park
RREP – Polimerização por emulsão em radicais redox
SUV – Vesícula unilamelar pequena
SEM/MEV – Microscopia eletrônica de varredura
TEM/MET – Microscopia eletrônica de transmissão
TG - Termogravimetria

RESUMO

O objetivo do presente trabalho foi inicialmente extrair e purificar o polissacarídeo fucana proveniente do *Sargassum cymosum* e utilizá-lo como agente de direcionamento de nanopartículas para macrófagos. A caracterização do material extraído e purificado foi efetuada utilizando análise elementar, espectroscopia de infravermelho (IV), ressonância magnética nuclear de hidrogênio (RMN H^1) e análise térmica e a determinação da massa molecular foi efetuada por cromatografia de permeação em gel. Em seguida, o material obtido foi utilizado na preparação das novas nanopartículas de Poli(cianoacrilato de isobutila) revestidas de fucana (FC-PIBCA-NP). As nanopartículas foram obtidas através da polimerização por emulsão aniônica (AEP) e polimerização por radicais redox (RREP) utilizando mistura de dextrana e fucana em diferentes proporções. As nanopartículas foram caracterizadas através do tamanho, potencial zeta, morfologia e estabilidade a longo prazo. Nanopartículas estáveis foram obtidas com até 100% de fucana pelo método AEP. Por outro lado, suspensões estáveis de nanopartículas foram obtidas com no máximo 25% de fucana por RREP. As suspensões obtidas com porcentagem mais elevadas de fucana não permaneceram estáveis. O potencial zeta das nanopartículas diminuiu com o aumento da quantidade de fucana, alcançando o valor de -44 mV para as nanopartículas preparadas por AEP com 100% de fucana. Estes resultados indicam que a fucana permanece localizada na superfície das nanopartículas. A análise morfológica por microscopia eletrônica de varredura (MEV) e microscopia eletrônica de transmissão (MET) mostrou nanopartículas com forma esférica. A estrutura interna das nanopartículas do tipo núcleo-coroa foi mostrada claramente por análise MET o que está de acordo com os resultados do potencial zeta que sugerem a localização do polissacarídeo na superfície das nanopartículas. As interações das nanopartículas com a linhagem celular de macrófagos J774 e com fibroblastos NIH-3T3 foram investigadas pela avaliação da citotoxicidade e pela observação da captura celular por microscopia confocal de fluorescência. As nanopartículas preparadas pelo método AEP mostraram uma maior citotoxicidade em macrófagos. Por exemplo, as nanopartículas obtidas pelo método AEP contendo 25% de fucana mostraram-se 3 vezes mais citotóxicas em macrófagos J774 comparadas às nanopartículas RREP ($IC_{50} = 4 \pm 2 \mu g/mL$ e $IC_{50} = 11 \pm 3 \mu g/mL$, respectivamente). As observações efetuadas por microscopia confocal de fluorescência para avaliar a captura e a distribuição das nanopartículas nas células não permitiram explicar o aumento da citotoxicidade das nanopartículas obtidas pelo método AEP nos macrófagos.

Todos os tipos de nanopartículas foram encontradas nos dois tipos de células, demonstrando que elas podem ser capturadas independentemente pelos macrófagos e fibroblastos. As diferenças na aparência da fluorescência no interior das células, i.e., pontual ou difusa, sugerem que as nanopartículas penetraram nas células por mecanismos distintos. No entanto, nenhuma correlação pode ser estabelecida entre o tipo de nanopartículas (AEP ou RREP) e o teor de fucana. As nanopartículas foram administradas em ratos por via oral com objetivo de investigar se elas poderiam ser direcionadas para os macrófagos do tecido linfóide intestinal. As propriedades bioadesivas e localização no tecido intestinal das nanopartículas fluorescentes foram investigadas por microscopia de fluorescência. Somente as nanopartículas revestidas com fucana obtidas pelo método RREP apresentaram uma redução na bioadesão comparada aos outros tipos de nanopartículas. A análise por microscopia de fluorescência revelou que as nanopartículas foram principalmente encontradas associadas ao muco residual. Apenas uma pequena quantidade de nanopartículas pôde ser visualizada na superfície das células epiteliais, do lado da luz intestinal onde estão localizadas as vilosidades e as placas de Peyer. Esses resultados sugerem a retenção das nanopartículas no muco e que uma pequena quantidade de nanopartículas chega à superfície do epitélio para ser eventualmente capturada pelos macrófagos. Embora o presente trabalho tenha permitido realizar a extração da fucana e a preparação de nanopartículas de PIBCA revestidas com fucana, não foi capaz, no entanto, de demonstrar que as novas nanopartículas podem ser utilizadas para aumentar a especificidade e direcionamento para macrófagos. Experimentos complementares serão, portanto, necessários para melhor compreender a origem da citotoxicidade das nanopartículas obtidas pelo método AEP em macrófagos J774. Além do mais, para o direcionamento das nanopartículas para macrófagos presentes em mucosas, as propriedades bioadesivas precisam ser ajustadas com vistas a reduzir a retenção pelo muco e de permitir um maior alcance na quantidade de nanopartículas a atingir a superfície das células epiteliais.

Palavras Chave: Nanopartículas, Fucana, Citotoxicidade, Macrófagos.

ABSTRACT

The aim of the present work was first to extract and purify the polysaccharide fucoidan from *Sargassum cymosum*, and to use it as targeting moiety of nanoparticles towards macrophages. The characterization of the purified material was achieved using elementary analysis, infrared spectroscopy (IR), proton nuclear magnetic resonance (^1H NMR), thermal analysis and the molecular weight was determined using size exclusion chromatography. Subsequently, the material was used to prepare new fucoidan-coated poly(isobutylcyanoacrylate) nanoparticles (FC-PIBCA-NP). The nanoparticles were prepared by anionic emulsion polymerization (AEP) and by redox radical emulsion polymerization (RREP) of isobutylcyanoacrylate using blends of dextran and fucoidan at different amount. The nanoparticles were characterized by their size, zeta potential, morphology and long term stability. Stable nanoparticles were obtained with up to 100% fucoidan by AEP. In contrast, stable suspensions of nanoparticles were only obtained with up to 25% fucoidan by RREP. Suspension obtained with higher percentages of fucoidan were very unstable. The zeta potential of fucoidan-coated PIBCA nanoparticles was decreased when the percentage of fucoidan was increased. It reached the value of -44 mV for nanoparticles prepared by AEP with 100% of fucoidan. This indicated that fucoidan was located on the nanoparticle surface. Morphology analysis by scanning electron microscopy and transmission electron microscopy highlighted nanoparticles with a spherical shape and a core-shell structure in agreement with the results of zeta potential, which suggested that the polysaccharide stranded at the nanoparticle surface. The interactions of the nanoparticles with a J774 macrophage cell line and NIH-3T3 fibroblasts were investigated by evaluating the cytotoxicity and observing the intracellular uptake by confocal fluorescent microscopy. All the nanoparticles prepared by AEP showed a high cytotoxicity on the macrophage cell line. For instance, the AEP nanoparticles containing 25% fucoidan appeared 3 folds more cytotoxic in J774 macrophages than the corresponding RREP

nanoparticles ($IC_{50} = 4 \pm 2\mu\text{g/mL}$ and $IC_{50} = 11 \pm 3\mu\text{g/mL}$, respectively). All types of nanoparticles were taken up by the two types of cells. Differences in the appearance of the fluorescence, i.e. punctuated or diffuse, suggested that the nanoparticles are associated with the cells following different pathways but no correlation could be established with the type of nanoparticles and/or their fucoidan content. The nanoparticles were then administered *in vivo* by the oral route in order to investigate if they can target macrophages of the intestinal lymphoid tissue. Fluorescent nanoparticles were used to investigate both their bioadhesive properties and their localization in the intestinal tissue by fluorescence microscopy. Only the fucoidan decorated RREP nanoparticles showed a reduction of bioadhesive properties compared with the other types of nanoparticles. By fluorescence microscopy, the nanoparticles were mainly found associated with the mucus remaining in the lumen. Only a very low amount of nanoparticles could be highlighted at the surface of the epithelial cells on the luminal side of both the epithelium containing villi and Peyer patches. These results suggested that the entanglement of the nanoparticles in the mucus compromised any eventual uptake of the nanoparticles by macrophages because the amount of nanoparticles reaching the surface of the epithelium was only marginal. Although fucoidan could be successfully extracted from the brown algae and associated with the PIBCA nanoparticle surface, the study failed to demonstrate that the new fucoidan-coated nanoparticles could be used to increase the specificity of the targeting of macrophages. Further experiments are needed to better understand the origin of the specific cytotoxicity found for the AEP nanoparticles on the J774 macrophage cell line and if one wants to target macrophages in a mucosa tissue, the bioadhesive properties of the nanoparticles need to be adjusted so that they will reach in large amount the surface of the epithelial cells.

Keywords: Nanoparticles, Fucoidan, Citotoxicity, Macrophages.

RÉSUMÉ

Les objectifs de notre travail ont été d'extraire et de purifier un polysaccharide, le fucane, à partir de l'algue *Sargassum cymosum* puis d'utiliser ce composé pour formuler des nanoparticules capable de cibler spécifiquement les macrophages. La caractérisation et la pureté du matériel extrait de l'algue ont été évaluées par analyse élémentaire, spectroscopie infrarouge, spectroscopie par résonance magnétique du proton, analyse thermique et la masse molaire a été mesurée par chromatographie d'exclusion stérique. Le fucane obtenu a été utilisé pour préparer des nanoparticules de poly(cyanoacrylate d'isobutyle) décorées par ce polysaccharide (FC-PIBCA-NP). Les nanoparticules ont été préparées par polymérisation du cyanoacrylate d'isobutyle en émulsion par voie anionique (AEP) et radicalaire (RREP) en présence de mélanges de dextrane et fucane en différentes proportions. Les nanoparticules ont été caractérisées par la mesure de leur diamètre, de leur potentiel zéta et leur morphologie a été étudiée par diverses méthodes de microscopie électronique. Des suspensions stables de nanoparticules ont pu être obtenues par la méthode AEP avec une composition en fucane de 100%. En revanche, par la méthode RREP, le pourcentage maximum de fucane qui peut être utilisé pour formuler des nanoparticules stables est de 25%. Les suspensions obtenues avec des pourcentages plus élevés de fucane ne sont pas stables. Le potentiel zeta des nanoparticules diminue lorsque la teneur en fucane augmente. Il atteint une valeur de -44 mV pour les nanoparticules préparées par la méthode AEP avec 100% de fucane. Ces résultats indiquent que le fucane est exposé à la surface des nanoparticules. L'analyse morphologique des nanoparticules par microscopie à balayage et par microscopie à transmission fait apparaître des particules de forme sphériques. Par microscopie électronique à transmission, une structure interne des nanoparticules de type core shell a été clairement mise en évidence ce qui est en accord avec les mesures de potentiel zeta qui suggéraient une localisation en

surface des polysaccharides. Les interactions des nanoparticules avec une lignée de cellules macrophagiques, les J774 et avec des fibroblastes, les NIH-3T3 a été étudiée par évaluation de la cytotoxicité et par observation de la capture par microscopie confocale à fluorescence. L'ensemble des nanoparticules préparées par la méthode AEP a montré une cytotoxicité élevée pour les macrophages J774. Par exemple, les nanoparticules AEP préparées avec 25% de fucane apparaissent 3 fois plus cytotoxiques pour les macrophages J774 que les nanoparticules RREP correspondantes ($IC_{50} = 4 \pm 2$ and $IC_{50} 11.3 \pm 3$, respectivement). Les observations réalisées en microscopie confocale de fluorescence pour évaluer la capture et la distribution des nanoparticules dans les cellules n'a pas permis d'expliquer l'augmentation de cytotoxicité des nanoparticules AEP pour les macrophages J774. Tous les types de nanoparticules ont été retrouvés à l'intérieur des deux types cellulaires montrant qu'elles peuvent être captées indépendamment par les macrophages et les fibroblastes. Les différences de fluorescence observées à l'intérieur des cellules, ponctuelle ou diffuse, suggèrent que les nanoparticules rentrent dans les cellules par différents mécanismes. Cependant, aucune corrélation n'a pu être établie avec le type de nanoparticules (AEP vs RREP) ou leur teneur en fucane. Les nanoparticules ont été administrées in vivo par la voie orale à des rats afin d'étudier le ciblage des macrophages du tissu lymphoïde associé au tractus intestinal. Des nanoparticules fluorescentes ont été utilisées pour évaluer la bioadhésion et la localisation des nanoparticules dans le tissu intestinal par microscopie à fluorescence. Seules les nanoparticules RREP recouvertes de fucane ont montré une réduction de la bioadhésion comparée aux autres nanoparticules étudiées. Par microscopie à fluorescence, les nanoparticules ont été retrouvées dans la lumière de l'intestin et associées à du mucus résiduel. Seulement une faible quantité de nanoparticules a pu être visualisée à la surface des cellules épithéliales du côté de la lumière intestinale dans du tissu contenant des villosités et des plaques de Peyer. Ces résultats suggèrent que les nanoparticules sont piégées dans le

mucus et que la quantité de nanoparticules qui arrive à la surface de l'épithélium est trop faible pour être éventuellement prises en charge par les macrophages. Si ce travail a permis de proposer une méthode d'extraction du fucane et d'associer avec succès ce polysaccharide à la surface de nanoparticules de PIBCA, il n'a pas permis de mettre en évidence une augmentation de spécificité de ciblage des nanoparticules recouvertes de fucane vers les macrophages. Des expériences complémentaires sont nécessaires pour comprendre l'origine de la cytotoxicité augmentée des nanoparticules AEP vis-à-vis des macrophages J774. Par ailleurs, si l'on souhaite poursuivre des travaux en vue de cibler des macrophages dans des tissus de type muqueuse, il sera d'abord nécessaire d'ajuster les propriétés de bioadhésion des nanoparticules en vue de réduire leur piégeage dans le mucus et de permettre à une plus grande quantité de nanoparticules d'atteindre la surface des cellules épithéliales.

Mots-clés : Nanoparticules, Fucoidan, Citotoxicité, Macrophages.

INTRODUÇÃO

1. INTRODUÇÃO

A nanotecnologia é definida como a ciência envolvida no desenvolvimento, síntese, caracterização e aplicação de materiais e dispositivos que se encontram na escala nanométrica (SAHOO, et al., 2007). A crescente aplicação da nanotecnologia na saúde, mais especificamente nas áreas farmacêutica e médica, originaram um novo campo de interesse denominado Nanomedicina. A nanomedicina consiste em processos de diagnóstico, tratamento e prevenção de doenças, no alívio da dor, na preservação e melhoria da saúde humana, através da utilização de dispositivos e sistemas na escala nanométrica (FREITAS, 2005). O desenvolvimento de sistemas de liberação controlada de substâncias é uma das áreas mais exploradas da nanomedicina. Lipossomas, nanopartículas poliméricas, nanotubos de carbono e dendrímeros são alguns exemplos de sistemas desenvolvidos. Esses sistemas têm como objetivo principal minimizar a toxicidade de certos fármacos utilizados atualmente na terapêutica, proporcionar uma melhoria no tratamento de doenças severas e, conseqüentemente, promover uma maior aceitação do tratamento por parte do paciente (HUGHES, 2005).

Dentre os vários tipos de sistemas, as nanopartículas poliméricas se destacam pela sua versatilidade estrutural e pela fácil obtenção. As nanopartículas poliméricas podem ser obtidas através de diversos métodos de preparação, no entanto, aqueles que não necessitam da utilização de solventes orgânicos são preferíveis porque não apresentam resíduos de solvente na formulação final. Dessa forma, a polimerização por emulsão aniônica (AEP) bem como a polimerização por emulsão em radicais redox (RREP) são técnicas que não necessitam de solventes orgânicos e são utilizados no preparo das nanopartículas poliméricas. Em ambos os métodos, a polimerização é realizada pela dispersão do monômero, em solução ácida de um polissacarídeo a ser associado à superfície da matriz polimérica das nanopartículas

(COUVREUR et al., 1979; CHAUVIERRE et al., 2003; BERTHOLON et al., 2006). Esses métodos são capazes de originar nanopartículas com diferentes características de organização das cadeias poliméricas do polissacarídeo na superfície das nanopartículas. Enquanto as nanopartículas obtidas por AEP apresentam superfície com aspecto de “cabeleira cacheada” as nanopartículas preparadas por RREP apresentam superfície em forma de “cabeleira alongada” (BERTHOLON-RAJOT et al., 2005). Além disso, a carga da superfície das nanopartículas pode ser moldada em ambos os métodos de acordo com a carga dos grupamentos químicos presentes no material utilizado. Assim, polissacarídeos negativos, neutros e positivos promovem a obtenção de nanopartículas com carga de superfície negativa, neutra ou positiva, respectivamente (CHAUVIERRE et al., 2003; BERTHOLON et al., 2006).

Desde a descoberta as nanopartículas vêm passando por várias modificações estruturais com objetivo de melhorar as propriedades de carreamento de fármacos ou substâncias biologicamente ativas. As nanopartículas convencionais não apresentam modificações em relação a sua superfície, sendo esta hidrofóbica, apresentando como característica principal o rápido reconhecimento pelas células do sistema fagocitário mononuclear. Consequentemente, essas nanopartículas são captadas e eliminadas da corrente circulatória após poucos minutos acumulando-se principalmente no fígado e no baço. Apesar das nanopartículas convencionais serem ideais para o tratamento de patologias que acometem esses órgãos, elas tornam-se ineficientes para o tratamento de outros órgãos alvo. Com objetivo de superar este inconveniente, as nanopartículas convencionais foram modificadas através da adição de moléculas hidrofílicas na sua superfície criando-se uma nuvem hidrofílica impedindo assim o seu reconhecimento pelo sistema fagocitário mononuclear (MPS). Consequentemente ocorre aumento no tempo de circulação, aumentando também as chances dessas nanopartículas atingirem outros órgãos alvo. O polietilenoglicol (PEG) é o polímero hidrofílico mais utilizado para obter nanopartículas de longa circulação, no entanto,

atualmente outras moléculas vêm sendo exploradas como alternativa ao PEG incluindo, por exemplo, os polissacarídeos.

Polissacarídeos são macromoléculas formadas por diferentes tipos de ligações químicas entre monossacarídeos e são encontrados em variadas fontes naturais: algas (alginato, fucana), plantas (pectina, agar-agar), microorganismos (dextrana, goma xantana) e de origem animal (quitosana e condroitina) (SINHA; RACHNA, 2001). A fucana é um polissacarídeo sulfatado extraído exclusivamente de algas marrons e de invertebrados marinhos como o ouriço e o pepino do mar (MULLOY et al., 2000). O aumento significativo no número de publicações sobre estudos relacionados à fucana é resultado do crescente interesse dado a este polissacarídeo, provavelmente devido as suas diversas atividades biológicas incluindo atividade anticoagulante, (CUMASHI et al., 2007; YOON et al., 2007), antiviral (HAYASHI et al. 2008), antiinflamatória (CUMASHI et al., 2007), antiangiogênica (CUMASHI et al., 2007), antitumoral (ITOH et al., 1993; ELLOUALI et al., 1993) e antiproliferativa (ELLOUALI et al., 1994). Além dessas atividades, a fucana tem a habilidade de seqüestrar metais pesados como Cd^{2+} , Cu^{2+} , Zn^{2+} , Pb^{2+} , Cr^{3+} e Hg^{2+} (DAVIS et al., 2003), ela também pode ser utilizada como suporte para imobilização de antibióticos (ARAÚJO et al., 2004) e ainda no tratamento de lesões cutâneas quando incorporada às microesferas (SEZER et al., 2008). Ademais, outra atividade explorada é a inibição da ativação do sistema complemento (BLONDIN et al., 1996; ZVYAGINTSEVA et al., 2003). Atualmente, em especial na área de nanocarreadores de liberação controlada, a inativação do sistema complemento é de grande interesse para aumentar o tempo de circulação desses sistemas. Finalmente, a fucana tem a capacidade de se ligar a receptores presentes nos macrófagos chamados “macrophages scavenger receptors” (MSR), do tipo AI e AII (HSU et al., 2001, KIM; JOO, 2003). Esta especificidade pode ser útil para proporcionar propriedades de direcionamento aos sistemas de liberação controlada recobertos com a fucana.

Dessa forma, neste trabalho foram associadas a utilização promissora de polissacarídeo sulfatado como material de revestimento e a nanotecnologia farmacêutica na obtenção de novas nanopartículas recobertas de fucana para liberação controlada de fármacos.

A tese consta da revisão da literatura sobre os temas a serem abordados como nanotecnologia e sistemas de liberação controlada, especificamente nanopartículas poliméricas com superfície modificada e por fim a fucana, polissacarídeo sulfatado como material de revestimento de nanopartículas. Os resultados obtidos nessa tese serão apresentados na forma de artigos, onde o primeiro aborda a extração, purificação e caracterização da fucana a partir de *Sargassum cymosum*. Por outro lado, o segundo artigo está relacionado à preparação e caracterização de novas nanopartículas revestidas de fucana observando-se os aspectos físico-químicos, a estabilidade a longo prazo, além da avaliação da citotoxicidade *in vitro* e captura celular das nanopartículas, através da quantificação da intensidade de fluorescência no interior das células. O terceiro artigo mostra os resultados obtidos no estudo *in vivo* de bioadesão de nanopartículas revestidas com fucana após administração oral em ratos.

REVISÃO DA LITERATURA

2. REVISÃO DA LITERATURA

2.1. Nanotecnologia e Sistemas de Liberação Controlada

O marco da nanotecnologia é bastante controverso. Alguns pesquisadores afirmam que as primeiras idéias relacionadas à nanotecnologia originaram-se nos anos 50, quando o físico Richard Feynman, em sua palestra intitulada “*There’s Plenty of Room at the Bottom*” (“Há mais espaço lá em baixo”), descreveu um novo campo da ciência que lida com “o problema de manipular e controlar materiais em escala pequena” (FREITAS, 2005; EMERICH; THANOS, 2006). No mesmo período, o cientista Paul Ehrlich inspirou o atual conceito de nanopartículas e o direcionamento de fármacos, através das “Magic Bullets”, sugerindo que a liberação de substâncias ativas em alvos terapêuticos poderia proporcionar melhorias significativas na terapia farmacológica (KREUTER, 1978; 2007).

O termo nanotecnologia somente foi utilizado em 1974 pelo pesquisador Norio Taniguchi da Universidade de Tokyo, o qual empregou o termo para aludir a habilidade de se desenvolver materiais precisamente na escala nanométrica (SAHOO et al., 2007). Atualmente, de acordo com a Iniciativa Nacional de Nanotecnologia (*The National Nanotechnology Initiative - NNI*) nos Estados Unidos, a nanotecnologia é definida como a compreensão e o controle da matéria nas dimensões nanométricas que variam de 1 a 100 nm (www.nano.gov) (Figura 1).

O advento da nanotecnologia é considerado como a maior inovação tecnológica desde a revolução industrial. Os investimentos na área da nanotecnologia Brasil ainda são poucos. Segundo o Ministério da Ciência e Tecnologia, entre 2004 e 2008 o ministério investiu 242 milhões de reais em pesquisas voltadas à nanotecnologia (www.mct.gov.br). Elas se espalham por áreas que incluem medicina, eletrônica, física, química, biologia e engenharia dos

materiais. Por outro lado, nos países desenvolvidos, os investimentos nesta área são bastante significativos. Em 2005 na Europa, o total de investimentos em nanotecnologia girou em torno de 5 bilhões de euros, e as estimativas para 2011-2015 no mercado mundial estão orçadas em mais de 1 trilhão de euros (STYLIOS, et al., 2005) o equivalente a 2,5 trilhões de reais. Indubitavelmente, a nanotecnologia vem proporcionando profundos impactos nas mais diversas áreas do conhecimento e, conseqüentemente, em vários aspectos da vida humana (GWINN; VALLYATHAN, 2006). As primeiras aplicações práticas da nanotecnologia são observadas nos avanços relacionados às áreas de comunicação, engenharia, robótica, física, química, biológica, farmacêutica e médica (FARAJI; WIPF, 2009).

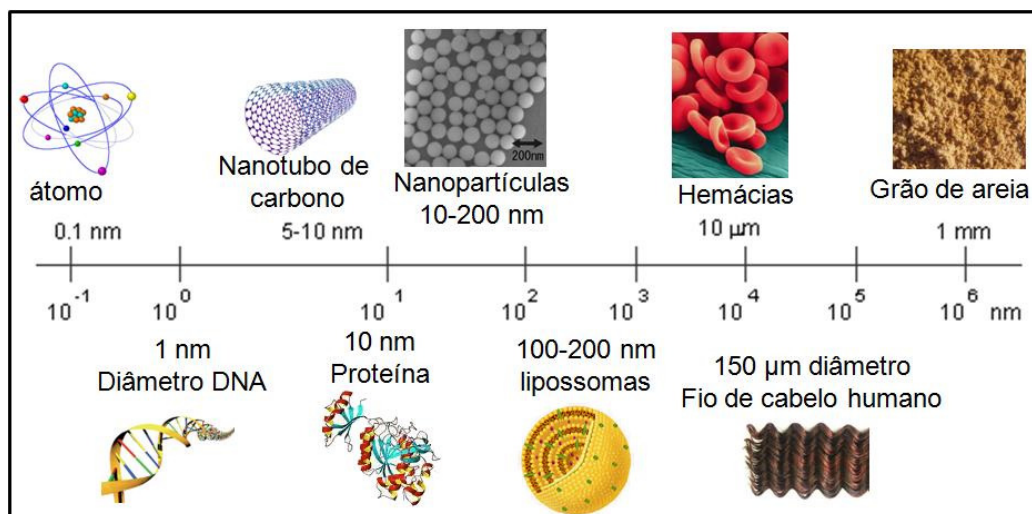


Figura 1. Esquema comparativo do tamanho de diversas estruturas (Criado por LIRA, M.C.B).

Os avanços da nanotecnologia na saúde, mais especificamente nas áreas farmacêutica e médica, impulsionaram o surgimento de um novo campo da nanotecnologia denominado nanomedicina. A nanomedicina engloba processos de diagnóstico, tratamento e prevenção de doenças, bem como preservação da saúde humana através da utilização de materiais na escala nanométrica (KAGAN et al., 2005).

Dentre as várias aplicações da nanomedicina, o desenvolvimento de sistemas de liberação controlada de substâncias como fármacos, material genético e proteínas, é uma das

áreas mais exploradas. Pesquisadores envolvidos no desenvolvimento de novos produtos farmacêuticos afirmam que os sistemas de liberação controlada são, atualmente, parte fundamental do desenvolvimento de novos medicamentos (SAHOO et al., 2007). Com isso, uma ampla gama de sistemas vem sendo estudados como as nanopartículas poliméricas, os lipossomas, as micelas, os nanotubos de carbono, os dendrímeros, entre outros (Figura 2) (FARAJI; WIPF, 2009).

SISTEMA	DEFINIÇÃO	FIGURA ESQUEMÁTICA	REFERÊNCIA
NANOESEFERAS	Sistemas matriciais na qual a substância ativa encontra-se adsorvida na superfície, dispersa ou solubilizada na matriz polimérica.		VAUTHIER; BOUCHEMAL, 2009
NANOCÁPSULAS	Sistemas reservatórios com uma cavidade interna oleosa (núcleo), rodeada por uma coroa polimérica onde a substância ativa pode encontrar-se dissolvida e dispersa respectivamente.		VAUTHIER; BOUCHEMAL, 2009
LIPOSSOMAS	Vesículas esféricas formadas por bicamadas concêntricas de fosfolípidos. Têm a capacidade de incorporar substâncias hidrofílicas no compartimento interno aquoso e lipofílicas na bicamada de fosfolípidos.		VOINEA; SIMIONESCU, 2002 TORCHILN, 2005.
MICELAS	Esferas formadas por moléculas de tensoativos dissolvidas em água. Apresenta um núcleo hidrofóbico rodeada por um córtex hidrofílico.		KATIME et al. 2003 KABANOV et al. 2007 NISHIYAMA; KATAOKA, 2006 TORCHILIN, 2002
NANOTUBOS DE CARBONO	São obtidos pelo enrolamento de uma ou mais folhas de grafeno originando nanotubos de parede única e múltiplas respectivamente.		BIANCO et al. 2005
DENDRÍMERO	Macromolécula globular contendo 3 partes distintas: núcleo, ramos que se originam do núcleo e grupamentos funcionais no exterior da macromolécula		DUTTA et al. 2009 DUCAN et al, 2005

Figura 2. Exemplos de nanossistemas de liberação controlada, definição, figura esquemática e referência (Adaptado de OUTTES-WANDERLEY, M.S., 2007).

Atualmente, alguns medicamentos na forma de sistema de liberação controlada de fármacos já se encontram disponíveis no mercado incluindo, por exemplo, DOXIL[®] e AMBISOME[®] que são a doxorubicina e a anfotericina na forma lipossomal, respectivamente.

Os sistemas de liberação controlada de fármacos, ao contrário dos convencionais, são aqueles que, além de apresentarem a habilidade de liberar de forma controlada a molécula ativa, ainda protegem a substância incorporada de possíveis degradações *in vivo* e melhoram a sua estabilidade frente a fatores externos como luz e oxidação (LEMARCHAND et al., 2004). A liberação controlada permite que a concentração plasmática da substância ativa mantenha-se dentro de uma faixa terapêutica ideal, ou seja, não ultrapasse limites que alcancem concentrações tóxicas e nem concentrações subterapêuticas (Figura 3). Consequentemente, ocorre diminuição dos possíveis efeitos adversos, aumento da adesão do paciente ao tratamento e melhoria da eficácia terapêutica. É importante que os sistemas de liberação controlada de fármacos, principalmente aqueles destinados a administração parenteral, sejam biodegradáveis, apresentem o tamanho de partícula na escala nanométrica, apresentem alta taxa de incorporação da substância ativa, sejam de fácil preparação e de baixo custo (TORCHILIN, 2007).

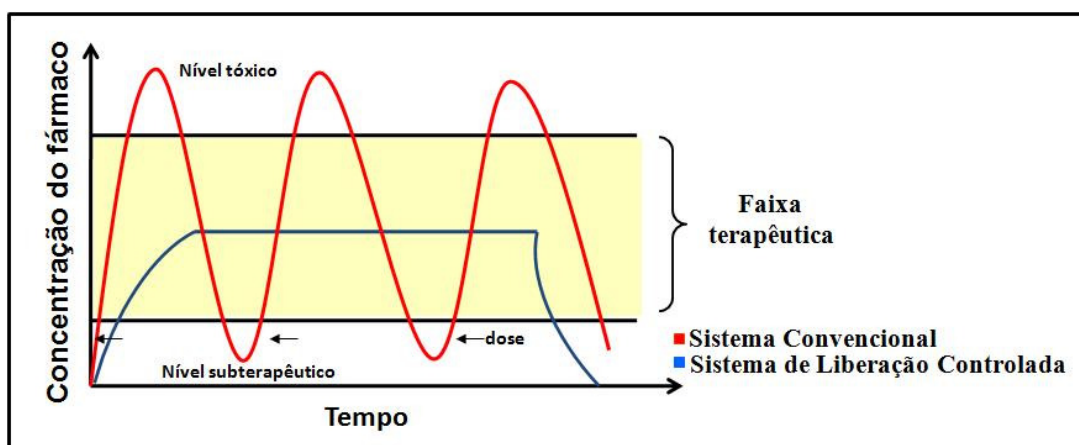


Figura 3. Farmacocinética comparativa dos sistemas terapêuticos convencionais e de liberação controlada (Criado por LIRA, M.C.B).

Atualmente, dentre os mais variados tipos de sistemas de liberação controlada de fármacos, as nanopartículas poliméricas se destacam por apresentarem vantagens como: estabilidade nos fluidos biológicos, fácil modificação da superfície e preparação, eficiência de encapsulação satisfatória, controle da cinética de liberação e facilidade de transposição de escala seguindo as normas de boas práticas de manipulação (BPM) (VLERKEN et al., 2007).

2.1.1. Nanopartículas poliméricas

O termo nanopartícula é uma denominação geral utilizada para se referir a dois sistemas distintos: nanoesferas e nanocápsulas. As nanoesferas são sistemas matriciais nos quais a substância ativa encontra-se dispersa ou solubilizada na matriz polimérica ou ainda adsorvida na superfície. Por outro lado, as nanocápsulas são sistemas reservatórios que apresentam uma cavidade interna (núcleo), geralmente oleosa, onde a substância ativa pode se encontrar dissolvida ou dispersa, rodeada por uma coroa polimérica como mostra a Figura 4 (VAUTHIER; BOUCHEMAL, 2009).

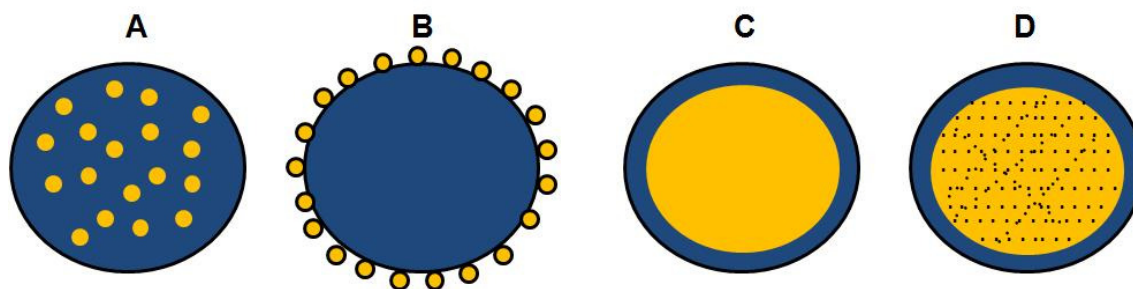


Figura 4. Representação esquemática das nanopartículas: (A) nanoesfera com princípio ativo na matriz polimérica, (B) nanoesfera com princípio ativo adsorvido a superfície, (C) nanocápsula com princípio ativo dissolvido, (D) nanocápsula com princípio ativo disperso na fase oleosa interna (Adaptado WEHRLÉ, 2007).

Tanto as nanoesferas como as nanocápsulas têm a capacidade de liberar a substância ativa incorporada de forma controlada. Essa liberação é realizada através de dois mecanismos que ocorrem de forma isolada ou associada: a difusão e a erosão. No primeiro mecanismo, a substância ativa que se encontra contida na matriz polimérica se dissolve e se difunde para fora do sistema. Por outro lado, na erosão a substância ativa é liberada em consequência da degradação natural do polímero pela hidrólise de suas ligações ésteres (FAROKHZAD; LANGER, 2006). Independentemente do mecanismo é imprescindível que a liberação da substância ativa ocorra de forma controlada com a concentração do fármaco dentro da faixa terapêutica.

Para a obtenção das nanopartículas poliméricas é importante conhecer as características dos polímeros empregados em sua preparação. Atualmente, devido aos avanços na química de polímeros é possível obter nanopartículas poliméricas com as mais variadas características (BRAUNECKER; MATYJASZEWSKI, 2007). Existe uma diversidade de polímeros que podem ser utilizados no desenvolvimento desses nanocarreadores incluindo os polímeros sintéticos: poliamidas, poliésteres, poliortoésteres, poliuretanos, poliacrilamidas (JAIN, 2000), os cianoacrilatos como poli(cianoacrilato de isobutila), poli(cianoacrilato de metila) e ainda os polímeros naturais como o alginato, a quitosana, gelatina, albumina entre outros (PILLAI; PANCHAGNULA, 2001). Os poliésteres, como poliácido lactico (PLA), poli(ϵ -caprolactona) (PCL), poliácido glicólico (PGA), poli(ácido láctico-co-glicólico) PLGA e poli(cianoacrilato de isobutila) (PIBCA), são os mais amplamente utilizados (FERNÁNDEZ-CARBALLIDO et al., 2008; GARBAYO et al., 2008; VAUTHIER; BOUCHEMAL, 2009).

Apesar da grande diversidade de polímeros, nem todos atualmente disponíveis podem ser utilizados. Para o uso seguro é necessário que eles atendam a alguns requisitos importantes. Primeiramente, é imprescindível que os polímeros escolhidos para preparação

das nanopartículas sejam biodegradáveis, ou pelo menos sejam totalmente eliminados da corrente sanguínea em um período relativamente curto. Assim, doses repetidas podem ser administradas evitando-se qualquer risco relacionado ao acúmulo do material no organismo. Além disso, tanto os polímeros como seus possíveis produtos de degradação não devem ser tóxicos nem imunogênicos e ainda devem ser oficialmente autorizados para uso em humano. Finalmente, é importante que os polímeros sintetizados e escolhidos na preparação proporcionem as características finais que se desejam obter nas nanopartículas como tamanho na escala nanométrica, forma esférica, perfil de liberação controlado do fármaco, entre outros fatores (VAUTHIER; BOUCHEMAL, 2009).

2.1.2. Métodos de preparação

Atualmente as nanopartículas poliméricas podem ser preparadas através de várias técnicas. A escolha do método, bem como o polímero a ser utilizado, depende das características físico-químicas da substância ativa a ser incorporada e das propriedades finais que se desejam obter nas nanopartículas (REIS et al., 2006; KUMARI et al., 2009). Os métodos de preparação das nanopartículas podem ser divididos em duas grandes classes: métodos utilizando polímeros pré-formados e métodos de preparação por polimerização *in situ* (SOPPIMATH et al., 2001). No primeiro caso destacam-se as técnicas de:

- *Emulsificação por evaporação de solvente*: Neste método, o polímero sintético e o princípio ativo são dissolvidos em uma fase orgânica imiscível na água. A solução obtida é dispersa na água formando uma emulsão grosseira. Em seguida a emulsão é levada ao homogeneizador (p. ex. ultrassom) com o objetivo de se obter gotículas finas e finalmente a fase orgânica é retirada dando origem a nanoesferas (WEHRLÉ, 2007; VAUTHIER; BOUCHEMAL, 2009).

- *Nanoprecipitação*: Por esta técnica, também conhecida como deslocamento de solvente, é possível se obter tanto nanoesferas como nanocápsulas. Primeiramente o princípio ativo e um óleo (no caso para obtenção de nanocápsulas) são dissolvidos em uma solução orgânica contendo o polímero. O solvente orgânico utilizado deve ser miscível na água em todas as proporções (geralmente utiliza-se acetona ou metanol). Em seguida, sob agitação moderada, a solução é injetada na fase aquosa, contendo geralmente por um surfactante hidrofílico. As nanopartículas são obtidas instantaneamente pelo efeito da difusão do solvente para a fase aquosa e da insolubilidade do polímero e do óleo no novo sistema solvente água. O polímero insolúvel na mistura água-solvente precipita na forma de nanoesferas incorporando o princípio ativo. No caso das nanocápsulas, com o deslocamento do solvente para a fase aquosa o polímero deposita-se na interface das gotículas emulsificadas formando uma parede polimérica. Finalmente o solvente orgânico é retirado por evaporação sob pressão reduzida (LIMAYEM BLOUZA et al., 2006; WEHRLÉ, 2007; VAUTHIER; BOUCHEMAL, 2009).

- *Emulsificação por difusão*: O princípio deste método se baseia no emprego de um solvente parcialmente miscível na água (p. ex. acetato de etila). O solvente e a água são saturados antes da utilização para a preparação da fase orgânica e aquosa. A fase orgânica contém o polímero, o princípio ativo e eventualmente um óleo (para obtenção das nanocápsulas) e na fase aquosa encontra-se o estabilizante. A emulsão é obtida sob agitação e sua diluição promove a formação das nanopartículas por difusão do solvente para a fase aquosa externa. Por fim, o solvente é eliminado por evaporação, ocorrendo a solidificação das nanopartículas (WEHRLÉ, 2007).

A segunda classe engloba os métodos de preparação por polimerização *in situ*. Quando o monômero a ser polimerizado é emulsionado em um solvente orgânico classifica-se em polimerização em dispersão, ao contrário, caso não ocorra utilização do solvente orgânico é

classificado como polimerização em emulsão (WEHRLÉ, 2007; VAUTHIER; BOUCHEMAL, 2009)

- *Polimerização em dispersão*: O fundamento da polimerização em dispersão consiste na dissolução do monômero hidrofílico em um meio aquoso contendo eventualmente um tensoativo não iônico. A polimerização é iniciada no meio aquoso por irradiação γ (gama) ou por agentes químicos (ex. peróxido de sulfato de amônio ou de sódio). A polimerização leva a formação de oligômeros que precipitam formando partículas primárias, estabilizadas pelo surfactante, as quais se combinam originando as nanopartículas. Caso o princípio ativo seja hidrofílico, ele é adicionado antes da formação das nanopartículas. Por outro lado, caso seja lipofílico é adicionado após a formação das nanopartículas (WEHRLÉ, 2007; VAUTHIER; BOUCHEMAL, 2009).

- *Polimerização em emulsão*: Este procedimento consiste na emulsificação de um monômero insolúvel ou parcialmente solúvel em água em uma fase aquosa contendo quantidade ideal de tensoativo. Este método apresenta a grande vantagem de não ser necessária a utilização de solventes orgânicos assim, evita a presença de resíduos de solvente na formulação final. Esta técnica vem sendo aplicada na preparação de nanopartículas utilizando-se, por exemplo, os poli(cianoacrilato de alquila). Os monômeros de cianoacrilato apresentam a notável vantagem de se polimerizarem espontaneamente quando entram em contato com os ânions presentes no meio, como é o caso da Polimerização Aniônica (AEP) descrita primeiramente por COUVREUR e colaboradores (1979). Os monômeros lipofílicos de cianoacrilatos de alquila são emulsificados em uma fase continua aquosa contendo o princípio ativo e um estabilizante (ex. polissacarídeo). O princípio ativo pode ser adicionado no meio reacional ou após a formação das nanoesferas. A polimerização é iniciada pelas hidroxilas (OH) presentes no meio aquoso se propagando ou na micela contendo o monômero (no caso do monômero ser bastante lipofílico), ou no meio aquoso e depois na

micela (no caso do monômero menos lipofílico). Neste método, o tempo de polimerização varia de 3 a 4 horas. A velocidade de polimerização e a ausência da formação de agregados depende do pH do meio, o qual para se obter partículas em torno de 200 nm deve ser ajustado para valores em torno 2-3. A acidez do meio reacional é o maior inconveniente deste método, pois pode afetar a estabilidade de certos princípios ativos até mesmo dificultar sua incorporação.

Além da polimerização por emulsão aniônica, a polimerização por emulsão radical redox (RREP) também é bastante utilizada na preparação das nanopartículas poliméricas. Neste método, o meio de polimerização é acidificado geralmente com ácido nítrico (0.2 M) e um par redox: um estabilizante (geralmente polissacarídeo) e íons cérico. O estabilizante e os íons cérico são solubilizados em solução aquosa ácida e em seguida o monômero é adicionado. A mistura permanece sob agitação vigorosa havendo imediatamente formação das nanopartículas. O tempo de polimerização é em torno de 1 hora. Ao contrário da polimerização aniônica que é iniciada pelas cadeias laterais do polissacarídeo, na polimerização em radical redox ela é iniciada pela parte final da cadeia. Esta diferença proporciona características de superfície distintas onde, no método AEP originam-se nanopartículas com superfície em forma de “cabeleira cacheada” e na RREP a superfície é em forma de “cabeleira alongada” (BERTHOLON-RAJOT et al., 2005) (Figura 5).

Métodos como o de polimerização por emulsão aniônica e radical redox, capazes de modificar as propriedades da superfície das nanopartículas, são de grande valia, pois essas propriedades são cruciais para se controlar o tempo e o destino final das nanopartículas após administração *in vivo* (VAUTHIER; BOUCHEMAL, 2009).

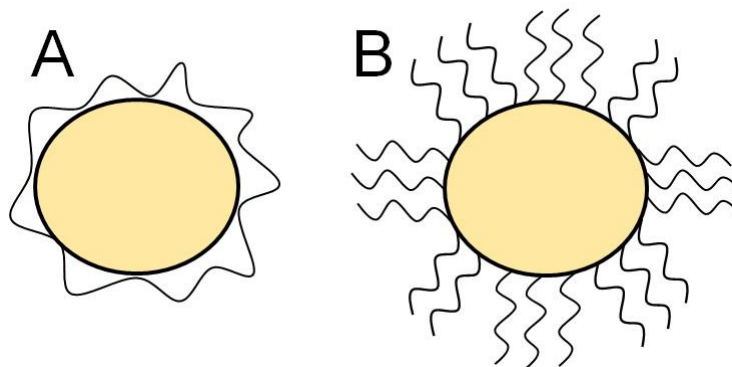


Figura 5. Representação esquemática da superfície das nanopartículas: (A) “cabeleira cacheada”, (B) “cabeleira alongada” (Adaptado de VAUTHIER et al., 2007).

2.1.3. Nanopartículas convencionais e Nanopartículas de superfície modificada

As primeiras nanopartículas obtidas são classificadas como convencionais ou de primeira geração. As nanopartículas convencionais apresentam uma superfície hidrofóbica a qual promove, após administração intravenosa, um fácil reconhecimento das nanopartículas pelas células do sistema fagocitário mononuclear (MPS). Como consequência, essas nanopartículas são captadas e rapidamente removidas da circulação sanguínea (KUMARI et al., 2009) acumulando-se principalmente no fígado e no baço. Tal peculiaridade faz das nanopartículas convencionais sistemas ideais para o tratamento, por exemplo, de doenças severas que acometem o fígado como hepatocarcinomas, metástase hepática (HALEY; FRENKEL, 2008). No entanto, o tratamento de doenças localizadas em outros órgãos fica comprometido, por não haver concentração suficiente da substância ativa no local de ação para promover o efeito farmacológico desejado. Com isso, a rápida eliminação torna-se a principal desvantagem das nanopartículas convencionais (LEMARCHAND et al., 2004).

O reconhecimento e eliminação de partículas estranhas da corrente sanguínea estão diretamente relacionados à habilidade das opsoninas em se ligarem à superfície dessas partículas. Neste contexto, com o objetivo de contornar tal limitação, várias modificações vêm sendo realizadas nas nanopartículas para camuflá-las, de modo a impedir seu reconhecimento

pelas células MPS (KUMARI et al., 2009). Uma modificação inclui alterações no tamanho das partículas, já que o tamanho desempenha um papel importante na biodistribuição e eliminação das mesmas. Partículas com tamanho superior a 200 nm são mais rapidamente eliminadas quando comparadas àquelas com diâmetro menor (OWENS; PEPPAS, 2006). Além do tamanho, uma abordagem de maior interesse relaciona-se com a modificação da superfície das nanopartículas através da inserção de moléculas hidrofílicas. As moléculas hidrofílicas têm a função de revestir as nanopartículas e bloquear as interações eletrostáticas e hidrofóbicas que auxiliam no reconhecimento e na ligação das opsoninas com a superfície das nanopartículas (OWENS; PEPPAS, 2006). Como consequência, há um bloqueio e atraso no primeiro passo do processo de opsonização (KUMARI et al., 2009). Assim, as nanopartículas revestidas apresentam um maior tempo de circulação, pois se encontram “camufladas”, ou seja, menos visíveis às células MPS não sendo reconhecidas, captadas e nem eliminadas facilmente.

Atualmente, diversas moléculas são descritas na literatura com a finalidade de revestir as nanopartículas e impedir a interação com as proteínas plasmáticas. Como exemplo dessas moléculas tem-se: poliacrilamida, poli(álcool vinílico), poli(N-vinil-2-pirrolidona), polietilenoglicol (PEG) e copolímeros de PEG. O PEG é a molécula mais empregada para modificar a superfície dos nanocarreadores. A inserção das moléculas de PEG é alcançada através da adsorção, da incorporação durante a preparação das nanopartículas ou ainda, através da formação de ligações covalentes entre o PEG e o polímero utilizado (MOGHIMI et al., 2001). A adição através de ligações covalentes é preferível, pois não apresenta o inconveniente encontrado na adsorção que é a fácil desestruturação *in vivo* da camada de revestimento (NEAL et al., 1998). As nanopartículas revestidas com PEG, denominadas de peguiladas, de longa-duração ou furtivas (Stealth[®]) são consideradas de segunda geração.

Vários trabalhos descritos na literatura expõem a utilização do PEG com a finalidade aumentar o tempo de circulação das nanopartículas. Há uma década, PERACCHIA e colaboradores (1999) já descreviam diferenças na biodistribuição de nanopartículas peguiladas e convencionais após administração intravenosa em ratos. Os autores observaram que as nanopartículas revestidas com moléculas de PEG apresentaram uma longa circulação, enquanto que aquelas sem o PEG foram removidas da circulação em poucos minutos. Mais recentemente, PARK e colaboradores (2009) compararam a eficácia de nanopartículas de PLGA-PEG contendo doxorrubicina, um potente fármaco antineoplásico, com o DOXIL[®] (forma lipossomal) e o fármaco livre, ou seja, não encapsulado. Os autores descreveram que as nanopartículas peguiladas contendo a doxorrubicina apresentaram maior tempo de circulação quando comparadas à forma lipossomal e à forma livre. Além disso, expuseram que tanto a eficácia terapêutica foi aumentada como a cardiotoxicidade do fármaco diminuída. Vários outros estudos também descrevem a utilização do PEG para aumentar o tempo de circulação das nanopartículas (RENOIR et al., 2006; VLERKEN et al., 2007; DANHIER et al., 2009).

O aumento do tempo de circulação das nanopartículas através da inserção do PEG permite uma melhoria no direcionamento passivo para as áreas onde há maior permeabilidade vascular, no local acometido pela doença, juntamente com aumento do extravasamento para tecidos vizinhos (EMERICH; THANOS, 2006). No entanto, para um direcionamento ativo, ou seja, para a liberação em um local específico da substância incorporada através do reconhecimento celular, é necessário que sejam adicionados ligantes à superfície das nanopartículas capazes de interagir com receptores nas células alvo (PERACCHIA et al., 1999; MOGHIMI et al., 2001). Neste contexto, as nanopartículas de terceira geração ou nanopartículas sítio-específicas vêm sendo estudadas.

Diferentes tipos de moléculas têm sido propostas para obtenção de nanocarreadores sítio-específicos como as lectinas (RODRIGUES et al., 2003; JEPSON et al., 2004), proteínas como a avidina (COESTER et al., 2000; PULKKINEN et al., 2008), anticorpos (KOCBEK et al., 2007), folato (DESTITO et al., 2007), entre outras. No entanto, o acoplamento de ligantes químicos nos nanocarreadores peguilados muitas vezes, torna-se difícil (STELLA, et al., 2000), sendo esta uma das razões pela qual o revestimento das nanopartículas com polissacarídeos têm sido considerado como uma alternativa à utilização do PEG (LEMARCHAND, et al., 2004). Ademais, polissacarídeos são capazes de alcançar sítios específicos *per si*, uma vez que apresentam receptores específicos em certas células e/ou tecidos (STAHN et al., 2000). Assim, é notável o interesse na utilização de polissacarídeos para revestimento de nanopartículas devido ao crescente número de publicações recentes relacionadas ao tema (LEMARCHAND et al., 2004; BERTHOLON et al., 2006; HE et al., 2009; MARTIMPREY et al., 2009; WU et al., 2009).

2.1.4. Métodos de caracterização das nanopartículas de superfície modificada

Além das técnicas usualmente utilizadas para caracterização dos nanocarreadores, várias outras têm sido desenvolvidas e empregadas para estudar especificamente a superfície das nanopartículas. A eficiência da modificação na superfície pode ser medida tanto pela determinação da carga, como pela densidade dos grupos funcionais e ainda através da mudança na hidrofobicidade da superfície (SOPPIMATH et al.; 2001).

Uma das técnicas mais utilizadas para avaliar as modificações na superfície dos nanocarreadores é a determinação da carga da superfície avaliada através da mobilidade da carga das partículas carregadas positiva ou negativamente, a qual é monitorada pela aplicação de um potencial elétrico. O valor do potencial pode ser positivo ou negativo, dependendo da natureza do polímero ou do material utilizado para revestir a nanopartícula. CHAUVIERRE e

colaboradores (2003) analisaram nanopartículas de poli(cianoacrilato de alquila) contendo polissacarídeos com diferentes cargas e uma mistura entre eles. Os resultados mostraram que o potencial zeta das nanopartículas revestidas com heparina, polissacarídeo de carga negativa, foi negativo. Por outro lado, aquelas recobertas com dextrana, polissacarídeo positivo, mostraram um potencial zeta positivo. E finalmente nas nanopartículas formadas pela mistura de polissacarídeos a carga da superfície apresentou-se como um valor intermediário. Posteriormente, a mesma tendência foi observada por BERTHOLON e colaboradores (2006).

Além do potencial zeta, a espectroscopia eletrônica para análise química (ESCA), também chamada de espectroscopia fotoeletrônica de raios-X, e ainda a determinação da hidrofobicidade da superfície através de cromatografia de interação hidrofóbica são técnicas de grande valia para caracterizar a superfície das nanopartículas (SOPPIMATH et al., 2001). A primeira técnica se baseia na emissão de elétrons do material em resposta a uma irradiação gerada. Como os fótons possuem baixa energia de penetração, apenas aqueles elétrons pertencentes a átomos na superfície ou próximo a superfície das nanopartículas são ejetados e podem ser quantificados. Desta forma, análise elementar da superfície pode ser realizada. Por outro lado, a determinação da hidrofobicidade envolve a utilização de coluna cromatográfica, a qual é capaz de separar o material baseando-se na interação com uma matriz de gel hidrofóbica. A interação das nanopartículas e do gel é função da hidrofobicidade da superfície (SOPPIMATH et al., 2001).

Independente do método utilizado é de extrema importância que a inserção dos polissacarídeos na superfície das nanopartículas seja confirmada.

2.2. Polissacarídeos - Fucana

Os polissacarídeos são polímeros de monossacarídeos muitas vezes de baixo custo. Podem ser facilmente encontrados na natureza em diversas fontes como: algas (alginato,

fucana), plantas (pectina, goma arábica), microrganismos (dextrana, goma xantana) e animais (quitosana, condroitina) (SINHA; RACHNA, 2001). Os polissacarídeos apresentam um grande número de grupos reativos, uma variedade de pesos moleculares, de composição química e de carga, podendo esta ser negativa, neutra ou positiva. Tais características contribuem para a grande diversidade na estrutura e nas propriedades dos polissacarídeos (LIU et al., 2008) e, como consequência, contribuem para a sua ampla utilização.

Atualmente há um grande interesse no isolamento e identificação de novos polissacarídeos bioativos especialmente os polissacarídeos sulfatados, os quais apresentam diversas e potentes atividades biológicas (HUANG et al., 2008).

A fucana é um exemplo de polissacarídeo sulfatado. Apresenta carga negativa devido aos grupamentos sulfato e é composta por resíduos de L-fucose ligadas por ligações $\alpha(1-2)$ e $\alpha(1-4)$ (ZVYAGINTSEVA et al., 2003) e outros monossacarídeos tais como xilose, galactose, manose e glicose. A fucana foi isolada pela primeira vez em 1913 por Kylin, o qual a denominou de “Fucoidin”. Atualmente, é nomeada de Fucoidana, contudo também é conhecida como fucana, fucosana e fucana sulfatada (LI et al., 2008).

A fucana é extraída da parede celular de espécies de algas marrons (Phaeophyceae) incluindo, *Fucus vesiculosus* (VEENA et al., 2007), *Fucus serratus* (BILAN et al., 2006), *Laminaria japonica* (WANG et al., 2008), *Sargassum cymosum* (PARDO; SOLÉ, 2007), *Stoechospermum marginatum* (LI et al., 2008) e várias outras espécies. Como mostra a Figura 6, além das algas marrons, a fucana também é extraída de invertebrados marinhos como o ouriço e o pepino do mar (BERTEAU; MULLOY, 2003; CINELLI et al., 2009). No entanto, a fucana não foi encontrada em espécies de algas verdes (Chlorophyceae), algas vermelhas (Rhodophyceae), algas douradas (Xanthophyceae) e nem tampouco nas algas de água doce e nas plantas terrestres (BERTEAU; MULLOY, 2003).

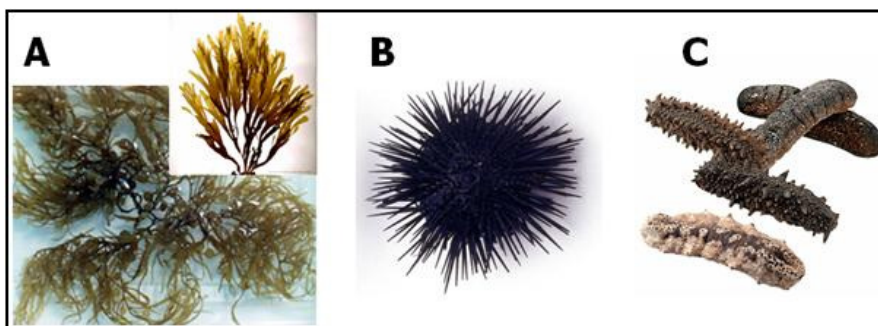


Figura 6. Diferentes fontes da fucana. (A) Algas marrons, (B) ouriço do mar, (C) pepino do mar (Criado por LIRA, M.C.B).

No caso dos equinodermos, a fucana extraída é conhecida como homofucana constituída por resíduos de L-fucose mono ou dissulfatada nos carbonos 2, 3 ou 4 (MULLOY, 1994). Por outro lado, a fucana extraída das algas marrons apresenta padrão heterogêneo apresentando além da fucose, uma pequena quantidade de outros monossacarídeos como xilose, galactose, manose e glicose (BILAN et al, 2004) (Figura 7).

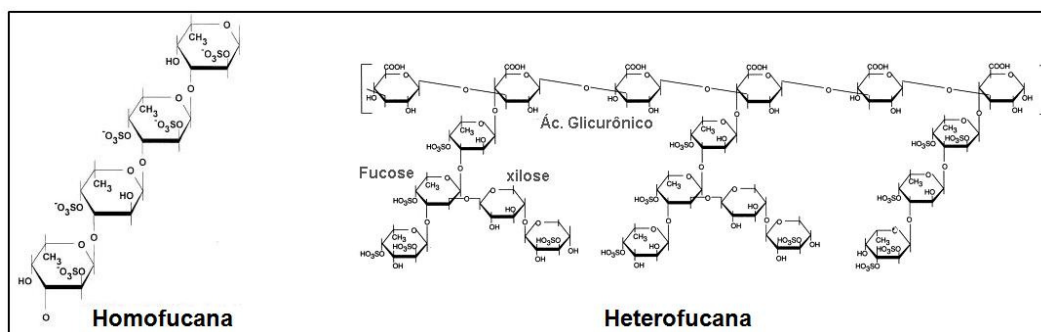


Figura 7. Estrutura química da homofucana e heterofucana (Adaptado de MULLOY et al., 2000 e LEITE et al, 1998).

Certas características da fucana como a massa molecular, a presença de grupamentos sulfato e as propriedades biológicas dependem diretamente da fonte de extração, seja alga marrom, seja dos invertebrados marinhos, e ainda dos métodos de extração e de purificação utilizados.

O crescimento significativo, nas últimas décadas, no número de publicações é reflexo do aumento do interesse pela fucana, devido a suas diversas atividades biológicas (HOLTKAMP et al., 2009). Essas atividades biológicas da fucana estão relacionadas com o grau de sulfatação, com a estrutura química e com o peso molecular (ZVYAGINTSEVA et al., 2003). Como a fucana nativa (extrato cru) é heterogênea e apresenta estrutura complexa, a elucidação da relação entre as atividades biológicas e estrutura química ainda não está esclarecida. Entretanto, é fato que as atividades biológicas da fucana não são meramente efeito da carga negativa, mas devido a estrutura (BERTEAU; MULLOY, 2003).

Dentre as várias atividades biológicas da fucana, a atividade anticoagulante é a mais estudada. Recentemente, USHAKOVA e colaboradores (2009), estudaram a atividade anticoagulante de onze extratos oriundos de diferentes espécies de algas marrons. O estudo mostrou que 8 das 11 fucanas analisadas apresentaram atividade anticoagulante significativa, embora diferente uma das outras. A diferença observada na atividade é atribuída às diferenças na fonte e na estrutura das fucanas analisadas. Outro estudo realizado por CUMASHI e colaboradores (2007) avaliou as propriedades anticoagulantes de nove espécies diferentes de algas marrons. Todas as fucanas avaliadas exceto aquela obtida da *Cladosiphon okamuramus* exibiram atividade anticoagulante. Ademais, também foi estudada a atividade anticoagulante da α -L-galactana e α -L-fucana extraída de invertebrados marinhos (PEREIRA et al. 2002). Os resultados obtidos indicaram que os requisitos estruturais necessários para a interação do polissacarídeo sulfatado com os fatores de coagulação são estereoespecíficos e não apresentam relação com a carga negativa do polissacarídeo. O estudo apresentou a relação entre a atividade

anticoagulante da fucana, a presença e posição dos grupamentos sulfato, a massa molecular e ainda da presença de outros monossacarídeos.

Além da potente atividade anticoagulante, a fucana apresenta atividade antioxidante (SOUZA, et al., 2007; WANG et al., 2008), antiviral (HAYASHI et al., 2008), antiinflamatória (CUMASHI et al., 2007), antiangiogênica (CUMASHI et al., 2007), antitumoral (ITOH et al., 1993; ELLOUALI et al., 1993), antiproliferativa (ELLOUALI et al., 1994) e imunoestimulante (KIM; JOO, 2008; TERUYA et al., 2009). Além disso, possui a habilidade de sequestrar metais pesados tóxicos como Cd^{2+} , Cu^{2+} , Zn^{2+} , Pb^{2+} , Cr^{3+} e Hg^{2+} (DAVIS et al., 2003) e pode ser utilizada como suporte para imobilização de antibióticos (ARAÚJO et al., 2004).

No contexto deste trabalho, o início da utilização do polissacarídeo em sistemas de liberação controlada bem como algumas atividades biológicas da fucana devem ser detalhadas.

Em 2008, SEZER e colaboradores publicaram trabalho pioneiro com a utilização da fucana associada a um sistema de liberação controlada. Primeiramente em 2007, o grupo utilizou um filme formado por complexo de fucana-quitosana para o tratamento de lesões cutâneas promovidas por queimaduras em coelhos (SEZER et al., 2007). Os autores observaram que após 14 dias de tratamento tópico utilizando o filme de fucana/quitosana, houve uma contração significativa na lesão com maior aceleração do processo de fechamento e cura comparado aos grupos controles (filme de quitosana e solução de fucana). A partir daí, os autores propuseram a incorporação da fucana em microesferas de quitosana, denominadas Fucoesferas, para a sua utilização em processos de cicatrização em coelhos (SEZER et al., 2008). O mesmo tratamento foi realizado e novamente se observou uma diminuição nas lesões e melhoria no processo de cicatrização. Além disso, foi observado um aumento na bioadesão das microesferas quando houve um aumento da concentração de quitosana e fucana na preparação de microesferas.

Uma atividade biológica da fucana explorada é a de inibição da ativação do sistema complemento (ZVYAGINTSEVA et al., 2003). O sistema complemento é o principal

componente da imunidade e está envolvido na resposta inata e humoral. A ativação do sistema complemento, regulada em vários níveis pelas células brancas do sangue, leva à atividades biológicas que dependem da interação entre as proteínas do complemento ou fragmentos ativados com receptores específicos nas células. Atualmente, em especial nos sistemas de liberação controlada, a inativação do sistema complemento é de grande interesse para obtenção de carreadores furtivos ou de longa circulação sistêmica. Assim, estudos têm se focalizado no desenvolvimento de moléculas com esta propriedade. Geralmente, são utilizados compostos sintéticos para a inibição do sistema complemento, porém muitas vezes esses compostos apresentam efeitos tóxicos. Por outro lado, algumas moléculas semi-sintéticas e compostos naturais têm sido descritos como inibidores do sistema complemento não apresentando toxicidade. A fucana vem sendo estudada como inibidor do sistema complemento há mais de uma década (BLONDIN et al., 1996), sendo observado que a atividade anticomplementar é influenciada pela massa molecular e presença de grupamentos sulfato. ZVYAGINTSEVA e colaboradores (2003) investigaram a atividade anticomplementar *in vitro* da fucana extraída de três diferentes espécies de algas marrons. Os autores descreveram que a fucana obtida da *L. Cichorioides*, da *L. japonica* e da *F. evanescens* apresenta atividade inibidora do sistema complemento humano.

Outra atividade da fucana de grande interesse é a capacidade de se ligar aos receptores de membrana do tipo AI e AII encontrados nos macrófagos chamados “macrophages scavenger receptors” (MSR) (YOKOTA et al.; 1998; HSU, et al.; 2001; SULAHIAN et al.; 2008). Receptores deste grupo são capazes de reconhecer vários ligantes incluindo moléculas quimicamente modificadas, em particular as lipoproteínas modificadas que estão relacionadas com o desenvolvimento de doença vascular, além de polissacarídeos como a dextrana sulfatada e a fucana, lipopolissacarídeos e sílica (PLATT; GORDON, 2001).

Assim, a utilização da fucana como molécula anticomplementar descrita na literatura, bem como sua capacidade de se ligar aos macrófagos, pode ser uma alternativa na obtenção de novos nanocarreadores de longa circulação e/ou sítio-específicos.

3. REFERENCIAS BIBLIOGRAFICAS

- ARAÚJO, P.M.; OLIVEIRA, G.B.; CÓRDULA, C.R.; LEITE, E.L.; CARVALHO JR. L.B.; SILVA, M.P.C. Sulfated fucan as support for antibiotic immobilization. *Brazilian Journal of Medical and Biological Research*. v. 37, p. 301-305, 2004.
- BERTEAU, O.; MULLOY, B. Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology*. v. 13, p. 29R-40R, 2003.
- BERTHOLON-RAJOT, I.; LABARRE, D.; VAUTHIER, C. Influence of the initiator system, cerium-polysaccharide, on the surface properties of poly(isobutylcyanoacrylate) nanoparticles. *Polymer*. v. 46, p. 1407-1415, 2005.
- BERTHOLON, I.; HOMMEL, H.; LABARRE, D.; VAUTHIER, C. Properties of polysaccharides grafted on nanoparticles investigated by EPR. *Langmuir: The ACS journal of surfaces and colloids*. v. 22, p. 5485-90, 2006.
- BILAN, M. I.; GRACHEV. A.A.; USTUZHANINA, N.E.; SHASHKOV, A.S.; NIFANTIEV, N.E.; USOV, A.I. A highly regular fraction of a fucoidan from the brown seaweed *Fucus distichus* L. *Carbohydrate Research*, v. 339, p. 511-517, 2004.
- BLONDIN, C.; CHAUBET, F.; NARDELLA, A.; SINQUIN, C.; JOZEFONVICZ, J. Relationship between chemical characters and anticomplementary activity of fucans. *Biomaterials*, v. 17, p. 597-603, 1996.
- BRAUNECKER, W. A.; K. MATYJASZEWSKI. Controlled/living radical polymerization: features, developments and perspectives. *Progress Polymer Science*. v. 33, pp. 93-146, 2007.
- CHAUVIERRE, C.; LABARRE, D.; COUVREUR, P.; VAUTHIER, C. Novel Polysaccharide-Decorated Poly(isobutylcyanoacrylate) Nanoparticles. *Pharmaceutical Research*, v. 20, p. 1786-1793, 2003.

- CINELLI, L.P.; VILELA-SILVA, A.C. E.S.; MOURÃO; P.A.S. Seminal fluid from sea urchin (*Lytechinus variegatus*) contains complex sulfated polysaccharides linked to protein. *Comparative Biochemistry and Physiology, Part B*. v.154, p. 108–112, 2009.
- COESTER, C.; KREUTER, J.; BRIESEN, H.; LANGER, K. Preparation of avidin-labelled gelatin nanoparticles as carriers for biotinylated peptide nucleic acid (PNA). *International Journal of Pharmaceutics*. v.196, p. 147–149, 2000.
- COUVREUR P.; KANTE B.; ROLAND M.; GUIOT P.; BAUDUIN P.; SPEISER P. Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: preparation, morphology and sorptive properties. *Journal of Pharmacy and Pharmacology*. v.31, p. 331–2, 1979.
- CUMASHI, A.; USHAKOVA, N. A.; PREOBRAZHenskAYA, M. E.; D'INCECCO, A.; PICCOLI, A.; TOTANI, L.; TINARI, N.; MOROZEVICH, G. E.; BERMAN, A. E.; BILAN, M. I.; USOV, A. I.; USTYUZHANINA, N. E.; GRACHEV, A. A.; SANDERSON, C. J.; KELLY, M.; RABINOVICH, G. A.; IACOBELLI, S.; NIFANTIEV, N. E. A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. *Glycobiology*. v. 17, p. 541-552, 2007.
- DANHIER, F.; LECOUTURIER, N.; VROMAN, B.; JÉRÔME, C.; MARCHAND-BRYNAERT, J.; FERON, O.; PRÉAT, V. Paclitaxel-loaded PEGylated PLGA-based nanoparticles: In vitro and *in vivo* evaluation. *Journal of Controlled Release*. v. 133, p.11–17, 2009.
- DAVIS, T.A.; VOLESKYA, B.; MUCCI, A. A review of the biochemistry of heavy metal biosorption by brown algae. *Water Research*. v. 37, p. 4311–4330, 2003.
- DESTITO, G.; YEH, R.; RAE, C.S.; FINN, M.G.; MANCHESTER, M. Folic acid-mediated targeting of cowpea mosaic virus particles to tumor cells. *Chemical Biology*. v. 14, p. 1152–1162, 2007.
- DUNCAN, R.; IZZO, L. Dendrimer biocompatibility and toxicity. *Adv. Drug Del. Rev.* 57, p. 2215-2237, 2005.

- DUTTA, T.; JAIN, N.K.; MCMILLAN, N.A.J.; PAREKH, H.S. Dendrimer nanocarriers as versatile vectors in gene delivery. *Nanomedicine*. v. xx, p. xxx–xxx xx, (2009) Article in press
- EMERICH, D.F.; THANOS, C.G. The pinpoint of nanoparticles-based drug delivery and molecular diagnosis. *Biomolecular Engineering*. Vol. 23, pp. 171-184, 2006.
- ELLOUALI, M.; BOISSON-VIDAL, C.; DURAND, P. ; JOZEFONVICZ, J. Antitumor activity of low molecular weight Fucoidans extracted from Brown seaweed *Ascophyllum nodosum*. *Anticancer Research*. v. 13, p. 2011-2020, 1993.
- ELLOUALI, M.; BOISSON-VIDAL, C.; JOZEFONVICZ, J. Antiproliferative effect and interaction of Fucoidans with cells. *Colloids and Surfaces B: Biointerfaces*. v. 2, p. 305-314, 1994.
- FARAJI, A.H.; WIPF, P. Nanoparticles in cellular drug delivery. *Bioorganic & Medicinal Chemistry*. v. 17, p. 2950–2962, 2009.
- FAROKHZAD, O.C.; LANGER, R. Nanomedicine: Developing smarter therapeutic and diagnostic modalities. *Advanced Drug Delivery Reviews*. v. 58, pp. 1456–1459, 2006.
- FERNÁNDEZ-CARBALLIDO, A.; PASTORIZA, P.; BARCIA, E.; MONTEJO, C.; NEGRO, S. PLGA/PEG-derivative polymeric matrix for drug delivery system applications: Characterization and cell viability studies. *International Journal of Pharmaceutics*. v. 352, p. 50–57, 2008.
- FREITAS, R.A. What Is Nanomedicine? *Nanomedicine: Nanotechnology, Biology, and Medicine*. v. 1, p. 3-9, 2005.
- GARBAYO, E.; ANSORENA, E.; LANCIEGO, J.L.; AYMERICH, M.S.; BLANCO-PRIETO, M.J. Sustained release of bioactive glycosylated glial cell-line derived neurotrophic factor from biodegradable polymeric microspheres. *European Journal of Pharmaceutics and Biopharmaceutics*. v. 69, p. 844–851, 2008.

- GWINN, M.R.; VALLYATHAN, V. Nanoparticles: Health Effects—Pros and Cons. *Environ Health Perspect.* v. 114, p. 1818–1825, 2006.
- HALEY, B.; FRENKEL, E. Nanoparticles for drug delivery in cancer treatment. *Urologic Oncology: Seminars and Original Investigations.* v. 26, p. 57–64, 2008.
- HAYASHI, K.; NAKANO, T.; HASHIMOTO, M.; KANEKIYO, K.; HAYASHI, T. Defensive effects of a fucoidan from brown alga *Undaria pinnatifida* against herpes simplex virus infection. *International Immunopharmacology.* v. 8, p. 109-116, 2008.
- HE, M.; ZHAO, Z.; YIN, L.; TANG, C.; YIN, C. Hyaluronic acid coated poly(butyl cyanoacrylate) nanoparticles as anticancer drug carriers. *International Journal of Pharmaceutics.* v. 373, p. 165-173, 2009.
- HOLTKAMP, A.D.; KELLY, S.; ULBER, R.; LANG, S. Fucoidans and fucoidanases—focus on techniques for molecular structure elucidation and modification of marine polysaccharides. *Apply Microbiology Biotechnology.* v. 82, p. 1–11, 2009.
- HUANG, X.; WANG, D.; HU, Y.; LU, Y.; GUO, Z.; KONG, X.; SUN, J. Effect of sulfated astragalus polysaccharide on cellular infectivity of infectious bursal disease virus. *International Journal of Biological Macromolecules.* v. 42, p. 166–171, 2008.
- HUGHES, G.A. Nanostructure-mediated drug delivery. *Nanomedicine: Nanotechnology, Biology, and Medicine.* v. 1, p. 22– 30, 2005.
- HSU, H.Y.; CHIU, S.L.; WEN, M.H.; CHEN, K.Y.; HUA, K.F. Ligands of Macrophage Scavenger Receptor Induce Cytokine Expression via Differential Modulation of Protein Kinase Signaling Pathways. *The Journal of Biological Chemistry.* v. 276, p. 28719–28730, 2001.
- ITOH, H.; NODA, H.; AMANO, H.; ZHUAUG, C.; MIZUNO, T. Antitumor-activity and immunological properties of marine algal polysaccharides, especially fucoidan, prepared from *Sargassum-Thunbergii* of Phaeophyceae. *Anticancer Research,* v. 13, p 2045-2052, 1993.

- JAIN, R.A. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. *Biomaterials*. v. 21, p. 2475-2490, 2000.
- JEPSON, M.A.; CLARK, M.A.; HIRST, B.H. M cell targeting by lectins: a strategy for mucosal vaccination and drug delivery. *Advanced Drug Delivery Reviews*. v. 56, p. 511 – 525, 2004.
- KABANOV, A.V.; H.E. Gendelman Nanomedicine in the diagnosis and therapy of neurodegenerative disorders. *Progr. Poly. Sci.* v. 32, p. 1054-1082, 2007.
- KAGAN, V.E.; BAYIR, H.; SHVEDOVA, A.A. Nanomedicine and nanotoxicology: two sides of the same coin. *Nanomedicine: Nanotechnology, Biology, and Medicine*. v. 1, p. 313– 316, 2005.
- KIM, M.H.; JOO, H.G. Immunostimulatory effects of fucoidan on bone marrow-derived dendritic cells. *Immunology Letters*. v.115, p. 138–143, 2008.
- KREUTER, J. Nanoparticles and nanocapsules New dosage forms in the nanometer size range. *Pharmaceutica Acta Helvetiae*. v. 53, p. 33-39, 1978.
- KREUTER, J. Nanoparticles — a historical perspective. *International Journal of Pharmaceutics*. v. 331, p. 1–10, 2007.
- KOCBEK, P.; OBERMAJER, N.; CEGNAR, M.; KOS,J.; KRISTL, J. Targeting cancer cells using PLGA nanoparticles surface modified with monoclonal antibody. *Journal of Controlled Release*, v. 120, p. 18–26, 2007.
- KUMARI, A.; YADAV, S.K.; YADAV, S.C. Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids and Surface (2009) Accepted manuscript*.
- LEITE E. L.; MEDEIROS M. G. L.; ROCHA H. A. O.; FARIAS G. G. M.; DA SILVA L. F.; CHAVANTE S. F.; DE ABREU L. D.; DIETRICH C. P.; NADER H. B. Structure and

- pharmacological activities of a sulfated xylofucoglucuronan from the alga *Spatoglossum schröederi*. *Plant Science*. v. 132, 215–228, 1998.
- LEMARCHAND, C.; GREF, R.; COUVREUR, P. Polysaccharide-decorated nanoparticles. *European Journal of Pharmaceutics and Biopharmaceutics*.v. 58, p. 327-41, 2004.
- LI, B.; LU, F.; WEI, X.; ZHAO, R. Fucoidan: Structure and Bioactivity. *Molecules*. v. 13, p. 1671-1695, 2008.
- LIMAYEM BLOUZA, I.; CHARCOSSET, C.; SFAR, S.; FESSI, H. Preparation and characterization of spironolactone-loaded nanocapsules for paediatric use. *International Journal of Pharmaceutics*. v. 325, p. 124–131, 2006.
- LIU, Z.; JIAO, Y.; WANG, Y.; ZHOU, C.; ZHANG, Z. Polysaccharides-based nanoparticles as drug delivery systems. *Advanced Drug Delivery Reviews*. v. 60, pp. 1650–1662, 2008.
- MARTIMPREY, H.; VAUTHIER, C.; MALVY, C.; COUVREUR, P. Polymer nanocarriers for the delivery of small fragments of nucleic acids: Oligonucleotides and siRNA *European Journal of Pharmaceutics and Biopharmaceutics*. v. 71, p. 490-504, 2009.
- MOGHIMI, S.M.; HUNTER, A.C.; MURRAY, J.C. Long-Circulating and Target-Specific Nanoparticles: Theory to Practice. *Pharmacological Reviews*. v. 53, p. 283-318, 2001.
- MULLOY, B. ; RIBEIRO, A.C. ; ALVES, A.P.; VIEIRA, R.P.; MOURÃO, P.A. Sulfated Fucans from Echinoderms Have a Regular Tetrasaccharide Repeating Unit Defined by Specific Patterns of Sulfation at the 2 and 4 Positions *Journal of Biological Chemistry*, v. 269, p. 22113–22123, 1994.
- MULLOY, B.; MOURAO, P.A.S.; GRAY, E. Structure/function studies of anticoagulant sulphated polysaccharides using NMR. *Journal of Biotechnology* v 77, p. 123-135, 2000.
- NEAL, J.C.; STOLNIK, S.; SCHACHT, E.; KENAWY, E.R.; GARNETT, M.C.; DAVIS, S.S.; ILLUM, L. *In vitro* displacement by rat serum of adsorbed radiolabeled poloxamer and

- poloxamine copolymers from model and biodegradable nanospheres. *Journal of Pharmaceutical Science*. v. 87, p. 1242–1248, 1998.
- NISHIYAMA, N.; KATAOKA, K. Current state, achievements, and future prospects of polymeric micelles as nanocarriers for drug and gene delivery. *Pharmacol. Ther.* v. 112, p. 630–648, 2006.
- OWENS, D.E.; Peppas, N.A. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *International Journal of Pharmaceutics*. v. 307, p. 93–102, 2006.
- PARDO, P.; SOLÉ, M.A. Marine flora of the Peninsula of Macanao, Margarita Island, Venezuela. I. Chlorophyta and Phaeophyceae. *Acta Botánica Venezuelica*. v. 30, p. 291–325, 2007.
- PARK, P.; FONG, P.M.; LU, J.; RUSSELL, K.S.; BOOTH, C.J.; SALTZMAN, W.M.; FAHMY, T.F. PEGylated PLGA nanoparticles for the improved delivery of doxorubicin. *Nanomedicine: Nanotechnology, Biology, and Medicine* (2009) doi:10.1016/j.nano.2009.02.002 *article in press*
- PERACCHIA, M.T.; FATTAL, E.; DESMAELE, D.; BESNARD, M.; NOEL, J.P.; GOMIS, J.M.; APPEL, M.; D'ANGELO, J.; COUVREUR, P. Stealth Ø PEGylated polycyanoacrylate nanoparticles for intravenous administration and splenic targeting. *Journal of Controlled Release*. v. 60, p. 121–128, 1999.
- PEREIRA, M.S.; VILELA-SILVA, A.C.E.S.; VALENTE, A.P.; MOURÃO, P.A.S. A 2-sulfated, 3-linked α -L-galactan is an anticoagulant polysaccharide. *Carbohydrate Research*. v. 337, p. 2231–2238, 2002.
- PLATT, N. AND GORDON, S. Is the class A macrophage scavenger receptor (SR-A) multifunctional? — The mouse's tale. *Journal of Clinical Investigation*, v. 108, p. 649–654, 2001.
- PILLAI, O. AND PANCHAGNULA, R. Polymers in drug delivery. *Current Opinion in Chemical Biology*, v. 5, p. 447–451, 2001.

- PULKKINEN, M.; PIKKARAINEN, J.; WIRTH, T.; TARVAINEN, T.; HAAPA-AHO, V.; KORHONEN, H.; SEPPÄLÄ, J.; JÄRVINEN, K. Three-step tumor targeting of paclitaxel using biotinylated PLA-PEG nanoparticles and avidin–biotin technology: Formulation development and in vitro anticancer activity. *European Journal of Pharmaceutics and Biopharmaceutics*. v. 70, p. 66–74, 2008.
- REIS, C.P.; NEUFELD, R.J.; RIBEIRO, A.J.; VEIGA, F. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. *Nanomedicine: nanotechnology, Biology and Medicine*. v. 2, p. 8-21, 2006.
- RENOIR, J.M.; STELLA, B.; AMELLER, T.; CONNAULT, E.; OPOLON, P.; MARSAUD, V. Improved anti-tumoral capacity of mixed and pure anti-oestrogens in breast cancer cell xenografts after their administration by entrapment in colloidal nanosystems. *Journal of Steroid Biochemistry & Molecular Biology*.v. 102, p. 114–127, 2006.
- RODRIGUES, J.S.; SANTOS-MAGALHÃES, N.S.; COELHO, L.C.B.B.; COUVREUR, P.; PONCHEL, G.; GREF, R. Novel core(polyester)-shell(polysaccharide) nanoparticles: protein loading and surface modification with lectins. *Journal of Controlled Release*. v. 92, p. 103–112, 2003.
- SAHOO, S.K.; PARVEEN,S.; PANDA, J.J. The present and future of nanotechnology in human health care. *Nanomedicine: Nanotechnology, Biology, and Medicine*. v. 3, pp. 20–31, 2007.
- SEZER, A.D.; HATIPOĞLU, F.; CEVHER, E.; OĞURTAN, Z.; BAŞ, A.Z.; AKBUĞA, J. Chitosan Film Containing Fucoïdan as a Wound Dressing for Dermal Burn Healing: Preparation and In Vitro/In Vivo Evaluation. *AAPS PharmSciTech*. v. 8, p. E1-E8, 2007.
- SEZER, A.D.; CEVHER, E.; HATIPOĞLU, F.; OĞURTAN, Z.; BAŞ, A.L.; AKBUĞA, J. The use of fucosphere in the treatment of dermal burns in rabbits. *European Journal of Pharmaceutics and Biopharmaceutics*. v. 69, p. 189–198, 2008.

- SINHA, V.R. AND RACHNA, K. Polysaccharides in colon-specific drug delivery. *International Journal of Pharmaceutics*. v. 224, p. 19–38, 2001.
- SOPPIMATH, K.S.; AMINABHAVI, T.M.; KULKARNI, A.R.; RUDZINSKI, W.E. Biodegradable polymeric nanoparticles as drug delivery devices. *Journal of Controlled Release*, v. 70, p. 1–20, 2001.
- SOUZA, M.C.R.; MARQUES, C.T.M.; DORE, C.M.G.; SILVA, F.R.F.; ROCHA, H.A.O.; LEITE, E.L.L. Antioxidant activities of sulfated polysaccharides from brown and red seaweeds. *Journal of Applied Phycology*. v.19, p. 153–160, 2007.
- STAHN, R.; ZEISIG, R. Cell adhesion inhibition by glycoliposomes: effects of vesicle diameter and ligand density. *Tumor Biology*. v. 21, p. 176–186, 2000.
- STELLA, B.; ARPICCO, S.; PERACCHIA, M.T.; DESMAELE, D.; HOEBEKE, J.; RENOIR, V.; D'ANGELO, J.; CATTEL, L.; P. COUVREUR. Design of folic acidconjugated nanoparticles for drug targeting. *Journal of Pharmaceutical Science*. v. 89, p. 1452-1464, 2000.
- STYLIOS, G.K.; GIANNOUDIS, P.V.; WAN, T. Applications of nanotechnology in medical practice. *International Journal of the Care of the Injured*. v. 36S, p. S5-S13, 2005.
- SULAHIAN, T.H.; IMRICH, A.; DELOID, G.; WINKLER, A.R.; KOBZIK, L. Signaling pathways required for macrophage scavenger receptor-mediated phagocytosis: analysis by scanning cytometry. *Respiratory Research*. v. 9, p 59-74, 2008.
- TERUYA, T.; TATEMOTO, H.; KONISHI, T. TAKO, M. Structural characteristics and in vitro macrophage activation of acetyl fucoidan from *Cladosiphon okamuranus*. *Glycoconjugate Journal*. DOI 10.1007/s10719-008-9221-x 2009.
- TORCHILIN, V.P. PEG-based micelles as carriers of contrast agents for different imaging modalities. *Adv. Drug Del. Rev.* v. 54, p. 235-252, 2002.

- TORCHILIN, V.P. Targeted pharmaceutical nanocarriers for cancer therapy and imaging. *The AAPS Journal*. v. 9, p. E128-147, 2007.
- USHAKOVA, N. A.; MOROZEVICH, G. E.; USTYUZHANINA, N. E.; BILAN, M. I.; USOV, A. I.; NIFANTIEV, N. E.; PREOBRAZHENSKAYA, M. E. Anticoagulant Activity of Fucoidans From Brown Algae. *Biochemistry Supplement Series B: Biomedical Chemistry*. v. 3, p. 77–83, 2009.
- VAUTHIER, C. AND BOUCHEMAL, K. Methods for the preparation and manufacture of polymeric nanoparticles. *Pharmaceutical Research*, v. 26, 2009. DOI: 10.1007/s11095-008-9800-3
- VEENA, C.K.; JOSEPHINE, A.; PREETHA, S.P.; VARALAKSHMI, P. Beneficial role of sulfated polysaccharides from edible seaweed *Fucus vesiculosus* in experimental hyperoxaluria. *Food Chemistry*. v. 100, p. 1552–1559, 2007.
- VLERKEN, L.E.; VYAS, T.K.; AMIJI, M.M. Poly(ethylene glycol)-modified nanocarriers for tumor-targeted and intracellular delivery. *Pharmaceutical Research* (# 2007) DOI: 10.1007/s11095-007-9284-6
- VOINEA, M.; SIMIONESCU, M. Designing of 'intelligent' liposomes for efficient delivery of drugs. *Journal of Cellular and Molecular Medicine* v. 6, pp. 456-474, 2002.
- WANG, J.; ZHANG, Q.; ZHANG, Z.; LI, Z. Antioxidant activity of sulfated polysaccharide fractions extracted from *Laminaria japonica*. *International Journal of Biological Macromolecules*. v. 42, p. 127–132, 2008.
- WEHRLE, P. Pharmacie Galénique: Formulation et Technologie Pharmaceutique. Chap 2. p. 231-238, Editions Maloine, 2007.
- WU, M.; DELLACHERIE, E.; DURAND, A.; MARIE, E. Poly(n-butyl cyanoacrylate) nanoparticles via miniemulsion polymerization (1): Dextran-based surfactants. *Colloids and Surfaces B: Biointerfaces*. v. 69, p. 141-146, 2009.

YOKOTA, T.; EHLIN-HENRIKSSON, B.; HANSSON, G.K. Scavenger receptors mediate adhesion of activated B lymphocytes. *Experimental Cell Research*. v. 239, p. 16–22, 1998.

YOON, S.J.; PYUN, Y.R.; HWANG, J.K.; MOURÃO, P.A.S. A sulfated fucoidan from the brown alga *Laminaria cichorioides* has mainly heparin cofactor II-dependent anticoagulant activity. *Carbohydrate Research*, v. 342, p 2326–2330, 2007.

ZVYAGINTSEVA, T. N.; SHEVCHENKO, N.M., NAZAROVA, I.V.; SCOBUN; A.S.; LUKYANOV, P.A.; ELYAKOVA, L.A. Inhibition of complement activation by water-soluble polysaccharides of some far-eastern brown seaweeds. *Comparative Biochemistry and Physiology Part C*. v. 126 p. 209–215 pp. 2003.

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OBJETIVOS

4. OBJETIVOS

Geral: Extrair, purificar e caracterizar fucana de alga marrom *S. cymosum* para utilização na preparação de nanopartículas. Preparar nanopartículas de poli(cianoacrilato de isobutila) (PIBCA) revestidas com fucana, caracterizar e avaliar sua citotoxicidade *in vitro* de em macrófagos e fibroblastos. Avaliar a biodesão de nanopartículas em células epiteliais do trato gastrointestinal após administração oral.

Específicos:

- Extrair e purificar a fucana de alga marrom *S. cymosum*;
- Caracterizar o material purificado por: espectroscopia de infravermelho (IV), ressonância magnética nuclear de hidrogênio (RMN H^1), análise elementar, cromatografia de permeação em gel (GPC) para determinação da massa molecular, análise térmica (DSC e TG);
- Preparar nanopartículas de PIBCA revestidas de fucana pelos métodos de polimerização em emulsão por radicais redox (RREP) e polimerização por emulsão aniônica (AEP)
- Preparar nanopartículas de PIBCA com marcador fluorescente, contendo ou não a fucana na superfície, pelos métodos RREP e AEP;
- Caracterizar as nanopartículas RREP e AEP quanto aos aspectos macroscópicos, aspectos microscópicos utilizando microscopia eletrônica de varredura (MEV) e microscopia eletrônica de transmissão (MET), tamanho de partícula, carga da superfície e estabilidade a longo prazo;
- Avaliar a citotoxicidade das nanopartículas revestidas de fucana *in vitro* em macrófagos J774 e Fibroblastos NIH 3T3;

- Avaliar a captura das nanopartículas revestidas de fucana por macrófagos J774 e fibroblastos NIH 3T3 utilizando microscopia confocal de fluorescência;
- Quantificar a intensidade de fluorescência nas células após incubação das nanopartículas revestidas de fucana.
- Avaliar a bioadesão *in vivo* das nanopartículas revestidas de fucana após administração oral em ratos Wistar.
- Estudar a biodistribuição das nanopartículas revestidas de fucana no trato gastrointestinal mais especificamente a interação das nanopartículas com placas de Peyer através de microscopia confocal de fluorescência.

ARTIGO I

Fucoidan from *Sargassum cymosum*: extraction, purification and characterization

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Abstract

Fucoidan is a water soluble sulfated polysaccharide obtained from brown algae known for its various biological activities. In this study, fucoidan was extracted from *Sargassum cymosum* collected on the northeast coast of Brazil. The crude material was purified using Sepharose CL 4B column and the second elution peak, corresponding to the fraction of the low molecular weight fucoidan, was collected. Subsequently, molecular weight, elementary analysis, infrared (IR) and ^1H NMR spectroscopy characterized this fraction. Furthermore, the thermal properties of fucoidan were evaluated through DSC and TG analyses. Fucoidan presented 14.15% of carbon, 2.8% of hydrogen and 3.4% of sulfur. No traces of nitrogen were detected (0%). The infrared spectrum of fucoidan showed a band at 3415 cm^{-1} corresponding to O-H and a peak at 1197 cm^{-1} related to C-O stretching vibration. The peaks at 1633 cm^{-1} , 1199 cm^{-1} and 1124 cm^{-1} are related to stretching vibration of C=O and C-O, respectively. Also, two relevant peaks were found at 1079 cm^{-1} and 615 cm^{-1} that are corresponding to S=O and S-O, respectively. The ^1H NMR spectrum of fucoidan in deuterated water presented the complex pattern characteristic of the heterogeneous and branched structures of fucans from algae. Intense signals from methyl protons H-6 appear at 1.31-1.29 ppm and signals from ring protons (H-2 – H-5) are seen between 3.4 and 4.2 ppm. Thermal analysis of fucoidan showed three significant mass loss processes. The first at 62°C and the second at 95°C corresponding to the initial weight loss due to water evaporation. The fucoidan weight loss starting around 160°C is ascribed to a complex process including dehydration and disintegration of the polysaccharide chains. Suitable purified and characterized fucoidan from *S. cymosum* is now available to be used as coating material of nanoparticles to improve drug delivery.

Keywords: Fucoidan, *Sargassum cymosum*, purification, characterization, spectroscopy, thermal analysis.

Introduction

Polysaccharides are the polymers of monosaccharide easily and abundantly found in nature. They can be found in different sources such as algae (e.g. alginate, fucoidan), plants (e.g. cellulose, pectin, guar gum), microorganisms (e.g. dextran, xanthan gum), and animals (chitosan, chondroitin) (Sinha; Rachna, 2001). This important macromolecule has a large number of reactive groups, a wide range of molecular weight (MW), varying chemical composition and charge. These characteristics contribute to their diversity in structure and biological properties (Liu et al. 2008) thus enlarge their utilization.

A renewed interest has been improved the isolation and identification of new bioactive polysaccharides, especially sulfated ones, which present different and stronger biological activities (Huang et al. 2008). Fucoidan is an example of water-soluble sulfated polysaccharide composed mainly of L-fucose residues (Zvyagintseva, et al. 2000) and of some minor sugars such as xylose, galactose, mannose and glucose. The main constituents are $\alpha(1-4)$ and $\alpha(1-2)$ linked L-fucose sulfates and the proportion of fucan ranges between 5 and 20 wt% of algae dry weight (Davis et al, 2003).

Fucoidan is extracted from the cell wall of brown algae species (Phaeophyceae) as *Fucus vesiculosus* (Veena et al. 2007), *Laminaria japonica* (Wang et al. 2008), *Sargassum cymosum* (Pardo; Solé, 2007), *Stoechospermum marginatum* (Li et al. 2008) and many others. Fucoidan seem to be absent from green algae (Chlorophyceae), red algae (Rhodophyceae), golden algae (Xanthophyceae) and from freshwater algae and terrestrial plants (Berteau; Mulloy, 2003). On the other hand, fucoidan can be found in some marine invertebrates as the egg of sea urchin and body wall of sea cucumber (Berteau; Mulloy, 2003).

Each year the number of works related to fucoidan is higher as described by Holtkamp et al. (2009). The significant number of recent publications about fucoidans is a result of their

biological activities including: anticoagulant (Cumashi et al. 2007, Yoon et al. 2007), antiviral (Hayashi et al. 2008), anti-inflammatory and antiangiogenic (Cumashi et al. 2007), antioxidant (Wang et al. 2008), antitumor (Itoh et al. 1993, Ellouali et al. 1993) and antiproliferative effects (Ellouali et al. 1994).

Moreover, polysaccharides are currently been used in nanotechnology mainly in drug delivery systems as coating material (He et al. 2009, Martimprey et al. 2009, Wu et al. 2009). In this context, fucoidan can be used as a promising molecule to coat the surface of nanoparticles to confer new properties as long-circulation and specificity to targeted tissues.

The present study aims to obtain fucoidan from *Sargassum cymosum* and characterize the material for further use in nanotechnology as a coating molecule.

Materials and Method

Fucoidan extraction

The brown algae *S. cymosum* was collected on the south coast of Recife, PE, Brazil. Fucoidan was extracted from the brown algae following the method adapted from Araújo et al. (2004). The algae were first triturated and then the material was washed twice with acetone under magnetic stirring for 12 h, in order to remove pigments and lipids. The solid residue was dried. Then it was dispersed in NaCl 0.15 M, the pH was adjusted to 8.0 with NaOH (0.1 M) and proteolysis was performed by adding papain (15 mg/g of solid residue) at 40°C. The mixture was maintained under magnetic stirring for 24 h. Subsequently, the material was separated by filtration, the filtrate was centrifuged (10.000 g, 4°C, 15 min), and the supernatant was collected and lyophilized.

Fucoidan purification

The brown lyophilized powder obtained was used to get a pure fucoidan. First, the powder was dissolved in acetic acid (2%) and purified by gel filtration on a Sepharose CL 4B column (Sigma-Aldrich, US) (20 cm x 2 cm), using acetic acid 2% as eluent. Fractions of 1 ml were collected and analyzed by spectrophotometry at 265 nm.

Fucoidan Characterization

The fucoidan sample, corresponding to the second eluted peak, was characterized by molecular weight determination, elementary analysis, infrared (IR) and proton nuclear magnetic resonance (^1H NMR) spectroscopy.

Molecular weight was determined by gel permeation chromatography (Viscotek, Houston, USA) using commercial fucoidan from *Fucus vesiculosus* (SIGMA) as a reference. Elementary analysis was performed using Carlo Erba EA equipment (Milan, Italy). IR spectra were recorded on a IFS66 spectrophotometer (Bruker, Karlsruhe, Germany) at a range of $4.000\text{--}400\text{ cm}^{-1}$ using the KBr disc method. ^1H NMR analysis was carried out with samples dissolved in deuterated water (D_2O) as solvent using a Varian Unity Plus 300 MHz (Palo Alto, CA, USA) apparatus.

Simultaneous thermogravimetric (TGA) and differential thermal analysis (DTA) experiments were performed in Netzsch STA 409 PC/PG equipment coupled to a Bruker Tensor 27 Fourier Transformed infrared spectrometer, which analyzed the released gases. A Netzsch Phoenix 204F1 equipment was used to obtain Differential Scanning Calorimetry (DSC) thermograms of the investigated samples. The measurements were performed under nitrogen flow using an open aluminum pan, in which approximately 5 mg of the sample was placed. The temperatures varied from room temperature up to 500°C , at $10^\circ\text{C}\cdot\text{min}^{-1}$.

Results and Discussion

The eluting chromatographic profile of the crude fucoidan is shown in Figure 1. Two separate peaks were obtained both containing fucoidan. The fractions corresponding to the second elution peak, corresponding to the low molecular weight fucoidan, were pooled and lyophilized. The lyophilized powder was then characterized and stored at room temperature until use.

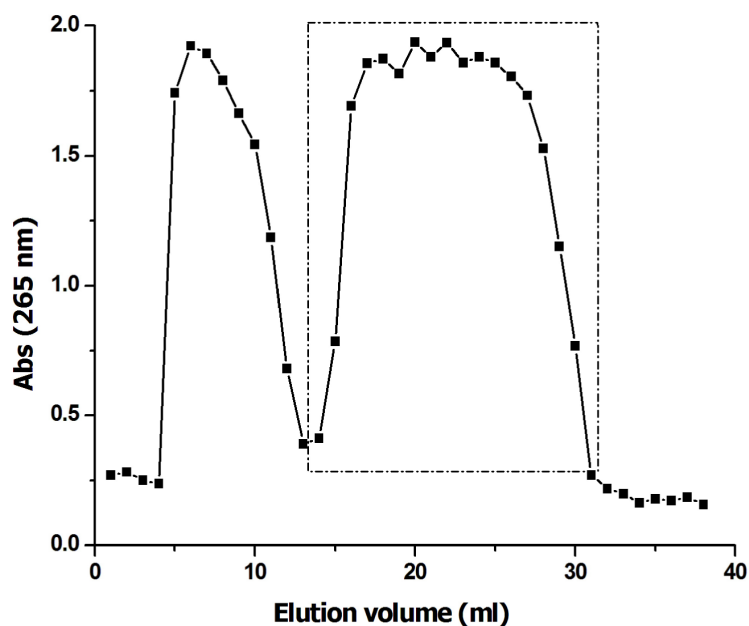


Figure 1. Chromatography of extracted polysaccharide from *S. cymosum* on Sepharose CL 4B column eluted with 2% acetic acid.

The fucoidan from the algae *S. cymosum* used in the present study presented a molecular weight value of 53kDa. As described in literature, the molecular weight of fucoidans wide-ranging, depending on the brown algae species and the method used for their extraction and purification. In previous works, fucoidan molecular weight has been varied from 13kDa, 40kDa, 100-180kDa to 950kDa, corresponding to fucoidan extracted from *Ascophyllum nodosum*,

Stoechospermum marginatum, *Fucus vesiculosus* (Sigma) and *Hizikia fusiforme*, respectively (Senni et al. 2006; Adhikari, et al. 2006; Holtkamp et al. 2009).

Elementary analysis of fucoidan from the algae *S. cymosum* showed the presence of carbon (14.15%), hydrogen (2.8%) and sulphur (3.4%). In contrast, no traces of nitrogen were detected (0%) indicating the absence of proteins. As observed in molecular weight determinations, the amount of sulfur present in the fucoidan molecule can also be different from the algae species and varied according to the method of extraction. Chandía and Matsuihiro (2008) recently described that fucoidan from *Lessonia nigrescens* presents a sulfur content varying from 6 to 13%.

Figure 2 shows the infrared spectrum of fucoidan from *S. cymosum*. It can be observed a peak at 3415 cm^{-1} corresponding to O-H and a peak at 1197 cm^{-1} related to C-O stretching vibration, which are in accordance with Patankar et al (1993), who attributed the last one to hemiacetal stretching. The peaks at 1633 cm^{-1} , 1199 cm^{-1} and 1124 cm^{-1} are related to stretching vibration of C=O and C-O, respectively. Also, two relevant bands were found at 1079 cm^{-1} and 615 cm^{-1} that are corresponding to S=O and S-O, respectively. According to Matsuihiro (1996), the band at a ranges $1100\text{-}1060\text{ cm}^{-1}$ and $900\text{-}610\text{ cm}^{-1}$ are related to sulfate groups. These results are also in agreement with Bilan et al (2004), and Chandía; Matsuihiro (2008) who previously reported the bands corresponding to S=O at 1245 cm^{-1} and O-S-O at 600 cm^{-1} indicating the presence of sulfated groups.

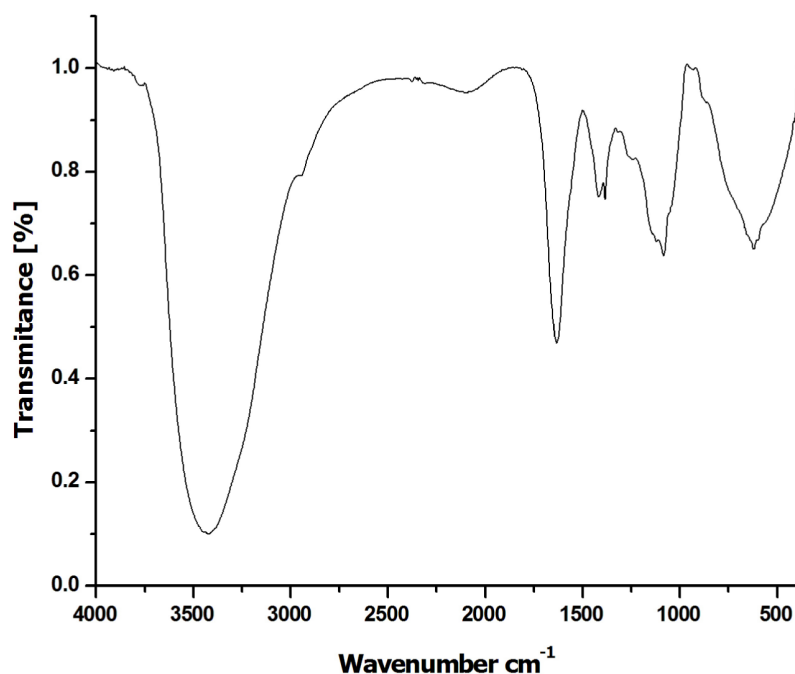


Figure 2. Infrared spectrum of fucoidan extracted from the brown algae *S. cymosum*.

The ^1H NMR spectrum of fucoidan from *S. cymosum* in deuterated water (Fig. 3) presented the complex pattern characteristic of the heterogeneous and branched structures of algae fucans as previously reported (Mulloy et al., 2000). Intense signals from methyl protons H-6 appear at 1.31-1.29 ppm and signals from ring protons (H-2 – H-5) are seen between 3.4 and 4.2 ppm (Chandía and Matsuhira, 2008; Adhkira et al., 2006). The anomeric proton signals, usually in the range of 4.9 to 5.5 ppm, were not observed due to an intense HDO signal present in the spectrum.

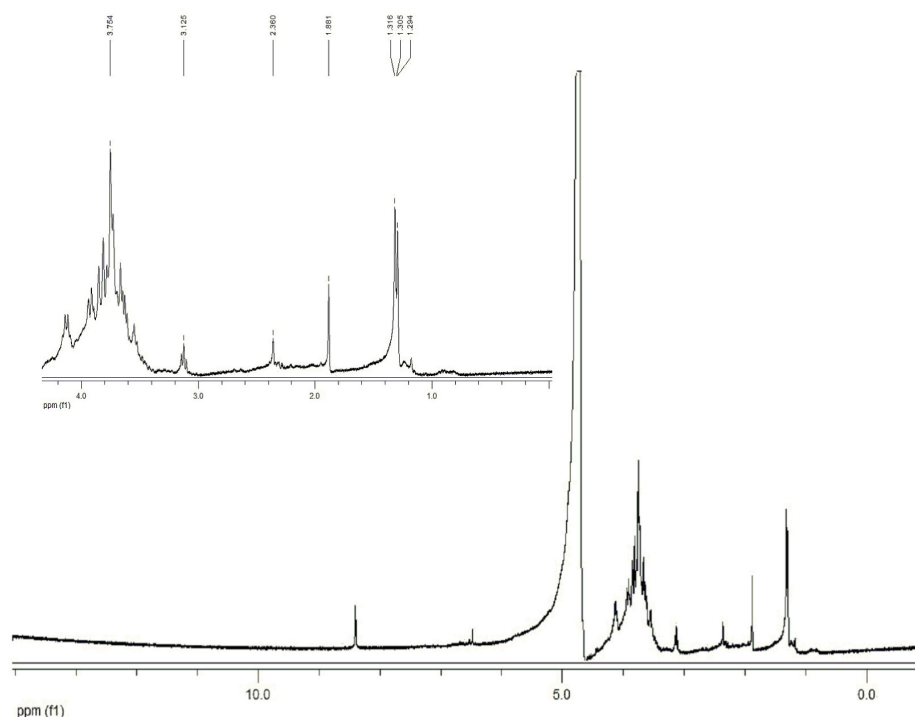


Figure 3. ^1H NMR spectrum of fucoidan from the brown algae *S. cymosum*, recorded at 300 MHz in D_2O solution at 60°C .

The TG/DTG and DSC thermograms of fucoidan are plotted in Figure 4 (a) and (b), respectively. Fucoidan from *S. cymosum* shows three significant mass loss processes. The first event occurred at 62°C and the second at 95°C , corresponding to the initial weight loss due to water evaporation. The fucoidan weight loss starting around 160°C is ascribed to a complex process including dehydration and disintegration of the polysaccharide chains, as previously reported (Zhong-Ling, 2009). As described in literature, the thermal analysis of fucoidan from *M. pyrifera* showed similar decomposition steps (Ross et al, 2009). All the events observed in the TG/DTG curve of fucoidan from *S. cymosum* are associated with endothermic peaks in the DSC thermograms. However, the DSC peak at 137°C has no correspondence in the TG curve. Thus, this event could be related to the fusion of the dehydrated sample.

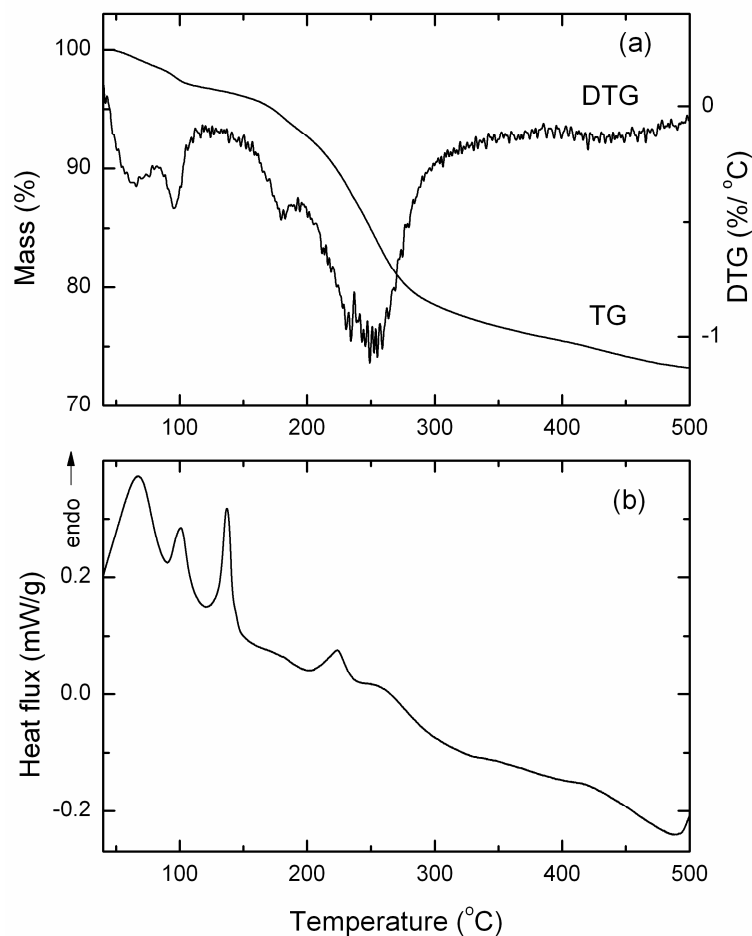


Figure 4. Thermal analysis of fucoidan from the brown algae *S. cymosum*: (a) Thermogravimetry and (b) Differential scanning calorimetry.

Conclusion

Fucoidan extracted from the brown algae *S. cymosum* was extracted and purified using chromatography. Elementary analysis confirmed the presence of sulfur groups in the purified material. Fucoidan characterization by infrared and ^1H NMR showed characteristic bands and peaks of this sulfated polysaccharide. In addition, the thermal analysis showed that fucoidan from *S. cymosum* showed three endothermic peaks corresponding to three different weight loss events.

Suitable purified and characterized fucoidan from *S. cymosum* is now available to be used in the development of targeted drug delivery systems.

Acknowledgments

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References

- Adhikari, U.; Mateu, C.G.; Chattopadhyay, K.; Pujol, C.A.P; Damonte, E.B.; Ray, B Structure and antiviral activity of sulfated fucans from *Stoechospermum marginatum*. *Phytochemistry*. v. 67, p. 2474–2482, 2006.
- Araújo, P.M.; Oliveira, G.B.; Córdula, C.R.; Leite, E.L.; Carvalho Jr. L.B.; Silva, M.P.C. Sulfated fucan as support for antibiotic immobilization. *Brazilian Journal of Medical and Biological Research*. v. 37, p. 301-305, 2004.
- Berteau, O.; Mulloy, B. Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology* v. 13 p. 29R-40R, 2003.
- Bilan, M. I.; Grachev. A.A.; Ustuzhanina, N.E.; Shashkov, A.S.; Nifantiev, N.E.; Usov, A.I. A highly regular fraction of a fucoidan from the brown seaweed *Fucus distichus* L. *Carbohydrate Research*, v. 339, p. 511-517, 2004.

- Chandía, N.P.; Matsuhira, B. Characterization of a fucoidan from *Lessonia vadosa* (Phaeophyta) and its anticoagulant and elicitor properties. *International Journal of Biological Macromolecules*. v. 42, p. 235–240, 2008.
- Cumashi, A.; Ushakova, N. A.; Preobrazhenskaya, M. E.; D'Incecco, A.; Piccoli, A.; Totani, L.; Tinari, N.; Morozevich, G. E.; Berman, A. E.; Bilan, M. I.; Usov, A. I.; Ustyuzhanina, N. E.; Grachev, A. A.; Sanderson, C. J.; Kelly, M.; Rabinovich, G. A.; Iacobelli, S.; Nifantiev, N. E. A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. *Glycobiology*. v. 17 p. 541-552, 2007.
- Davis, T. A.; Volesky, B.; Mucci, A. A review of the biochemistry of heavy metal biosorption by brown algae. *Water Research*. v. 37, p. 4311–4330, 2003
- Ellouali, M.; Boisson-Vidal, C.; Durand, P. ; Jozefonvicz, J. Antitumor activity of low molecular weight Fucoidans extracted from Brown seaweed *Ascophyllum nodosum*. *Anticancer Research*, v. 13, p. 2011-2020, 1993.
- Ellouali, M.; Boisson-Vidal, C.; Jozefonvicz, J. Antiproliferative effect an interaction of Fucoidans with cells. *Colloids and Surfaces B: Biointerfaces*. v. 2, p. 305-314, 1994.
- Hayashi, K.; Nakano, T.; Hashimoto, M.; Kanekiyo, K.; Hayashi, T. Defensive effects of a fucoidan from brown alga *Undaria pinnatifida* against herpes simplex virus infection. *International Journal of Immunopharmacology*. v. 8, p. 109-116, 2008.
- He, M.; Zhao, Z.; Yin, L.; Tang, C.; Yin, C. Hyaluronic acid coated poly(butyl cyanoacrylate) nanoparticles as anticancer drug carriers. *International Journal of Pharmaceutics*. v. 373, p. 165-173, 2009.
- Holtkamp, A.D.; Kelly, S.; Ulber, R.; Lang, S. Fucoidans and fucoidanases—focus on techniques for molecular structure elucidation and modification of marine polysaccharides *Apply Microbiology Biotechnology*. v. 82, p. 1-11, 2009.

- Huang, X. ; Wang, D.; Hu, Y.; Lu, Y.; Guo, Z.; Kong, X.; Sun, J. Effect of sulfated astragalus polysaccharide on cellular infectivity of infectious bursal disease virus. *International Journal of Biological Macromolecules*. v. 42, p. 166–171, 2008.
- Itoh, H.; Noda, H.; Amano, H; Zhuaug, C.; Mizuno, T. Antitumor-Activity and Immunological Properties of Mariane Algal Polysaccharides, Especially Fucoidan, Prepared from *Sargassum-Thunbergii* of Phaeophyceae. *Anticancer Research*. v. 13, p 2045-2052, 1993.
- Li, S.D.; Huang L. Pharmacokinetic and biodistribution of nanoparticles. *Molecular Pharmaceutics*. v. 5, p. 496-504, 2008.
- Liu, Z.; Jiao, Y.; Wang, Y.; Zhou, C.; Zhang, Z. Polysaccharides-based nanoparticles as drug delivery systems. *Advanced Drug Delivery Reviews*. v. 60, p. 1650–1662, 2008.
- Martimprey, H.; Vauthier, C.; Malvy, C.; Couvreur, P. Polymer nanocarriers for the delivery of small fragments of nucleic acids: Oligonucleotides and siRNA *European Journal of Pharmaceutics and Biopharmaceutics*. v. 71, p. 490-504, 2009.
- Matsuhiro, B. Vibrational spectroscopy of seaweed galactans. *Hydrobiologia*. v. 326/327, p. 481-489, 1996.
- Mulloy, B.; Mourao, P.A.S.; Gray, E. Structure/function studies of anticoagulant sulphated polysaccharides using NMR. *Journal of Biotechnology* v. 77, p. 123-135, 2000.
- Pardo, P.; Solé, M.A.; Marine flora of the Peninsula of Macanao, Margarita Island, Venezuela. I. Chlorophyta and Phaeophyceae. *Acta Botanic Venezuelan*. v. 30, p. 291-325, 2007.
- Patankar, M.S.; Oehninger, S.; Barnett, T.; Williams, R.L.; Clark, G.F. A Revised Structure for Fucoidan May Explain Some of Its Biological Activities. *The Journal of Biological Chemistry*. v. 268, p. 21770-21776, 1993.

- Ross, A.B.; Anastasakis, K.; Kubacki, M.; Jones, J.M.. Investigation of the pyrolysis behaviour of brown algae before and after pre-treatment using PY-GC/MS and TGA. *Journal of Analytical and Applied Pyrolysis*. v. 85, p. 3–10, 2009.
- Senni, K., Gueniche, F., Foucault-Bertaud, A., Igondjo-Tchen, S., Fioretti, F., Collicec-Jouault, S., Durand, P., Guezennec, J., Godeau, G., Letourneur, D. Fucoïdan a sulfated polysaccharide from brown algae is a potent modulator of connective tissue proteolysis. *Archive Biochemical Biophysical*. v. 445, p. 56-64, 2006.
- Sinha, V.R. and Rachna, K. Polysaccharides in colon-specific drug delivery. *International Journal of Pharmaceutics*. v. 224, p. 19–38, 2001.
- Veena, C.K.; Josephine, A.; Preetha, S.P.; Varalakshmi, P. Beneficial role of sulfated polysaccharides from edible seaweed *Fucus vesiculosus* in experimental hyperoxaluria. *Food Chemistry*. v. 100, p. 1552–1559, 2007.
- Yoon, S.J.; Pyun, Y.R.; Hwang, J.K.; Mourão, P.A.S. A sulfated Fucoïdan from the brown alga *Laminaria cichorioides* has mainly heparin cofactor II-dependent anticoagulant activity *Carbohydrate Research*. v. 342, p 2326–2330, 2007.
- Wang, J.; Zhang, Q.; Zhang, Z.; Li, Z. Antioxidant activity of sulfated polysaccharide fractions extracted from *Laminaria japonica*. *International Journal of Biological Macromolecules*. v. 42, p. 127–132, 2008.
- Wu, M.; Dellacherie, E.; Durand, A.; Marie, E. Poly(n-butyl cyanoacrylate) nanoparticles via miniemulsion polymerization (1): Dextran-based surfactants. *Colloids and Surfaces B: Biointerfaces*. v. 69, p. 141-146, 2009.
- Zhong-Ling, C. Physical and chemical characteristics of silk fibroin/fucoïdan composite films. *Journal of Clinical Rehabilitative Tissue Engineering Research*. v.13, p. 1589-1592, 2009.
- Zvyagintseva, T. N.; Shevchenko, N.M., Nazarova, I.V.; Scobun; A.S.; Lukyanov, P.A.; Elyakova, L.A. Inhibition of complement activation by water-soluble polysaccharides of

some far-eastern brown seaweeds. *Comparative Biochemistry and Physiology Part C*, v. 126
p. 209–215, 2000.

ARTIGO II

**Cytotoxicity and cellular uptake of new fucoidan-coated
poly(isobutylcyanoacrylate) nanoparticles**

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Abstract

The aim of this study was to synthesize and characterize new fucoidan-coated poly(isobutylcyanoacrylate) (PIBCA) nanoparticles. The nanoparticles were prepared by anionic emulsion polymerization (AEP) and by redox radical emulsion polymerization (RREP) of isobutylcyanoacrylate using fucoidan as a new coating material. The nanoparticles were characterized and their cytotoxicity was evaluated *in vitro* on J774 macrophage and NIH-3T3 fibroblast cell lines. Cellular uptake of labeled nanoparticles was investigated by confocal fluorescent microscopy. Stable nanoparticles were obtained with up to 100% fucoidan as coating material using AEP. In contrast, stable suspensions of nanoparticles were only obtained with up to 25% fucoidan by RREP. The zeta potential of fucoidan-coated PIBCA nanoparticles was decreased depending on the percentage of fucoidan used as coating material. It reached the value of -44 mV for nanoparticles prepared by AEP with 100% of fucoidan. Nanoparticles made by AEP appeared 3 folds more cytotoxic on macrophages ($IC_{50} = 4 \pm 2$) compared with nanoparticles made by RREP ($IC_{50} 11 \pm 3$). On fibroblasts, both types of nanoparticles showed similar cytotoxicity. Confocal fluorescent microscopy observations highlighted that all types of nanoparticles were taken up by both cell lines. The distribution of the fluorescence in the cells varied with the type of nanoparticles. However, these observations could not explain the superior cytotoxicity observed with the AEP nanoparticles on J774 macrophages.

Keywords: Poly(isobutylcyanoacrylate), fucoidan, nanoparticles, cytotoxicity, cellular uptake.

Introduction

Polymer nanoparticles are considered as nanomedicine presenting high potential for diagnosis and therapeutical purposes with possibility to achieve site-specific drug delivery (Couvreur and Vauthier 2006, Li and Huang 2008, Farokhzad and Langer 2009). It is now well established that the *in vivo* fate of such nanocarriers is governed by their surface properties (Vonabourg et al. 2006, Dobrovolskaia et al. 2008). Several data have already been collected from nanoparticles coated with hydrophilic chains such as poly(ethyleneglycol) (PEG), which have been designed to enhance blood circulation time and achieve drug targeting from the intravenous route in treatments of severe diseases—such as cancer (Avgoustakis 2004, Renoir et al. 2006, Vlerken et al. 2007). In general, PEG coating allows the nanoparticles to escape the massive uptake by macrophages of the defense system of the organism while the further attachment of a ligand on the nanoparticle surface increases cell recognition specificity (Peracchia et al. 1999, Moghimi et al. 2001, Stella et al. 2000).

Instead of PEG, polysaccharides can be used to make nanoparticles with tunable surface properties. Oligo and polysaccharides are spread at the surface of many living entities including eukaryote cells, bacteria, viruses where they play a major role in recognition and signaling mechanisms (Sheen et al. 1999). They have communication skills that may be interesting to associate with nanoparticles to design drug-targeting strategies based on biomimetism (Vauthier and Labarre, 2008). Only a few studies has been addressed despite the fact that biomimetic drug delivery technologies can be designed using polysaccharides as coating material. However, the interest of this approach is growing according to the increasing number of recent publications reporting the design of nanoparticles coated with polysaccharides for drug delivery (Lemarchand et al. 2004, Bertholon et al. 2006a,b, Bravo Osuna et al. 2007a, He et al. 2009, De Martimprey et al. 2009, Wu et al. 2009).

We are still at the very beginning of the development of such an approach as well as on the understanding of the potential of what it can bring to improve the specificity of drug delivery to cells. The incorporation of glycomics in nanomedicine appears challenging because of the wide variety of oligo and polysaccharides and the various functionalities they display. Moreover, recent data have suggested that the arrangement of polysaccharides on the surface of nanoparticles might induce dramatic changes in the biological response (Betholon et al. 2006a, Lemarchand et al. 2006, Bravo-Osuna et al. 2007b). For instance, the complement system was strongly activated with particles covered with polysaccharides taking a side-on conformation while long enough polysaccharide chains grafted on the end-on conformation did not activate the complement system (Bertholon et al. 2006a). Varying the chain conformation and the nature of the polysaccharide on the nanoparticle surface can tune mucoadhesive properties of the nanoparticles (Bravo-Osuna et al. 2007). Elsewhere, a modification of chain conformation and of the type of polysaccharide on the surface of nanoparticles was found to affect greatly the cytotoxicity of the nanoparticles against cancer cells (Chauvierre et al. 2007). Thus, progresses are still needed to better understand the effect of those parameters on the interactions of polysaccharide-coated nanoparticles with biological systems.

Until now, dextran, chitosan and eventually heparin were used as coating material of nanoparticles. However, other types of polysaccharides may be interested to be used. Among those, fucoidan, a sulfated polysaccharide, isolated from brown algae displays many interesting biological properties. This polysaccharide is named in this manuscript as fucoidan, but it also known as fucan and sulfated fucan (Li et al. 2008). Its biological activities include antioxidant (Wang et al. 2008), anticoagulant (Ushakova et al. 2009, Becker et al. 2007, Cumashi et al. 2007, Yoon et al. 2007), antiviral (Hayashi et al. 2008, Kazuya et al. 2008), antiinflammatory (Cumashi et al. 2007), antiangiogenic (Cumashi et al. 2007), antitumor (Itoh et al. 1993, Ellouali et al. 1993) activities, inhibition of complement activation (Zvyagintseva

et al. 2000) and antiproliferative effect (Ellouali et al. 1994, Logeart et al. 1997). Fucoidan can also be applied for its ability to sequester toxic heavy metals such as Cd^{2+} , Cu^{2+} , Zn^{2+} , Pb^{2+} , Cr^{3+} and Hg^{2+} (Davis et al. 2003). For pharmaceutical applications, it was used as support for antibiotic immobilization (Araújo et al. 2004) and in the treatment of dermal burns as fucospheres (Sezer et al. 2008).

Interestingly fucoidan can bind to type A I and II transmembrane glycoprotein receptors, called macrophage scavenger receptors (MSR), which are found on the macrophage surface. These receptors show upon the macrophage surface after fixation of a ligand, modulating protein kinase signal transduction pathways, which regulate atherogenesis-related inflammatory cytokines TNF and IL-1 (Hsu et al. 2001, Kim et al. 2003). Thus fucoidan it may be interesting to use fucoidan to promote specific interactions of a drug carrier with this receptor to achieve highly specific delivery of antibiotics to *Mycobacterium tuberculosis*-infected macrophages. Indeed, such an approach could be worth to deliver large amount of antibiotics to infected macrophages.

The aim of the present work was to design new poly(isobutylcyanoacrylate) (PIBCA) nanoparticles coated with fucoidan. In this study, fucoidan was selected as a promoter of interactions between the nanoparticles and macrophages. The cytotoxicity and the potential of the fucoidan-coated PIBCA nanoparticles to be preferentially taken up by macrophages were evaluated *in vitro* in comparison with a fibroblast cell line known for its low capability of phagocytosis.

Materials

Isobutylcyanoacrylate (IBCA) was kindly provided as a gift by Henkel Biomedical (Dublin, Ireland). Dextran (Mw 66,900) was purchased from Sigma (Saint-Quentin Fallavier, France). Cerium (IV) from cerium ammonium nitrate and trisodium citrate dihydrate were

purchased from Fluka (Saint Quentin Fallavier, France). PolyFluor[®] 570: methacryloxyethyl thiocarbamoyl rhodamine B (N-[9-(2-carboxy-x-methacryloxy-ethylthiolcarbamoyl-phenyl)-6-diethylamino-3H-xanthen-3-ylidene]-N-ethyl-ethanaminium chloride) was supplied from Polyscience (Biovalley, Marne la Vallée, France). Fucoidan from *Sargassum cymosum* was a gift from Dr. M.P.C. Silva (Federal University of Pernambuco, Recife, Brazil).

Methods

Extraction and purification of fucoidan

Fucoidan was extracted from the brown algae *S. cymosum* collected on the south coast of Recife, PE, Brazil. The method was adapted from Araújo et al. (2004). Briefly, the algae were triturated and washed twice with acetone under magnetic stirring for 12 h to remove pigments and lipids. The solid residue was dried and dispersed in NaCl 0.15 M at pH 8.0 and digested with papain (15 mg/g of solid residue) at 40°C for 24h. After filtration, the filtrate was centrifuged (1000 rpm, 4°C, 15 min) and the supernatant was lyophilized. Finally, the lyophilized powder obtained was dissolved in acetic acid (2%) and purified by gel filtration on a Sepharose CL 4B column (Sigma-Aldrich, US) (20 cm x 2 cm), using acetic acid at 2% as eluent. Fractions of 1 ml were collected and analyzed by spectrophotometry at 265 nm. Fractions of the second elution peak, corresponding to the low molecular weight of fucoidan, were pooled and lyophilized.

Preparation of fucoidan-coated poly(isobutylcyanoacrylate) nanoparticles

Redox Radical Emulsion Polymerization (RREP)

The methods described by Chauvierre et al. (2003) and Bertholon et al. (2006) were adapted to prepare fucoidan-coated nanoparticles. A total amount of fucoidan and dextran of 0.11 g was dissolved in nitric acid (4 ml, 0.2M) varying the ratio between fucoidan and dextran in the polysaccharide blend from 0 to 100% in fucoidan. The solution was placed under magnetic

stirring at 40°C with nitrogen bubbling. After 10 min, 1 ml of cerium (IV) ammonium-nitrate solution (8×10^{-2} M) in 0.2 M nitric acid was added to the solution containing the polysaccharide and immediately after, 250 µl of the monomer IBCA were added under vigorous magnetic stirring (1000 rpm). The system was maintained under magnetic stirring for 1 h. After cooling down to room temperature, 1 ml of an aqueous solution of trisodium citrate dihydrate (1 M) was added to complex the remaining cerium ions. The nanoparticles prepared by this method were named RREP-Fuc-X%. The X indicates the percentage of fucoidan in the total amount of polysaccharides used in the synthesis of the nanoparticles. To prepare fluorescent-labeled nanoparticles, 1 ml of a PolyFluor[®] 570 solution (rhodamine) dissolved in acetonitrile (4 mg/ml) was added to the polymerization medium 2 min after the addition of the monomer (IBCA).

Anionic Emulsion Polymerization (AEP)

The preparation of fucoidan-coated PIBCA nanoparticles by AEP was adapted from the previously reported method (Couvreur et al. 1979). Briefly, fucoidan was dissolved in pure water (5 ml). The solution was used to dissolve dextran to reach a total amount in polysaccharide of 0.05 g. Subsequently, the pH was decreased to 2.5 with HCl (1 N). Then, 50 µl of monomer IBCA were dropwise added under vigorous stirring. The system was left to polymerize under vigorous stirring for 3 h at room temperature. The nanoparticles prepared by this method were named AEP-Fuc-X%, where the X indicates the percentage of fucoidan in the total amount of polysaccharides used in the synthesis of the nanoparticles. The labeled-nanoparticles were prepared following the same method but by adding 50 µl of a PolyFluor[®] 570 solution in acetonitrile (5 mg/ml) 5 min after the addition of the monomer (IBCA).

Purification of nanoparticles

All nanoparticle suspensions were purified by dialysis (Spectrapor[®] membrane 100,000 g/mol molecular weight cut off (MWCO), Biovalley, Marne la Vallée, France) twice against 1 L of distilled water for 30 min and once overnight. This dialysis was aimed to eliminate all reagents including polysaccharides that have not reacted during the nanoparticle synthesis. The purified suspensions were stored at 4°C until use.

Evaluation of particle size and zeta potential

The diameter of the nanoparticles was measured at 25°C by quasi-elastic light scattering using a Malvern Zetasizer (Malvern Instrument, UK) operating at the angle of 90°. Samples were diluted in deionized water (Milli Q[®], Millipore, USA) and the whole suspension was filtered through a 0.22 µm (Millipore filter[®], Millipore, USA). The polydispersity index (PI) was evaluated to verify nanoparticle dispersion homogeneity (values closer to zero indicate a homogeneous dispersion). The coefficient of variation (CV) was calculated from the following equation: $CV = \sigma / SD \times 100$ where, σ was the polydispersity index and SD was the standard deviation.

The zeta potential of the nanoparticles was deduced from the electrophoretic mobility using a Malvern Zetasizer (Malvern Instrument, UK). Dilution of the nanoparticle suspensions was performed in KCl 1 mM. Results corresponded to the average of three determinations.

Morphology of the nanoparticles

The morphological analysis of AEP and RREP fucoidan-coated nanoparticles was assessed by transmission electron microscopy (TEM) (FEI, Tecnai20) at the Centro de Tecnologias do Nordeste (CETENE), Recife, PE, Brazil). For TEM analysis, the samples were diluted in water

and deposited on, a 200 mesh copper grid coated with Formvar[®]. The grid was dried in a desiccator before observations.

Cell culture

Two cell lines were used in this study. They were chosen among a macrophage cell line known to phagocyte nanoparticles: the macrophages J774, and a fibroblast cell line, which does not have strong phagocytic activity: NIH-3T3.

Macrophages J774 were grown in RPMI medium containing 10% decompemented bovine fetal serum (SFB) (Gibco, Cergy-Pontoise, France) and antibiotics (penicillin 100 IU/ml – streptomycin 100 µg/ml), in a 5% CO₂ atmosphere at 37°C. Experiments were performed between the fourth and the fiftieth passages. The cells were detached mechanically with a cell scraper and adjusted to the required concentration of viable cells.

Fibroblasts NIH-3T3 cells were routinely grown in high glucose DMEM (4.5 g/ml), 10% SFB (Gibco, Cergy-Pontoise, France) and antibiotics (penicillin 100 IU/ml and streptomycin 100 µg/ml), in a 5% CO₂ atmosphere at 37°C. Experiments were performed between the fourth and the fiftieth passages. The cells were detached with trypsin. Then they were counted in a haemocytometer. Finally, the cell concentration was adjusted with culture medium to the required concentration in viable cells.

Cytotoxicity studies

Cytotoxicity of fucoidan-coated PIBCA nanoparticles was evaluated on J774 macrophages and NIH-3T3 cells using the MTT method (Alley et al. 1988). The number of viable cells was determined by the estimation of their mitochondrial reductase activity. Briefly, cells were seeded onto 96-wells microtiter plates (NIH-3T3: 2000 /200 µl culture medium/well, J774 macrophages: 5000 /200µl culture medium/well) and grown in a humidified 5% CO₂ incubator at 37°C. After overnight incubation, samples of nanoparticles (10 µl) at different concentrations (0-150 µg/ml)]

were added to the medium and the cells were incubated for 48 h. Subsequently, 20 μ l of MTT (5 mg/ml) were added in the cell growth medium and then, the cells were re-incubated for two hours. The medium was discarded and replaced by 200 μ l of DMSO to dissolve the insoluble crystals of formazan formed by living cells. The absorbance was then read at 570 nm using a microplate reader (Labsystems Multiskan MS). Non-treated cells were used as a control. The cytotoxicity was expressed as the concentration required to inhibit fifty per cent of cell proliferation (IC_{50}). To evaluate the influence of the presence of fucoidan and also the preparation method of nanoparticles (AEP and RREP) on the cytotoxicity of PIBCA-nanoparticles on J774 macrophages and NIH 3T3 fibroblasts, statistical analysis of variance (ANOVA) of three different assays was performed.

Cellular uptake of fucoidan-coated nanoparticles

The uptake of nanoparticles was investigated on J774 macrophages and NIH-3T3 fibroblasts using confocal fluorescent microscopy (Plateforme d'imagerie cellulaire, IFR 141, UFR Châtenay-Malabry). Round glass microscope cover-slip ($\varnothing = 12$ mm) were carefully cleaned with ethanol (70%) and dried. They were placed in 12-wells culture plates before cell addition. Cells were added to the wells (10^5 and 2×10^4 cells/well of J774 macrophages and NIH-3T3 fibroblasts, respectively) and incubated for 48 h. Then fluorescent labeled-nanoparticles with and without fucoidan at the surface were added in each well as follow: 50 μ l of AEP labeled-nanoparticles previously diluted in culture medium (320 in 480 μ l/ml obtaining 4 μ g/ml) and 50 μ l of RREP labeled-nanoparticles previously diluted in culture medium (80 in 720 μ l/ml obtaining 4 μ g/ml). The volume of each well was filled to 1 ml to adjust the final concentration in nanoparticles to 0.2 μ g/ml and finally, plates were incubated for 1, 2, 3, 4 and 24 h at 37°C. After incubation, the cells were washed with culture medium and then fixed by addition of 800 μ l of 4% paraformaldehyde in phosphate buffer saline (PBS) (w/v) followed by an incubation

time of 20 minutes. Later, the cells were washed with culture medium. In order to block the reactivity of the aldehyde, 1 ml of 50 mM ammonium chloride (NH_4Cl) was added and incubated for at least 10 min. The cells were then washed again with culture medium. Finally, 10 μl of Vectashield[®] mounting medium for fluorescence (Vector Laboratories, Inc., Burlingame, CA, USA) were laid on a microscope glass slide and cover-slip with cells were over laid on this medium and sealed with nail polish. The mounted cells were kept at 4°C until observations. Observations by optical and fluorescent microscopy (Leitz Diaplan microscope, France) were performed in order to verify the shape of the cells and the absence of cell lysis.

The uptake of nanoparticles by the cells was then analyzed using confocal fluorescent microscopy (LSM 510 microscope, Zeiss, Germany). Confocal acquisitions were performed with a helium neon laser (excitation wavelength 543 nm) and a long pass emission filter LP 560 nm. Fluorescent images were obtained using a Plan Apochromat 63 $\times$, 1.4 numerical aperture oil immersion objective lens and the pinhole was set at 1.0 Airy unit (0.8 mm optical slice thickness). Cells were analyzed by acquiring and evaluating 0.41 μm -thick image slices with cross-sectional slices perpendicular to the plane of the cell monolayer midpoint (z-axis). The confocal images were analyzed by Carl Zeiss LSM image browser (version 4,2,0,121). Additionally, the area under the fluorescence quantification curve and the fluorescence maximum peak were determined by analyzing the image of the fluorescent cells using Image-J 1.40g and Origin[®] 6.0 software. Briefly, Image-J software was used to drawn a straight line in each cell image crossing the cytoplasm and the nucleus as illustrated in Figure 1. Then, a fluorescence profile was plotted and the corresponding data collected. Calculation of area under the curve was performed with the Origin[®] 6.0 software and the fluorescence maximum peak was determined as the intensity of the highest peak shown on the fluorescence profile.

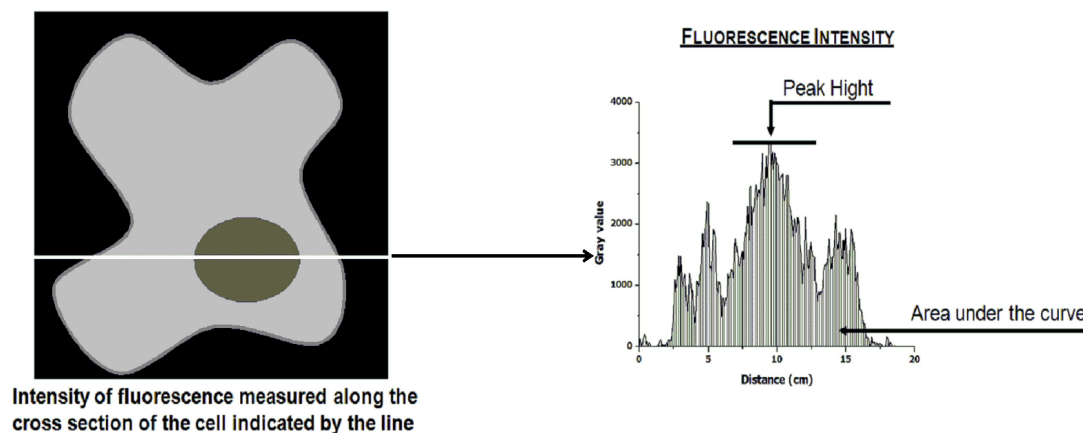


Figure 1. Scheme of fluorescence quantification (area under curve and fluorescence maximum peak).

Results and Discussion

Preparation of fucoidan-coated PIBCA nanoparticles

Emulsion polymerizations are interesting methods to prepare PIBCA-nanoparticles as one pot and one step procedure and as organic solvent free method. In the RREP and AEP methods, the emulsion polymerizations are performed by dispersing the monomer, IBCA, in acid solutions of polysaccharide(s) to be associated with the nanoparticle surface. Fucoidan-coated PIBCA nanoparticles were prepared by both methods varying the percentage of fucoidan from 0 to 100% in a blend of fucoidan and dextran. As indicated in Table 1, the AEP method made possible the obtaining of stable formulations of fucoidan-coated PIBCA-nanoparticles at all percentage of fucoidan. In contrast, PIBCA-nanoparticles prepared by the RREP method with 50 to 100% of fucoidan (RREP-Fuc-50, RREP-Fuc-75 and RREP-Fuc-100, respectively) were very unstable. A light brownish precipitate and aggregates were formed at the end of the preparations. Considering the RREP method, only preparations containing either 0% fucoidan (100% dextran) or 25 % fucoidan could form stable colloidal dispersions.

Table 1. Physicochemical characteristics of PIBCA nanoparticles obtained by AEP and RREP with different ratios between fucoidan and dextran.

PIBCA-NP	Fucoidan:dextran Ratio w/w (%)	Stability of nanoparticle suspensions*	Mean size (nm)	Zeta potential (mV)
AEP-Fuc-0	0 : 100	stable	193 ± 4	-5.53 ± 1
AEP-Fuc-25	25 : 75	stable	195 ± 2	-9.60 ± 0.4
AEP-Fuc-50	50 : 50	stable	298 ± 1	-12.0 ± 1
AEP-Fuc-75	75 : 25	stable	298 ± 0.4	-27.0 ± 1
AEP-Fuc-100	100 : 0	stable	399 ± 0.7	-44.2 ± 1
RREP-Fuc-0	0 : 100	stable	307 ± 4	-6.39 ± 0.1
RREP-Fuc-25	25 : 75	stable	223 ± 1	-7.18 ± 0.6
RREP-Fuc-50	50 : 50	unstable	-----	-----
RREP-Fuc-75	75 : 25	aggregated	-----	-----
RREP-Fuc-100	0 : 100	aggregated	-----	-----

* The nanoparticles were considered as stable when the suspensions remained homogenous and did not show any sediment or precipitation over a period of 1 week after preparation.

When the concentration of fucoidan was increased from 25 to 100%, the size of nanoparticles prepared by AEP was increased from 193 ± 4 nm to 399 ± 0.7 nm, respectively. In contrast, in RREP nanoparticles the size decreased from 307 ± 4 nm to 223 ± 1 nm when preparations contained 0 and 25 % fucoidan, respectively. The low polydispersity index and the low value of the CV ($< 5\%$) indicated a narrow size distribution of the particles. The modifications in nanoparticle size between both method and nature of polysaccharide were in agreement with previous work (Bertholon et al. 2006a, Labarre et al. 2005, Bravo-Osuna et al. 2007a).

As shown in Table 1, the zeta potential of the nanoparticles obtained by the two methods became more negative when the amount of fucoidan was increased. The zeta potential decreased to reach a very low value (-44 mV) when the nanoparticles were prepared with 100% fucoidan (AEP-Fuc-100%). This result can be explained by the negative charge of fucoidan. It also agreed well with the value of zeta potential found in previous studies considering heparin and dextran-

sulfate-coated nanoparticles (Chauvierre et al. 2003, Bertholon et al. 2006a). Thus, results from zeta potential measurements confirmed that fucoidan molecules were located on the surface of PIBCA-nanoparticles. It is also noteworthy that the zeta potential varied continuously from -5 to -44 mV when the composition in fucoidan was gradually increased. This result showed the same tendency than that previously observed with a preparation of nanoparticles made with a blend of heparin and dextran (Chauvierre et al. 2003), indicating that the nanoparticle surface was composed of a blend of the two polysaccharides.

The size of the fluorescent labeled nanoparticles was increased compared with those of the non fluorescent nanoparticles (Fig. 2).

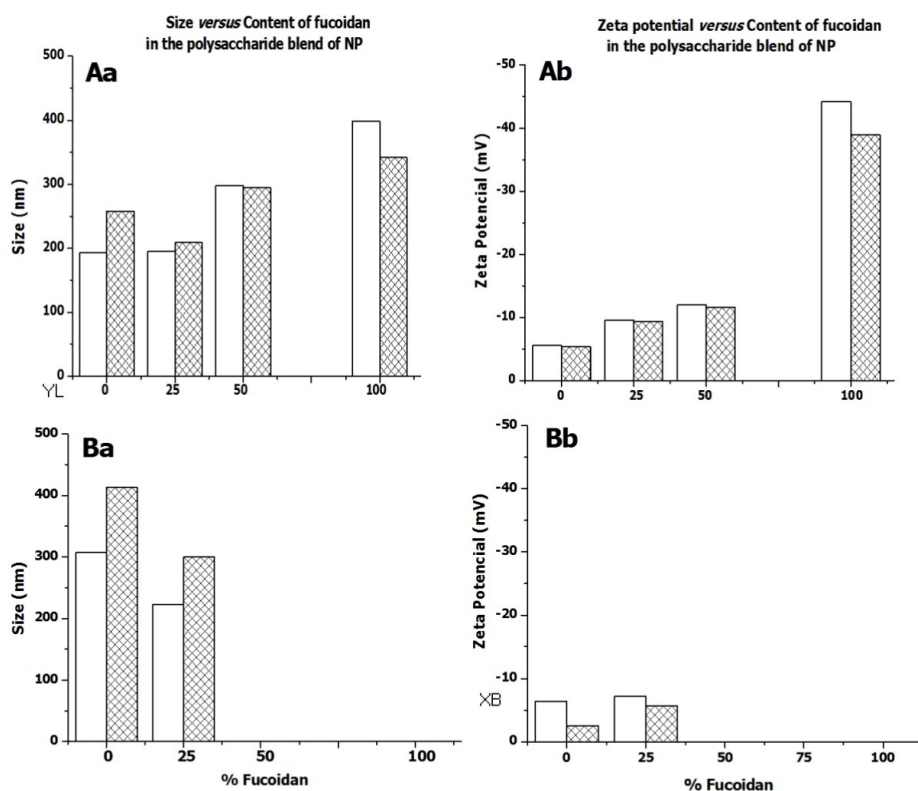


Figure 2. Size (a) and Zeta potential (b) of labeled and unlabeled nanoparticles prepared by AEP (A) and RREP (B) with different percentages of fucoidan (0, 25, 50 and 100%). White column for the unlabeled, and white sparse column for the labeled nanoparticles.

Such observations were also reported with chitosan-coated nanoparticles (Bravo-Osuna et al. 2007a). It can be suggested that the increase in size was due to the incorporation of rhodamine which is a large molecule in the nanoparticle core. The zeta potential of labeled nanoparticles was very similar to those of the corresponding non labeled nanoparticles (Fig. 2). At the pH of measurement, rhodamine was positively charged. The fact that the zeta potential of the nanoparticles was unchanged after labeling indicated that rhodamine was incorporated in the core of the nanoparticles and was not exposed on its surface. Data obtained from size measurements and zeta potential determinations were in agreement with the fact that the method of labeling was expected to promote the incorporation of the fluorescent probe in the nanoparticle core thanks to the copolymerization of the rhodamine-containing monomer with IBCA.

TEM microscopy analysis showed spherical fucoidan-coated PIBCA nanoparticles obtained by both AEP and RREP (Fig. 3).

It is noteworthy that the expected core shell structure was clearly seen on all types of nanoparticles obtained by both AEP and RREP whatever the fucoidan concentration was. Indeed, the internal core was well distinguished from the polysaccharide shell as illustrated on the examples presented in Figure 3.

and (E) RREP-Fuc-25 nanoparticles.

Long-term stability of the nanoparticle suspensions was evaluated upon storage at +4°C over a period of 6 months (Fig. 4). No significant changes in the size of nanoparticles were detected over this period indicating that the nanoparticle suspensions were stable. It is noteworthy that neither a modification of the surface composition, nor the method of preparation have affected the stability of the nanoparticle suspensions during storage.

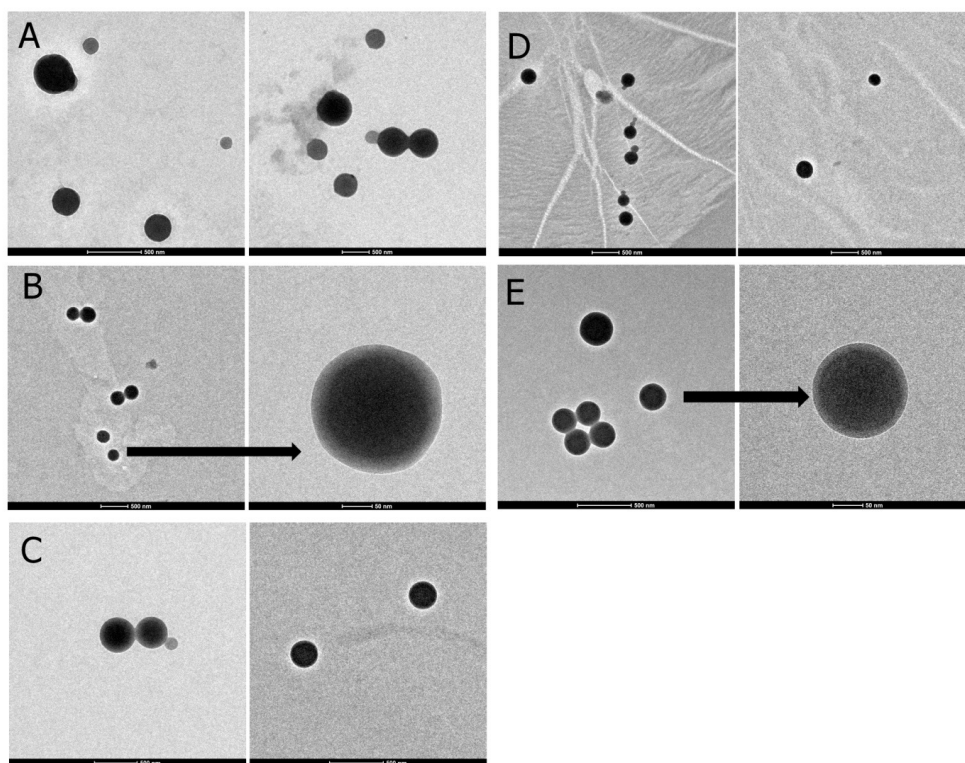


Figure 3. Electron transmission micrographs of (A) AEP, (B) AEP-Fuc-25, (C) AEP-Fuc-100, (D) RREP, (E) RREP-25.

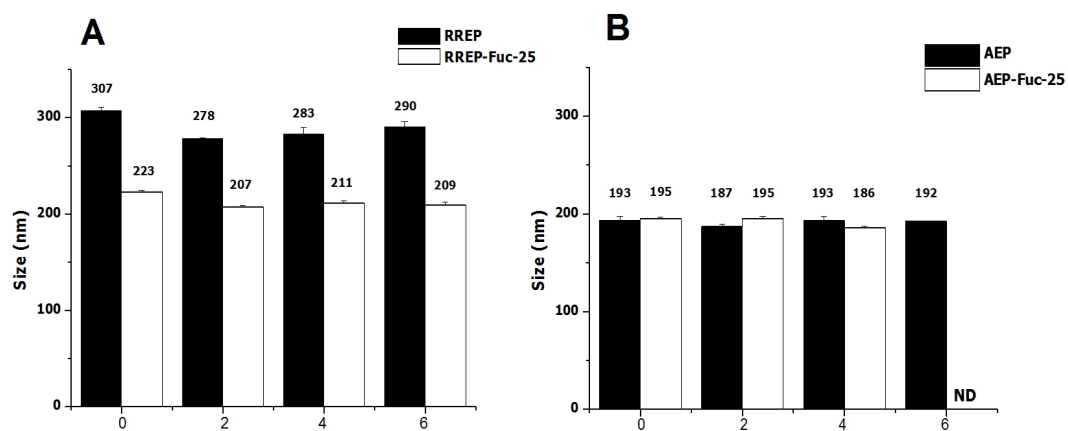


Figure 4. Size of poly(alkylcyanoacrylate) nanoparticles by RREP (A) and AEP (B) method after synthesis and until 6 months. Nanoparticles without fucoidan (black columns) and with 25% fucoidan (white columns) ND means no data.

Cytotoxicity study of fucoidan-coated PIBCA nanoparticles

By measuring the cytotoxicity of the nanoparticles, it was attempted to evaluate the capacity of fucoidan-coated PIBCA-nanoparticles to interact preferentially with J774 macrophages compared with NIH-3T3 fibroblasts. The Figures 5 A and B show the IC_{50} determined for the different types of nanoparticles made by AEP and RREP with different percentage of fucoidan. The IC_{50} of AEP and RREP nanoparticles on J774 macrophages were 2 ± 1 and 12.7 ± 0.6 , respectively. The IC_{50} of AEP-Fuc-25 was 1.3 ± 0.23 and 9.6 ± 0.5 for RREP-Fuc-25 nanoparticles on macrophages. By comparison, on fibroblasts the IC_{50} of the AEP and RREP nanoparticles, without fucoidan, were 9 ± 2 and 10 ± 2 , respectively indicating that the nanoparticles were far less cytotoxic on the NIH-3T3. However, the AEP-Fuc-25 nanoparticles appeared three fold more cytotoxic ($IC_{50} = 4 \pm 2$) than the corresponding nanoparticles synthesized by the RREP method ($IC_{50} = 11 \pm 3$) on the NIH-3T3 cell line.

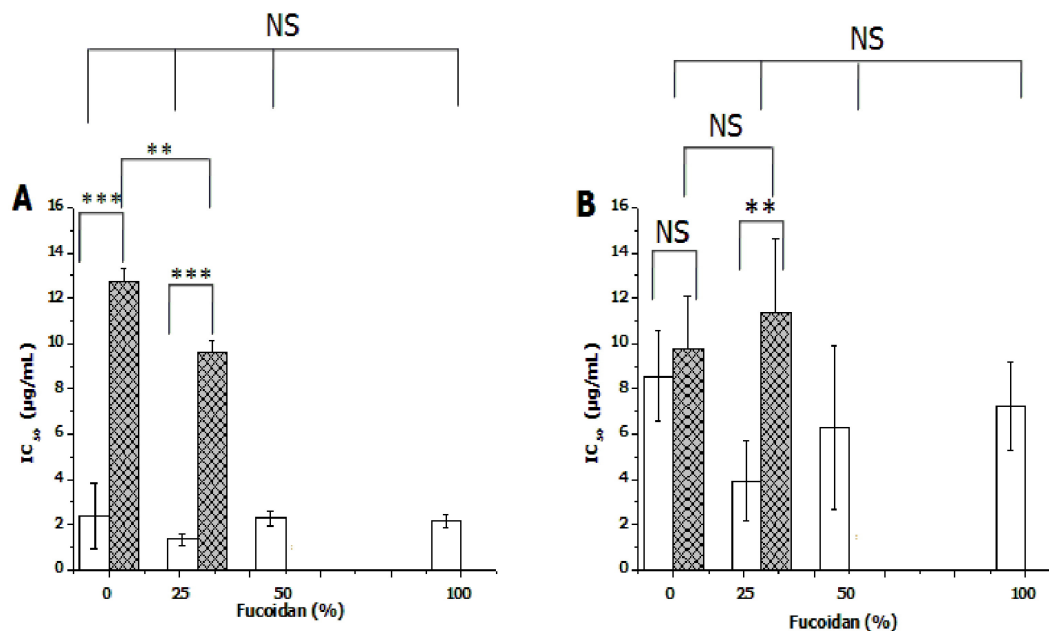


Figure 5. Comparative cytotoxicity (IC_{50}) of Fucoidan coated poly(alkylcyanoacrylate) nanoparticles in (A) J774 macrophage cells line and (B) NIH-3T3 fibroblast cells line, by AEP (white columns) and RREP (sparse columns) (ns) no statistically different; (**) statistically different with $p < 0.05$, (***) statistically different with $p < 0.001$.

Statistical analysis of the data evaluating the cytotoxicity of nanoparticles with different coating showed that there was no significant difference in the cytotoxicity of AEP nanoparticles coated with different content of fucoidan on J774 macrophage cells ($p > 0.05$). The cytotoxicity of RREP nanoparticles prepared with or without fucoidan were statistically different ($p < 0.05$). Related to the cytotoxicity of both AEP and RREP nanoparticles (with or without fucoidan) on fibroblast NIH-3T3 cells there was no statistical difference ($p > 0.05$).

Comparing the influence of the method of nanoparticle preparation, i.e. AEP vs RREP on the cytotoxicity of nanoparticles on J774 macrophages, a statistically significant difference ($p < 0.001$) was found. In contrast, no statistical difference was observed considering the cytotoxicity of the nanoparticles on fibroblast NIH 3T3.

The larger difference in cytotoxicity between nanoparticles was found considering the phagocytic cell line, i.e. J774, and the poor phagocytic cell line, i.e. NIH 3T3 fibroblasts. The fact that the cytotoxicity of the nanoparticles made by AEP was much above that of the nanoparticles made by the RREP on the J774 cell line suggested that the macrophages were able to distinguish both types of nanoparticles. This could actually occur thanks to the difference in the polysaccharide chain conformation on the nanoparticle surface which resulted from the polymerization mechanism involved during the synthesis of the nanoparticles. Indeed, the very subtle differences in the surface structure of the AEP and RREP nanoparticles was already reported to induce dramatic differences in the response of biological systems including cytotoxicity on a cancer cell line (Chauvierre et al. 2007), interactions with serum proteins (Labarre et al. 2005), bioadhesion on the gastrointestinal mucosa (Bravo-Osuna et al. 2007b) and complement activation capacity (Bertholon et al. 2006a). In the case of cytotoxicity, it can be

suggested that the difference in cytotoxicity observed with the different nanoparticles could be related to a difference in the level of their cellular uptake.

Cellular uptake of fucoidan-coated PIBCA nanoparticles

In order to better understand the cytotoxicity profile shown by the AEP and RREP nanoparticles, observations of the nanoparticle uptake by the cells were performed using fluorescence and confocal microscopy. These observations were performed after incubation of the cells with fluorescent labeled nanoparticles at a non cytotoxic concentration for the cells and for different duration.

Confocal analysis demonstrated that AEP and RREP PIBCA nanoparticles coated and not coated with fucoidan started to be taken up by macrophages and fibroblasts in less than one hour (Fig. 6).

The cross-sectional slices showed that nanoparticles were inside the cells and did not remain adsorbed on the cell surface because the fluorescence was only detected in the different planes throughout the cell thickness. The uptake of all types of nanoparticles by both cell lines increased with time as indicated by the increase in fluorescence intensity of the cells over a period of 24 hours (Fig.7).

At the early stages, time 1h and 3h, the fluorescence distributed in the cell cytoplasm as punctuations excepted for the nanoparticles AEP and RREP exempt of fucoidan which presented a homogeneous and diffuse distribution of the fluorescence (Fig. 6). To explain the difference of the fluorescence distribution in the cells shown by the AEP and RREP nanoparticles exempt of fucoidan, it can be suggested that these nanoparticles were taken up by the macrophages by a different pathway compared to that of the uptake of the fucoidan-coated nanoparticles. It is noteworthy that the nucleus of cells incubated with the AEP nanoparticles appeared already very fluorescent after 3 hours of incubation.

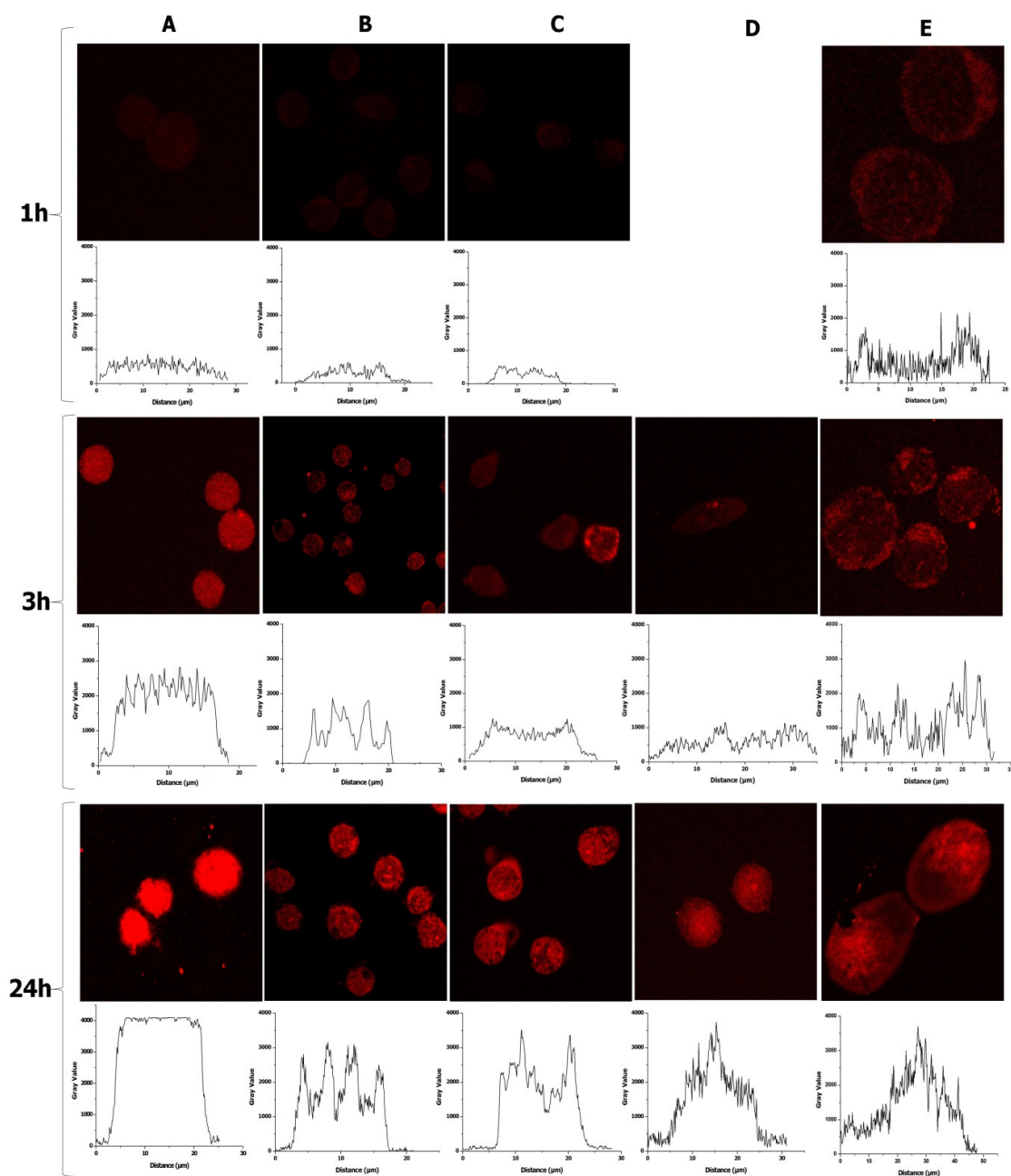


Figure 6. Confocal microscopy of macrophage J774 and fluorescence quantification (gray value versus distance (μm)) (A) AEP, (B) AEP-Fuc-25, (C) AEP-Fuc-100, (D) RREP, and (E) RREP-Fuc-25 nanoparticles after 1, 3 and 24 hours of incubation time.

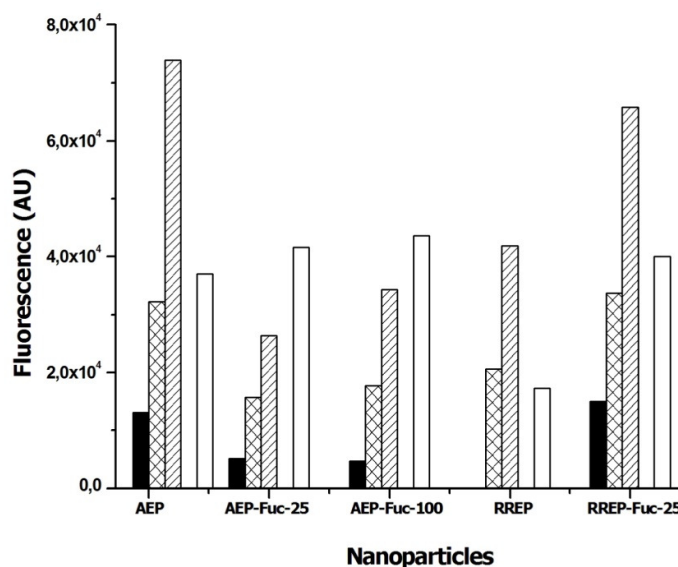


Figure 7. Fluorescence uptake of macrophage J774 after 1h (black column), 3h (sparse column), 24h (medium column) and fibroblast NIH 3T3 after 24 hours (white column).

After 24 hours, the fluorescence remained punctuated in the cytoplasm of cells incubated with AEP, AEP-Fuc25 and AEP-Fuc100 while the fluorescence in the nucleus was intensified and appeared as bright and condensed areas. The nucleus of the cells incubated with AEP nanoparticles was extremely fluorescent as shown in Figure 6 (column A time 24h). The fluorescence observed in cells incubated with the RREP and RREP-Fuc25 appeared more diffuse in both the cytoplasm and the nucleus (Figure 6 column D and E). In all cases, the finding of fluorescence due to the probe attached to the nanoparticles in the cell nucleus was unexpected because it was conditioned to the crossing of the nucleus membrane which has a highly selective permeability.

From the observations, it can be suggested that the fucoidan-coated nanoparticles penetrated in the cells through a different mechanism than those showing a surface exempt of fucoidan. More precisely and based on the literature, receptors able to interact with fucoidan are existing

on the surface of macrophages (Nakamura et al. 2006). Thus, the internalization of the fucoidan bearing nanoparticles could be mediated through a fucoidan receptor dependent mechanism resulting in their localization in intracellular compartments. This is in agreement with the occurrence of fluorescent punctuations in the cell cytoplasm. Unfortunately, the differences in the observed distribution of the fluorescence in the cells could not help the understanding of the cytotoxicity profiles shown by the nanoparticles on J774 as no specific intracellular distribution of the fluorescence due to the AEP nanoparticles coated or not with fucoidan could be highlighted while they were found to be the more cytotoxic nanoparticles.

Regarding the uptake of the nanoparticles by the fibroblasts, it is noteworthy that the NIH 3T3 were able to internalize nanoparticles within the cells. The uptake occurred almost at the same level than that found in J774 macrophages as shown by the amount of fluorescence found in the cells after 24 hours of incubation (Fig. 7, white bars). The fluorescence from the nanoparticles AEP-Fuc 25, AEP-Fuc 100, RREP and RREP-Fuc25 was diffuse and mainly located in the cell cytoplasm (Maximum Fluorescence intensity 4072 AU) and in a lower extend in the cell nucleus (Maximum Fluorescence intensity 3398 AU) (Fig. 8).

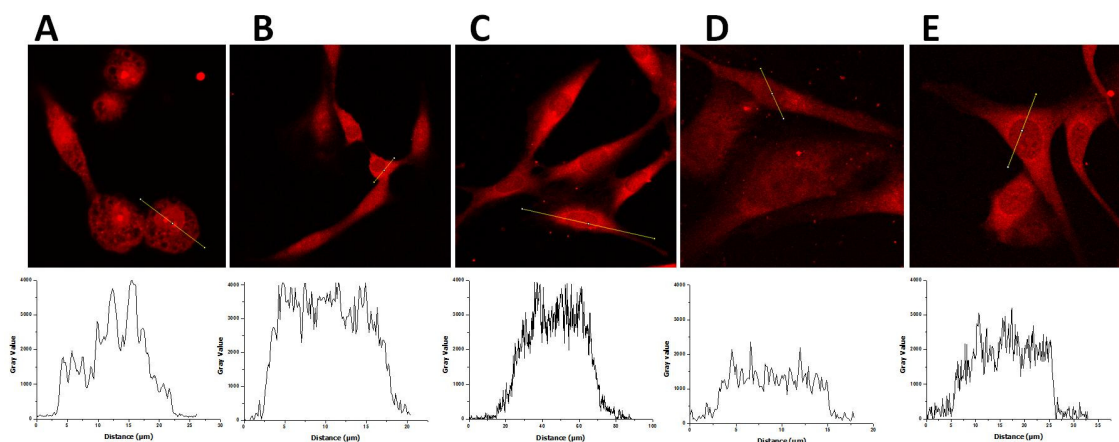


Figure 8. Confocal microscopy of NIH-3T3 fibroblasts and fluorescence quantification (gray value *versus* distance (μm)) after 24 h incubation of the nanoparticles with cells. (A) AEP, (B) AEP-Fuc-25, (C) AEP-Fuc-100, (D) RREP, and (E) RREP-Fuc-25 nanoparticles.

The typical shape of the NIH 3T3 fibroblasts was preserved. This is in contrast to what was observed when the cells were incubated with the AEP nanoparticles. The fluorescence was concentrated as punctuations in the cell cytoplasm and the cells have lost their typical elongated morphology to become more circular including vacuoles in the cytoplasm of the NIH-3T3 fibroblasts (Fig. 8A). The maximum intensity of the fluorescence measured in the cytoplasm was 3098 AU compared with that in the intracellular compartments which was 1.3 fold higher (4024 AU). This difference in the intracellular distribution of the fluorescence within the cells shown by the AEP nanoparticles compared to the other can be attributed to both the fact that the nanoparticle surface was exempt of fucoidan and that the polysaccharide chains was arranged in a different way compared to that found on the RREP nanoparticles. As indicated in the literature, the observed modification of the NIH-3T3 morphology can be explained by a loss of the structure of the actin fibers involved in the maintenance of the cytoskeleton of the cells (Dalby et al. 2003). As reported earlier, such a morphology modification did not interfere on cell viability on the time scale of the study (Gupta et al. 2004). This is in agreement with the absence of specific cytotoxicity profile found for the AEP nanoparticles on the NIH-3T3 cells.

Although different intracellular trafficking could be highlighted with the different nanoparticles depending on their method of synthesis and whether or not their surface were decorated with fucoidan chains, observations of the intracellular uptake by the J774 and the NIH-3T3 did not explain the difference in cytotoxicity found with the series of the AEP nanoparticles on J774.

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References

- Araújo, P.M.; Oliveira, G.B.; Córdula, C.R.; Leite, E.L.; Carvalho Jr. L.B.; Silva, M.P.C. Sulfated fucan as support for antibiotic immobilization. *Braz. J. Med. Biol. Res.* **37**:301-305 (2004).
- Alley, M.C.; Scudiero, D.A.; Monks, A.; Hursey, M.L.; Czerwinski, M.J.; Fine, D.L.; Abbot, B.J.; Mayo, J.G.; Shoemaker, R.H.; Boyd, M.R.. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* **48**:589–601 (1988).
- Avgoustakis K. Pegylated poly(lactide) and poly(lactide-co-glycolide) nanoparticles: preparation, properties and possible applications in drug delivery. *Curr. Drug Del.* **1**:321-33 (2004).
- Becker, C.F.; Guimarães, J.A.; Mourão, P.A.S.; Hugo Verli. Conformation of sulfated galactan and sulfated fucan in aqueous solutions: Implications to their anticoagulant activities. *J. Mol. Graph. Model.* **26**:391–399 (2007).
- Bertholon, I.; Vauthier, C.; Labarre, D. Complement activation by core-shell poly(isobutylcyanoacrylate)-polysaccharide nanoparticles: influences of surface morphology, length, and type of polysaccharide. *Pharm. Res.* **23**:1313–1323 (2006a).

- Bertholon, I.; Hommel, H.; Labarre, D.; Vauthier, C. Properties of polysaccharides grafted on nanoparticles investigated by EPR. *Langmuir: ACS J. Surf. Coll.* **22**:5485-90 (2006b).
- Bravo-Osuna, I.; Vauthier, C.; Farabollini, A.; Palmieri, G.F.; Ponchel, G. Mucoadhesion mechanism of chitosan and thiolated chitosan-poly(isobutyl cyanoacrylate) core-shell nanoparticles. *Biomaterials.* **28**:2233–2243 (2007a).
- Bravo-Osuna, I.; Ponchel, G.; Vauthier, C. Tuning of shell and core characteristics of chitosan-decorated acrylic nanoparticles. *Eur. J. Pharm. Sci.* **30**:143-154 (2007b).
- Chauvierre, C.; Labarre, D.; Couvreur, P.; Vauthier, C. Novel polysaccharide-decorated poly(isobutyl cyanoacrylate) nanoparticles. *Pharm. Res.* **20**:1786-1793 (2003).
- Chauvierre, C.; Leclerc, L.; Labarre, D.; Appel, M. Enhancing the tolerance of poly(isobutylcyanoacrylate) nanoparticles with a modular surface design. *Int. J. Pharm.* **338**:327–332 (2007).
- Couvreur P.; Kante B.; Roland M.; Guiot P.; Bauduin P.; Speiser P. Polycyanoacrylate nanocapsules as potential lysosomotropic carriers: preparation, morphological and sorptive properties. *J. Pharm. Pharmacol.* **31**: 331–2 (1979).
- Couvreur, P.; Vauthier, C. Nanotechnology: Intelligent design to treat complex disease. *Pharm. Res.* **23**:417–1450 (2006).
- Cumashi, A.; Ushakova, N. A.; Preobrazhenskaya, M. E.; D'Incecco, A.; Piccoli, A.; Totani, L.; Tinari, N.; Morozevich, G. E.; Berman, A. E.; Bilan, M. I.; Usov, A. I.; Ustyuzhanina, N. E.; Grachev, A. A.; Sanderson, C. J.; Kelly, M.; Rabinovich, G. A.; Iacobelli, S.; Nifantiev, N. E. A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. *Glycobiology.* **17**: 541-552 (2007).
- Dalby, M.J.; Riehle, M.O.; Sutherland, D.S.; Agheli, H.; Curti, A.S.G. Changes in fibroblast morphology in response to nano-columns produced by colloidal lithography. *Biomaterials* **25**:5415–5422 (2004).

- Davis, T. A.; Volesky, B.; Mucci, A. A review of the biochemistry of heavy metal biosorption by brown algae. *Water Res.* **37**:4311–4330 (2003).
- De Martimprey, H.; Vauthier, C.; Malvy, C.; Couvreur, P. Polymer nanocarriers for the delivery of small fragments of nucleic acids: Oligonucleotides and siRNA. *Eur. J. Pharm. Biopharm.* **71**:490-504 (2009).
- Dobrovolskaia, M.A.; Aggarwal, P.; Hall, J.B.; McNeil, S.E. Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution. *Mol. Pharm.* **5**:487–495 (2008).
- Ellouali, M.; Boisson-Vidal, C.; Durand, P. ; Jozefonvicz, J. Antitumor activity of low molecular weight fucoidans extracted from brown seaweed *Ascophyllum nodosum*. *Anticancer Res.* **13**:2011-2020 (1993).
- Ellouali, M.; Boisson-Vidal, C.; Jozefonvicz, J. Antiproliferative effect an interaction of Fucoidans with cells. *Coll. Surf. B: Biointerfaces.* **2**:305-314 (1994).
- Farokhzad O.C, Langer R. Impact of nanotechnology on drug delivery. *ACS Nano.* **27**:16-20 (2009).
- Gupta, A.K.; M.; Yarwood; S.J.; Curtis, A.S.G. Effect of cellular uptake of gelatin nanoparticles on adhesion, morphology and cytoskeleton organisation of human fibroblasts. *J. Control. Rel.* **95**:197– 207 (2004).
- Hayashi, K.; Nakano, T.; Hashimoto, M.; Kanekiyo, K.; Hayashi, T. Defensive effects of a fucoidan from brown alga *Undaria pinnatifida* against herpes simplex virus infection. *Int. Immunopharm.* **8**:109-116 (2008).
- He, M.; Zhao, Z.; Yin, L.; Tang, C.; Yin, C. Hyaluronic acid coated poly(butyl cyanoacrylate) nanoparticles as anticancer drug carriers. *Int. J. Pharm.* **373**:165-173 (2009).

- Hsu, H.Y.; Chiu, S.L.; Wen, M.H.; Chen, K.Y.; Hua, K.F. Ligands of macrophage scavenger receptor induce cytokine expression via differential modulation of protein kinase signaling pathways. *J. Biol. Chem.* **276**:28719–28730 (2001).
- Itoh, H.; Noda, H.; Amano, H.; Zhuaug, C.; Mizuno, T. Antitumor-activity and immunological properties of mariane algal polysaccharides, especially fucoidan, prepared from *Sargassum-Thunbergii* of phaeophyceae. *Anticancer Res.* **13**:2045-2052 (1993).
- Kazuya, H. I.P.J.; Takahashi, N.; Arihara, M.; Nagaoka, M.; Morita, K.; Suzuki, T. Structure and anti-dengue virus activity of sulfated polysaccharide from a marine alga. *Biochem. Biophys. Res. Commun.* **376**:91–95 (2008).
- Kim, W.S.; Ordija, C.M.; Freeman, M.W. Activation of signaling pathways by putative scavenger receptor class A (SR-A) ligands requires CD14 but not SR-A. *Biochem. Biophys. Res. Commun.* **310**:542–549 (2003).
- Labarre, D.; Vauthier, C.; Chauvierre, C.; Petri, B.; Müller, R.; Chehimi, M.M. Interactions of blood proteins with poly(isobutylcyanoacrylate) nanoparticles decorated with a polysaccharidic brush. *Biomaterials.* **26**:5075–5084 (2005).
- Lemarchand, C.; Gref, R.; Passirani, C.; Garcion, E.; Petrid, B.; Müller, R.; Costantini, D.; Couvreur, P. Influence of polysaccharide coating on the interactions of nanoparticles with biological systems. *Biomaterials* **27**:108–118 (2006).
- Lemarchand, C.; Gref, R.; Couvreur, P. Polysaccharide-decorated nanoparticles. *Eur. J. Pharm. Biopharm.* **58**:327-41 (2004).
- Li, B.; Lu, F.; Wei, X.; Zhao, R. Fucoidan: Structure and bioactivity. *Molecules* **13**:1671-1695 (2008).
- Li, S.D.; Huang L. Pharmacokinetic and biodistribution of nanoparticles. *Mol. Pharm.* **5**:496-504 (2008).

- Logeart, D.; Prigent-Richard, S.; Jozefonvicz, J.; Letourneur, D. Fucans, sulfated polysaccharides extracted from brown seaweeds, inhibit vascular smooth muscle cell proliferation. I. Comparison with heparin for antiproliferative activity, binding and internalization. *Eur. J. Cell Biol.* **74**:376–384 (1997).
- Moghimi, S.M.; Hunter, A.C.; Murray, J.C. Long-circulating and target-specific nanoparticles: theory to practice. *Pharm. Rev.* **53**:283–318 (2001).
- Nakamura, T.; Suzuki, H.; Wada, Y.; Kodama, T.; Doi, T. Fucoidan induces nitric oxide production via p38 mitogen-activated protein kinase and NF- κ B-dependent signaling pathways through macrophage scavenger receptors. *Biochem. Biophys. Res. Commun.* **343**:286–294 (2006).
- Peracchia, M.T.; Fattal, E.; Desmaele, D.; Besnard, M.; Noel, J.P.; Gomis, J.M.; Appel, M.; d'Angelo, J.; Couvreur, P. Stealth PEGylated polycyanoacrylate nanoparticles for intravenous administration and splenic targeting. *J. Controlled. Rel.* **60**:121–128 (1999).
- Renoir, J.M.; Stella, B.; Ameller, T.; Connault, E.; Opolon, P.; Marsaud, V. Improved anti-tumoral capacity of mixed and pure anti-oestrogens in breast cancer cell xenografts after their administration by entrapment in colloidal nanosystems. *J. Steroid Biochem. Mol. Biol.* **102**:114–127 (2006).
- Sezer, A.D.; Cevher, E.; Hatipoğlu, F.; Oğurtan, Z.;Baş, A.L.; Akbuğa, A.D. The use of fucosphere in the treatment of dermal burns in rabbits. *Eur. J. Pharm. Biopharm.* **69**:189-198 (2008).
- Sheen, J.; Zhou, L.; Jyun-Chyun, J. Sugars as signaling molecules. *Curr. Opin. Plant Biol.* **2**:410–418 (1999).
- Stella, B.; Arpicco, S.; Peracchia, M.T.; Desmaele, D.; Hoebeke, J.; Renoir, V.; d'Angelo, J.; Cattel, L.; P. Couvreur. Design of folic acid conjugated nanoparticles for drug targeting. *J. Pharm. Sci.* **89**:1452-1464, 2000.

- Ushakova, N. A.; Morozevich, G. E.; Ustyuzhanina, N. E.; Bilan, M. I.; Usov, A. I.; Nifantiev, N. E.; Preobrazhenskaya, M. E. Anticoagulant activity of fucoidans from brown algae. *Biom. Chem.* **3**:77–83, 2009.
- Vauthier, C.; Labarre, D. Modular biomimetic drug delivery systems. *J. Drug Del. Sci. Tech.* **18**:59-68 (2008).
- Vlerken, L.; Vyas, T.; Amiji, M. Poly(ethylene glycol)-modified nanocarriers for tumor-targeted and intracellular delivery. *Pharm. Res.* **24**:1405-1414 (2007).
- Vonarbourg, A.; Passirani, C.; Saulnier, P.; Benoit, J.P.; Parameters influencing the stealthiness of colloidal drug delivery systems. *Biomaterials.* **27**:4356–4373(2006).
- Wang, J.; Zhang, Q.; Zhang, Z.; Li, Z. Antioxidant activity of sulfated polysaccharide fractions extracted from *Laminaria japonica*. *Int. J. Biol. Macromol.* **42**:127–132 (2008).
- Wu, M.; Dellacherie, E.; Durand, A.; Marie, E. Poly(n-butyl cyanoacrylate) nanoparticles via miniemulsion polymerization (1): Dextran-based surfactants. *Coll. Surf. B: Biointerfaces* **69**: 141-146 (2009).
- Yoon, S.J.; Pyun, Y.R.; Hwang, J.K.; Mourão, P.A.S. A sulfated Fucoïdan from the brown alga *Laminaria cichorioides* has mainly heparin cofactor II-dependent anticoagulant activity *Carbohydrate Res.* **342**:2326–2330 (2007).
- Zvyagintseva, T. N.; Shevchenko, N.M., Nazarova, I.V.; Scobun; A.S.; Lukyanov, P.A.; Elyakova, L.A. Inhibition of complement activation by water-soluble polysaccharides of some far-eastern brown seaweeds. *Comp. Biochem Physiol. Part C.* **126**:209–215 (2000).

ARTIGO III

***In vivo* bioadhesive evaluation of new
fucoidan-coated poly(isobutylcyanoacrylate) nanoparticles**

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Abstract

The capacity of our recently developed fucoidan-decorated nanoparticles to target macrophages of the gastrointestinal tract was investigated *in vivo*. Results showed that the fluorescently labeled PIBCA nanoparticles could be successfully located in the gastrointestinal tract in *in vivo* conditions. Only the fucoidan decorated RREP nanoparticles showed a reduction of bioadhesive properties compared with the other types of nanoparticles. By fluorescent microscopy, the nanoparticles were mainly found associated with the mucus suggesting that they were retained in the mucus. Only a very low amount of nanoparticles could be highlighted at the surface of the epithelial cells on the luminal side of both the epithelium containing villi and Peyer patches. These results suggested that the entanglement of the nanoparticles in the mucus compromised any eventual uptake of the nanoparticles by macrophages accessible from the gastrointestinal tract.

Keywords: poly(isobutylcyanoacrylate), fucoidan, nanoparticles, *in vivo*, bioadhesion, fluorescence microscopy

Introduction

Nanoparticles composed by biodegradable polymers, such as poly(alkylcyanoacrylate) (PACA), are being widely investigated as delivery systems for small molecules, proteins, peptides and nucleic acids (Vauthier et al, 2007).

Nanoparticle surface properties can be changed to improve their biological properties such as the permeation enhancing properties, mucoadhesive or bioadhesive properties (Arbós et al. 2003) and their blood circulation time (Vlerken et al. 2007). Poly(ethylene glycol) (PEG) has been widely used to obtain surface modified nanoparticles. However, other molecules such as polysaccharides are alternative macromolecules to be used to cover the nanoparticle surface aiming to obtain biomimetic nanoparticles with new functionalities (Vauthier; Labarre, 2008). Different surface characteristics can be achieved simply by varying the nature of the polysaccharide attached on the nanoparticle surface and the conformation of the grafting chains by choosing suitable preparation method.

Fucoidan is a sulfated polysaccharide isolated from many species of brown algae and some marine invertebrates such as sea urchin (Berteau; Mulloy, 2003) being the most abundant and largely studied of all the sulfated polysaccharides from non-mammalian origin (Pereira et al. 2002). It was found to have anticoagulant (Ushakova, et al. 2009; Becker, et al. 2007; Yoo, et al. 2007), antiviral (Hayashi, et al. 2008), antitumor (Ellouali, et al. 1994; Ellouali et al. 1993; Itoh, et al. 1993), anti-inflammatory and antioxidant activities (Cumashi, et al. 2007; Souza et al. 2007; Wang, et al. 2007). It was also reported blood lipids reducing properties (Veena et al. 2007), inhibition of complement activation (Tissot, et al. 2003), and gastric protective effects (Li, et al. 2008). Another interesting issue is that fucoidan may be used as a ligand to confer the nanoparticle recognition properties to target macrophages thanks to the presence of receptors at the cell surface showing a high affinity for fucoidan.

The aim of the present work was to evaluate the capacity of our recently developed fucoidan-coated nanoparticles to target macrophages *in vivo*. The nanoparticles were obtained by emulsion polymerization of isobutylcyanoacrylate taking the advantage that polysaccharides can be grafted on different conformation, i.e. side-on (forming loops) or end-on (forming hairs or brushes), at the nanoparticle surface depending on the polymerization mechanism. The experimental strategy was to investigate the uptake of the targeted nanoparticles by macrophages accessible from the gastrointestinal tract. The experiments included *in vivo* bioadhesion evaluation and confocal fluorescent microscopy to visualize an eventual uptake of the nanoparticles by macrophages and to identify structures where the nanoparticles were located in the intestine.

Materials and Methods

Chemicals

Isobutylcyanoacrylate (IBCA) was kindly provided as a gift by Henkel Biomedical (Dublin, Ireland). Dextran (Mw 66,900) was purchased from Sigma (Saint-Quentin Fallavier, France). Cerium (IV) from cerium ammonium nitrate and trisodium citrate dihydrate were purchased from Fluka (Saint Quentin Fallavier, France). PolyFluor[®] 570: methacryloxyethyl thiocarbamoyl rhodamine B (N-[9-(2-carboxy-x-methacryloxy-ethylthiolcarbamoyl-phenyl)-6-diethylamino-3H-xanthen-3-ylidene]-N-ethyl-ethanaminium chloride) was supplied from Polyscience (Biovalley, Marne la Vallée, France). Fucoidan from *Sargassum cymosum* was a gift from Dr. M.P.C. Silva (Federal University of Pernambuco, Recife, Brazil). All other chemicals used were of reagent grade and were obtained from Merck (Darmsadt, Germany).

Labeled fucoidan-coated poly(isobutylcyanoacrylate) nanoparticles preparation

Fucoidan-coated poly(isobutylcyanoacrylate) nanoparticles were prepared by anionic emulsion polymerization (AEP) and redox emulsion polymerization (RREP) as described in Lira et al. (2009). In brief, in the RREP preparation, fucoidan and/or dextran (0.1356 g) were dissolved in nitric acid (4 ml, 0.2M) varying the ratio in the polysaccharide blend from 0 to 100% in fucoidan. The solution was placed under magnetic stirring at 40 °C with nitrogen bubbling. After 10 min, 1 ml of cerium (IV) ammonium-nitrate solution (8×10^{-2} M) in nitric acid 0.2 M was added to the solution containing the polysaccharide and immediately after, 250 μ l of the monomer IBCA were added under vigorous magnetic stirring (1000 rpm). After 2 min, 1 ml of a PolyFluor[®] 570 solution (rhodamine) dissolved in acetonitrile (4 mg/ml) was added to the polymerization medium. The polymerization system was maintained under magnetic stirring for 1 h. After cooling down to room temperature, 1 ml of an aqueous solution of trisodium citrate dihydrate (1 M) was added to complex the remaining cerium ions. In contrast, by AEP fucoidan was dissolved in pure water (5 ml) and the solution was used to dissolve the dextran. Subsequently, the pH was decreased to 2.5 with HCl (1 N). Then, 50 μ l of monomer IBCA were added drop wise under vigorous magnetic stirring. After 5 min, 50 μ l of a PolyFluor[®] 570 solution in acetonitrile (5 mg/ml) was added. The system was left to polymerize under vigorous stirring for 3 h at room temperature. All nanoparticle suspensions were purified by dialysis (Spectrapor[®] membrane 100,000 g/mol molecular weight cut off (MWCO), Biovalley, Marne la Vallée, France) twice against 1 L of distilled water for 30 min and once overnight. This dialysis was aimed to eliminate all reagents including polysaccharides that have not reacted during the nanoparticle synthesis. The purified suspensions were stored at 4 °C until use.

Labeled fucoidan-coated poly(isobutylcyanoacrylate) nanoparticles prepared by AEP and RREP were named Lab-AEP-Fuc-X% and lab-RREP-Fuc-X% respectively, where the X indicates the percentage of fucoidan used in the synthesis of the nanoparticles.

Labeled fucoidan-coated poly(isobutylcyanoacrylate) nanoparticles characterization

Fucoidan-coated poly(isobutylcyanoacrylate) nanoparticles preparations were characterized by macroscopic aspects, size and zeta potential (Lira et al., 2009). The diameter of the nanoparticles was measured at 25 °C by quasi-elastic light scattering using a Nanosizer N4 PLUS (Beckman-Coulter, Villepinte, France) operating at the angle of 90 °C. The samples were diluted in deionized water (Milli Q[®], Millipore, USA) and filtered through a 0.22 µm (Millipore filter[®], Millipore, USA). The zeta potential of the nanoparticles was deduced from the electrophoretic mobility using a Zetasizer 4 (Malvern Instruments, Orsay, France). Dilution of the suspensions was performed in KCl 1 mM. Results corresponded to the average of three determinations. Samples for SEM analysis were prepared from a nanoparticle suspensions diluted in water. The diluted suspension was dropped on a piece of silicium wafer and dried in a desiccators. After drying, the piece of silicium wafer was sealed with colloidal silver liquid on stubs for analysis (FEI, Quanta 200 FEG) at the Center of the Northeast Technology (CETENE, Recife, Brazil).

***In vivo* bioadhesion study**

The *in vivo* bioadhesion study was carried out according to Yoncheva et al. (2007). The study was in compliance of the responsible committee of the University of Navarra in line with the European Legislation on animal experiments (86/609/EU). Male Wistar rats (225 g), were placed in metabolic cages with free access to water 24 hours before the start of the experiment. Suspensions of fucoidan-coated poly(isobutylcyanoacrylate) labeled nanoparticles (10 mg) were orally administered (by gavage) to fasted rats. The animals were sacrificed by cervical dislocation after 0.5, 1, 3 and 8h. The abdominal cavity was opened and the stomach, small intestine and caecum were removed. Each mucosa segment was opened

lengthwise along the mesentery and rinsed with PBS buffer (pH 7.4). Finally each rinsed mucosa segment was cut into portions and digested in 1 ml of NaOH (3N) for 24 hours. Methanol (2 ml) was used to extract rhodamine from the tissue, the material was vortex for 1 min and centrifuged at 4000 rpm for 10 min. Aliquots of the supernatants were transferred to 96 well plate and assayed for rhodamine content by spectrofluorimetry at λ_{ex} 540 nm and λ_{em} 580 nm (GENion, TECAN, Groedig, Austria) to estimate the fraction of nanoparticles, which adhered to the mucosa. The absorbance of the tissue collected from rats that did not receive any nanoparticles was subtracted from the absorbance found in the samples, aiming to get the contribution from the rhodamine content.

Tissue fluorescence microscopy studies

The tissue distribution of fucoidan-coated poly(isobutylcyanoacrylate) nanoparticles in the GIT mucosa was observed by fluorescence microscopy. First, 10 mg of labeled nanoparticles were administered following the same protocol than that applied for the biodhesion study. After 2 h, the animals were sacrificed, the ileum was removed and rinsed with PBS buffer (pH 7.4). The rinsed ileum was cut into small portions treated with tissue processing medium O.C.T.TM (Sakura, Netherlands) and finally frozen in liquid nitrogen. Finally, the tissue samples were cut into 5 μ m section using a cryostat Leica CM1900 (2800 Frigocut E, Reichert-Jung, Germany) at -23°C and observed by fluorescence microscopy (Olympus CH40, Japan) with fluorescence source (Olympus U-RFLT50).

Results and discussion

Preparation of labeled fucoidan-coated PIBCA nanoparticles

In agreement with our previous study (Lira, et al. 2009), stable fucoidan-coated nanoparticles were obtained with up to 100% of fucoidan with the AEP method while the maximum percentage of fucoidan which lead to the preparation of stable nanoparticles by the RREP was only 25%. Regarding zeta potential, all fucoidan-coated nanoparticles presented a negative zeta potential value, which was decreased by increasing the percentage of fucoidan Table 1. No significant changes were observed between zeta potential of labeled and non labeled fucoidan-coated nanoparticles. In contrast, the size of the labeled nanoparticles was slightly increased compared to the non labeled nanoparticles. The results were suggested that fucoidan, a negatively charged polysaccharide, was located at the surface of nanoparticles whereas rhodamine, which was positive at the pH of the experiments, was incorporated in the core of the fucoidan-coated nanoparticles. As shown in the example of electron micrograph presented in Figure 1, nanoparticles were spherical with a homogenous size distribution.

Table 1. Physicochemical characteristics of labeled and unlabeled PIBCA nanoparticles prepared by AEP and RREP with different concentrations of fucoidan.

Preparations	Fucoidan:Dextran Ration (%)	Size (nm) unlabeled	Size (nm) labeled	Zeta Potential (mV) unlabeled	Zeta Potential(mV) labeled
AEP	0:100	193 ± 4	258 ± 5.7	-5.53 ± 1	- 5.32 ± 1.2
AEP-Fuc-25	25:75	195 ± 2	209 ± 5.7	-9.60 ± 0.4	-9.35 ± 0.2
AEP-Fuc-50	50:50	298 ± 1	295 ± 5.4	-12.0 ± 1	-11.6 ± 0.6
AEP-Fuc-100	100:0	298 ± 0.4	342 ± 5.9	-44.2 ± 1	- 39.0 ± 0.1
RREP	0:100	399 ± 0.7	413 ± 1	-6.39 ± 0.1	- 2.52 ± 0.4
RREP-Fuc-25	25:75	307 ± 4	300 ± 4.0	-7.18 ± 0.6	-5.67 ± 0.2
RREP-Fuc-50	50:50	----	----	----	----
RREP-Fuc-100	100:0	----	----	----	----

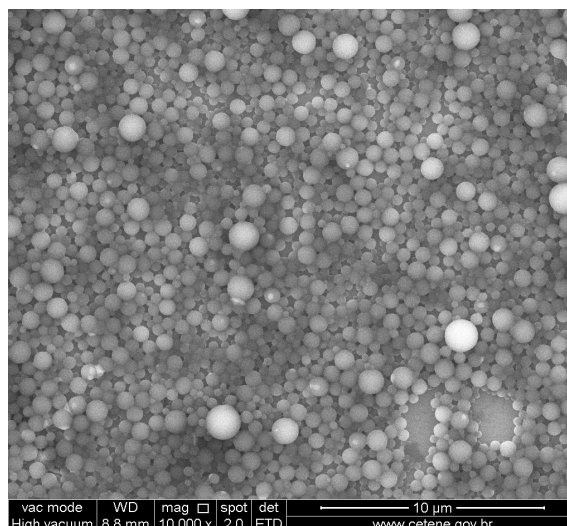


Figure . 1 Scanning electron microscopy for RREP-Fuc-25 nanoparticles.

Bioadhesive profile of fucoidan-coated PIBCA nanoparticles

The bioadhesive profile of the nanoparticles was investigated *in vivo* after oral administration of the fucoidan coated nanoparticles and compared to those given by the corresponding nanoparticles exempt of fucoidan. Figure 2 shows the bioadhesive profiles of AEP and RREP nanoparticles obtained after oral administration to Wistar rats. The graphs represent the fraction of nanoparticles adhering to the different gastrointestinal segments including the stomach (So), 4 fragments of the small intestine (portions from I1 to I4) and the caecum (Ce) at time 0.5, 1, 3 and 8 h after nanoparticle administration. Fluorescence due to the nanoparticles could be detected in the different portions of the GI tract and progressed towards the lower part of the intestine with time. In general, it can be observed that no fluorescence was detected in the stomach mucosa of animals, which have received the RREP and RREP-Fuc-25 nanoparticles. It can also be observed that both RREP and RREP-Fuc-25 nanoparticles were found in portion 3 of the intestine (I3) during 3 hours, but the presence of fucoidan (25%) decreased the amount of adhering nanoparticles. This finding was in agreement with the non bioadhesive property described earlier for fucoidan (Cumashi et al. 2007). AEP nanoparticles without fucoidan remained in the stomach

and were also found in the first intestine portion (I1) 30 min after administration. In contrast, the AEP nanoparticles containing fucoidan were not observed in the stomach but in I1, I2 and I3 intestine portions where they remained during 3 hours. These fucoidan-coated nanoparticles demonstrated a higher affinity for the intestinal mucosa rather than for the stomach wall. No significant amounts of AEP nanoparticles, with or without fucoidan, were found to adhere on the colon, excepted for AEP-Fuc-100.

With this series of nanoparticles it seems that the presence of fucoidan on the nanoparticle surface did not influence the adherent properties of the nanoparticles on the gastrointestinal tract in contrast to what was observed with the RREP nanoparticles. This can be explained by a different spatial configuration of the polysaccharide chains at the nanoparticle surface.

As seen from the first experiments the progression of the nanoparticles along the gastrointestinal tract was only slightly affected by the type of nanoparticles and fucoidan content. For a deeper analysis, the intestinal tissue was examined by fluorescent microscopy to investigate with which structures the nanoparticles were interacting.

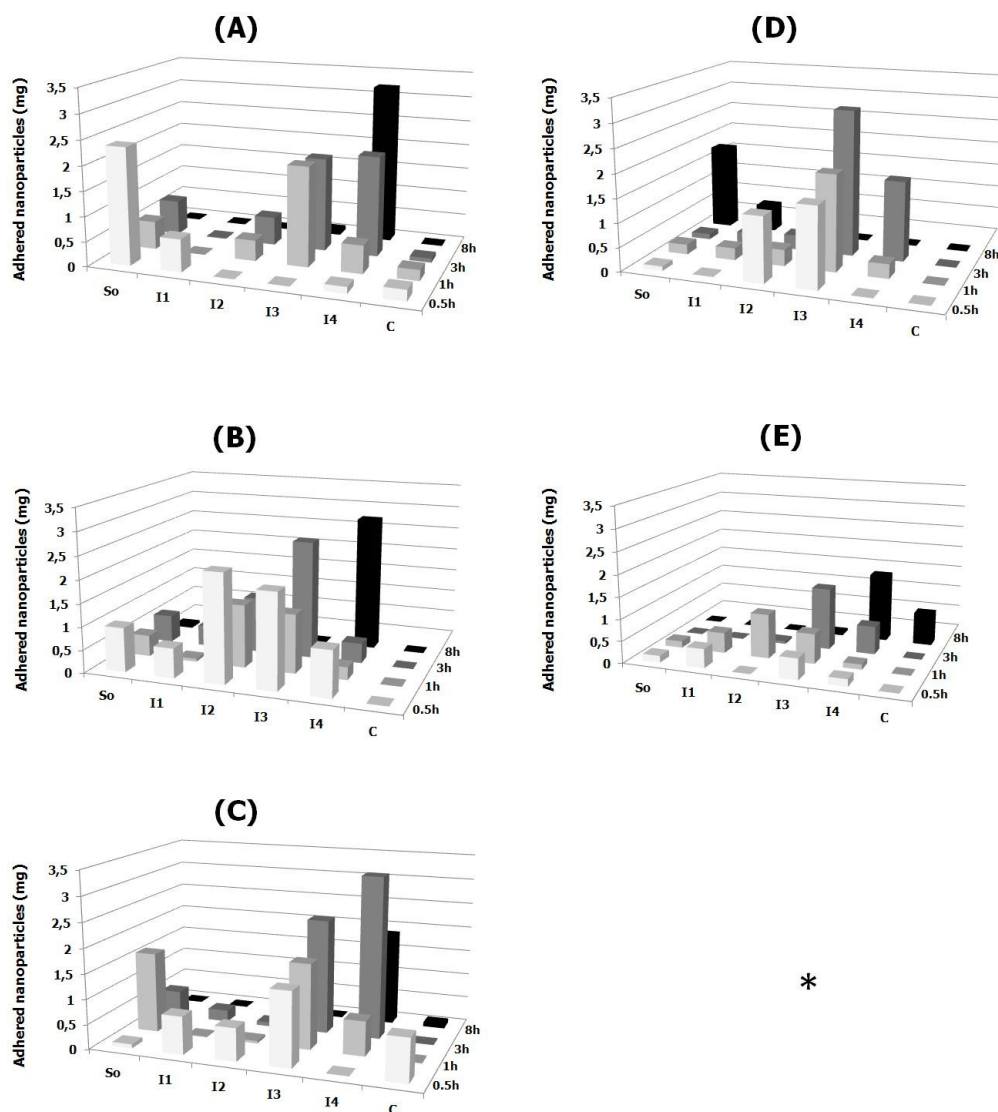


Figure 2. *In vivo* distribution of the adhered nanoparticles in rat gastrointestinal tract after oral administration of aqueous nanoparticle suspension. (A) AEP, (B) AEP-Fuc-25, (C) AEP-Fuc-100, (D) RREP, (E) RREP-Fuc-25. Plot: x-axis represents the adhered fraction (mg), y-axis illustrates the different gastrointestinal segments (Sto: stomach; I1, I2, I3, I4: parts of the small intestine; C: caecum) and z-axis marks the time of post-administration (h). Each value represents the mean of the results of three experiments.

Figure 3 shows the fluorescent microscopy of sectional views of intestinal tissue from rats who have received oral administration of labeled nanoparticles (10 mg). Observations suggested that the nanoparticles remained intact in the intestine as indicated by the concentration of the fluorescence in highly punctuated spots. The strongest fluorescence was associated with the mucus found in the lumen of the intestine of rats, which received fucoidan-coated nanoparticles. It appeared as a more or less aggregated spots of fluorescence in the mucus that varied with the type of nanoparticles suggesting that their surface properties were influencing their interactions with the mucus components.

Almost no fluorescence was observed on the epithelium. This very low fluorescence always appeared as very small spots associated with the luminal side of the cell epithelium surface including villi and Peyer patches (Figure 4). No evidence of nanoparticle absorption could be highlighted from these observations as the fluorescence remained located on the epithelium surface and no trace of fluorescence could be seen on the serosal side of the epithelium. In contrast to the observations made with the fucoidan-coated AEP and RREP nanoparticles, the fluorescence given by the nanoparticles exempt of fucoidan was extremely low.

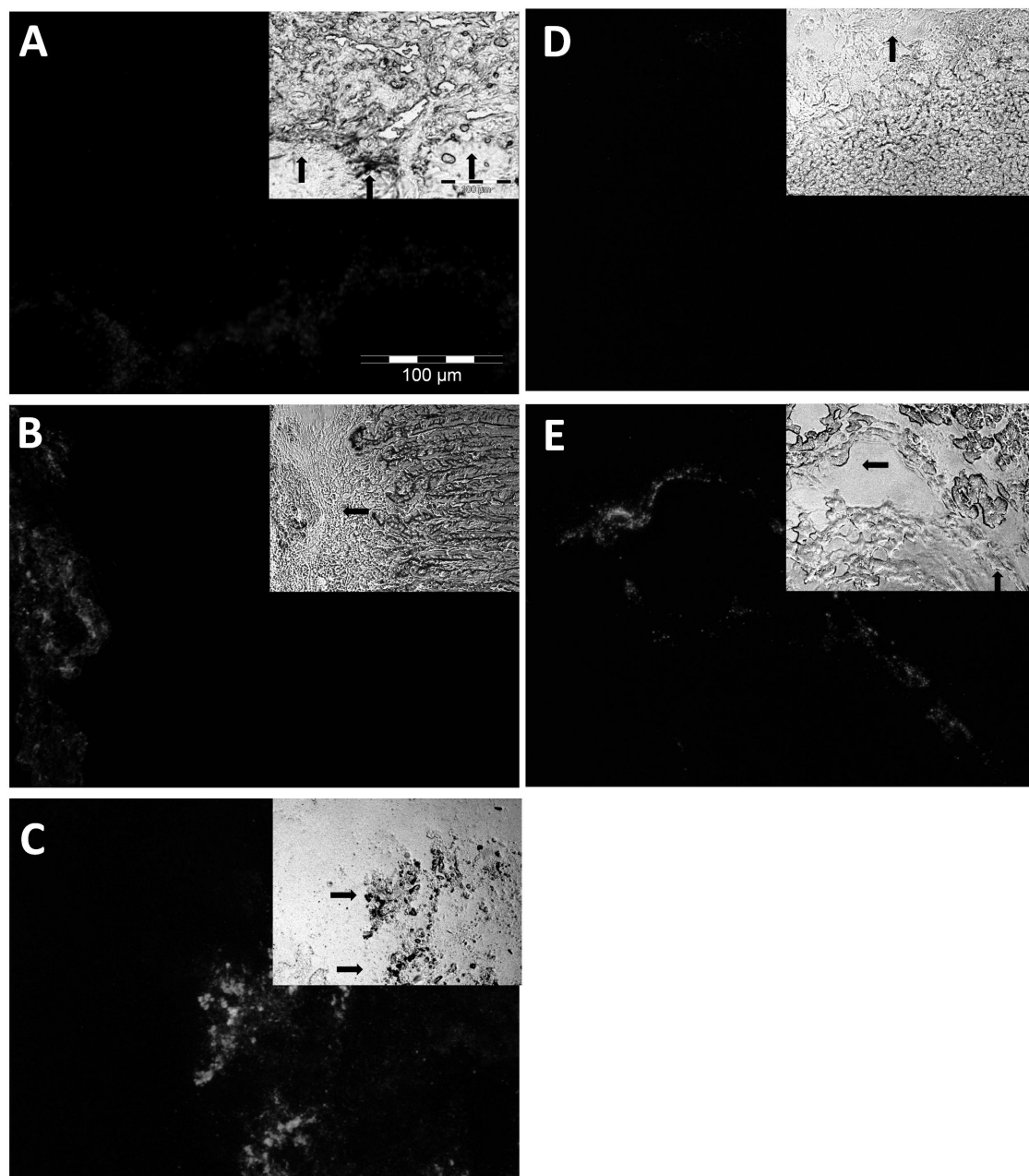


Figure 3. Nanoparticles remaining the small intestine (part I3) 2 h after administration to animals. (A) AEP, (B) AEP-Fuc-25, (C) AEP-Fuc-100, (D) RREP, (E) RREP-Fuc-25.

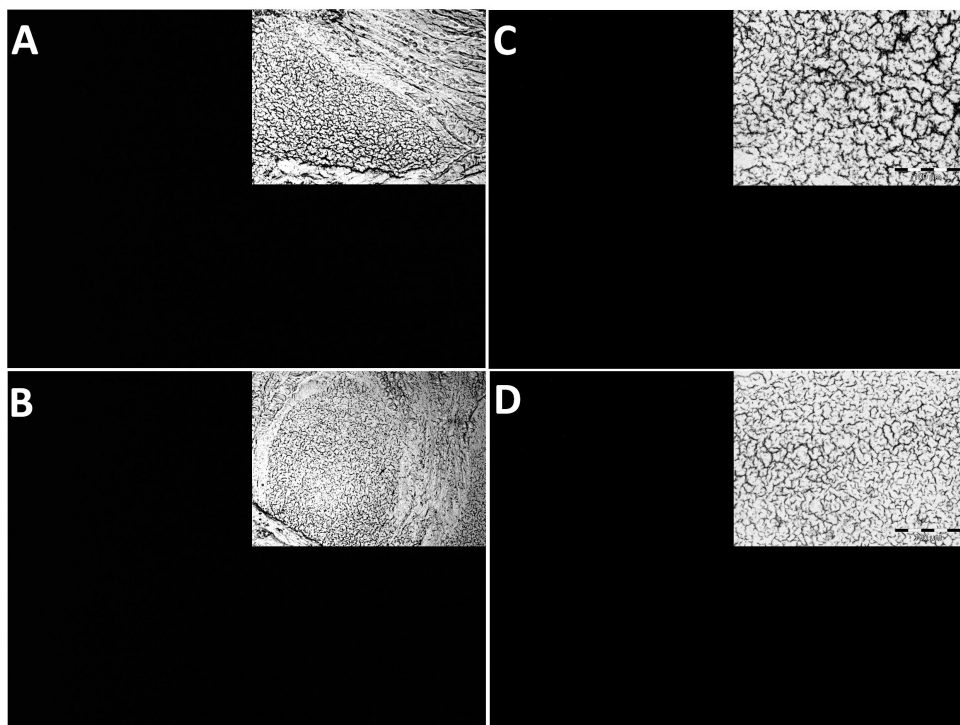


Figure 4. Nanoparticles remaining in Peyer patches (part I3) 2 h after administration to animals.

(A) AEP-Fuc-25, (B) AEP-Fuc-100, (C) RREP, (D) RREP-Fuc-25.

Conclusions

This study showed that PIBCA nanoparticles with surface decoration including dextran and fucoidan could be successfully located in the gastrointestinal tract in vivo conditions. Only the fucoidan decorated RREP nanoparticles showed a reduction of bioadhesive properties compared with the other types of nanoparticles. By fluorescence microscopy, the nanoparticles were mainly located in the mucus suggesting that they were retained in the mucus. Thus, only a very low amount of nanoparticles can reach the surface of the epithelial cells and could be seen on the luminal side of the epithelium containing villi and Peyer patches. These results suggested that the entanglement of the nanoparticles by the mucus compromised any eventual uptake of the nanoparticles by macrophages accessible from the lymphoid tissue of the gastrointestinal tract.

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References

- Arbos, P.; Campanero, M.A.; Arangoa, M.A.; Renedo, M.J.; Irache, J.M. Influence of the surface characteristics of PVM/MA nanoparticles on their bioadhesive properties *J. Control. Rel.* v. 89, p. 19–30, 2003.
- Becker, C.F.; Guimarães, J.A.; Mourão, P.A.S.; Verli, H. Conformation of sulfated galactan and sulfated fucan in aqueous solutions: Implications to their anticoagulant activities. *J. Mol. Graph. Model.* v. 26, p. 391–399, 2007.
- Berteau, O.; Mulloy, B. Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology.* v. 13, p. 29R-40R, 2003.
- Cumashi, A.; Ushakova, N. A.; Preobrazhenskaya, M. E.; D'Incecco, A.; Piccoli, A.; Totani, L.; Tinari, N.; Morozevich, G. E.; Berman, A. E.; Bilan, M. I.; Usov, A. I.; Ustyuzhanina, N. E.; Grachev, A. A.; Sanderson, C. J.; Kelly, M.; Rabinovich, G. A.; Iacobelli, S.; Nifantiev, N. E. A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic, and

- antiadhesive activities of nine different fucoidans from brown seaweeds. *Glycobiology*. v. 17 p. 541-552, 2007.
- Ellouali, M.; Boisson-Vidal, C.; Durand, P. ; Jozefonvicz, J. Antitumor activity of low molecular weight Fucoidans extracted from Brown seaweed *Ascophyllum nodosum*. *Anticancer Res.* v. 13, p. 2011-2020, 1993.
- Ellouali, M.; Boisson-Vidal, C.; Jozefonvicz, J. Antiproliferative effect an interaction of Fucoidans with cells. *Coll. Surf. B: Biointerf* v. 2, p. 305-314, 1994.
- Hayashi, K.; Nakano, T.; Hashimoto, M.; Kanekiyo, K.; Hayashi, T. Defensive effects of a fucoidan from brown alga *Undaria pinnatifida* against herpes simplex virus infection. *Inter. Immunopharmacol.* Vol. 8, pp. 109-116, 2008.
- Itoh, H.; Noda, H.; Amano, H; Zhuaug, C.; Mizuno, T. Antitumor-activity and immunological properties of marine algal polysaccharides, especially fucoidan, prepared from *Sargassum-Thunbergii* of Phaeophyceae. *Anticancer Res.* v 13, p 2045-2052, 1993.
- Li, B.; Lu, F.; Wei, X.; Zhao, R. Fucoidan: Structure and Bioactivity. *Molecules*. v 13, p. 1671-1695, 2008.
- Lira, M.C.B.; Santos-Magalhães, N.S.; Nicolas, V.; Marsaud, V.; Silva, M.P.C.; Ponchel, G.; Vauthier C. Cytotoxicity and cellular uptake of new fucoidan-coated poly(isobutylcyanoacrylate) nanoparticles. 2009 (submitted).
- Pereira, M.S.; Vilela-Silva, A.C.E.S.; Valente, A.P.; Mourão, P.A.S. A 2-sulfated, 3-linked α -L-galactan is an anticoagulant polysaccharide. *Carbohydr. Res.* v. 337, p. 2231–2238, 2002.
- Souza, M.C.R.; Marques, C.T.; Dore, C.M.G.; Silva, F.R.F.; Rocha, H.A.O.; Leite, E.L. Antioxidant activities of sulfated polysaccharides from brown and red seaweeds. *J. Appl. Phycol.* v. 19, p. 153–160, 2007.

- Tissot, B. ; Montdargent, B. ; Chevolot, L. ; Varenne, A. ; Descroix, S. ; Gareil, P. Daniel, R. Interaction of fucoidan with the proteins of the complement classical pathway. *Bioch. Biophys. Acta.* v.1651, p. 5 – 16, 2003.
- Ushakova, N. A.; Morozovich, G. E.; Bilan, M. I.; Usov, A. I.; Nifantiev, N. E.; Preobrazhenskaya, M. E. Anticoagulant Activity of Fucoidans From Brown Algae. *Supplement Series B: Biom. Chem.* v.3, p. 77–83, 2009.
- Vauthier, C. and Labarre, D. Modular biomimetic drug delivery systems. *J. Drug Del. Ssc. Tech.* v. 18, p. 59-68, 2008.
- Vauthier, C. Labarre, D.; Ponchel, G. Design aspects of poly(alkylcyanoacrylate) nanoparticles for drug delivery. *J. Drug Targ.* v. 15, p. 641–663, 2007.
- Veena, C.K.; Josephine, A.; Preetha, S.P.; Varalakshmi, P. Beneficial role of sulfated polysaccharides from edible seaweed *Fucus vesiculosus* in experimental hyperoxaluria. *Food Chem.* v. 100, p. 1552–1559, 2007.
- Vlerken, L.E.; Vyas, T.K.; Amiji, M.M. Poly(ethylene glycol)-modified nanocarriers for tumor-targeted and intracellular delivery. *Pharm. Res.* DOI: 10.1007/s11095-007-9284-6, 2007.
- Wang, J.; Zhang, Q.; Zhang, Z.; Li, Z. Antioxidant activity of sulfated polysaccharide fractions extracted from *Laminaria japonica*. *Int. J. Biol. Macromol.* v. 42, p. 127–132, 2008.
- Yoncheva, K.; Guembe, L.; Campanero, M.A.; Irache, J.M. Evaluation of bioadhesive potential and intestinal transport of pegylated poly(anhydride) nanoparticles. *Inter. J. Pharm.* v. 334, p. 156–165, 2007.
- Yoon, S.J.; Pyun, Y.R.; Hwang, J.K.; Mourão, P.A.S. A sulfated fucoidan from the brown alga *Laminaria cichorioides* has mainly heparin cofactor II-dependent anticoagulant activity. *Carbohydr. Res.* v. 342, p 2326–2330, 2007.

CONCLUSÕES

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Os resultados obtidos no presente trabalho permitem elaborar as seguintes conclusões:

- A fucana foi extraída da alga marrom *Sargassum cymosum* e purificada por cromatografia de permeação em gel com obtenção de dois picos de eluição distintos, correspondentes a fucanas com diferentes massas moleculares;
- A massa molecular da fucana referente ao segundo pico de eluição foi de 53 kDa;
- A análise elementar da fucana de menor massa molar revelou a presença de enxofre, confirmando que a fucana purificada é sulfatada;
- Os espectros de infravermelho bem como o de ressonância magnética nuclear de hidrogênio apresentaram picos característicos da molécula de fucana confirmando sua purificação;
- Ambos os métodos de polimerização *in situ* (AEP e RREP) utilizados foram adequados para obtenção de nanopartículas estáveis de poli-isobutilcianoacrilato revestidas com fucana;
- Os métodos também foram adequados na obtenção de nanopartículas marcadas com rodamina revestidas ou não com fucana;
- As nanopartículas apresentaram aspecto esférico e uniforme, com tamanho na escala nanométrica, conforme análise microscópica e granulométrica;
- A carga da superfície das nanopartículas diminuiu com o aumento do percentual de fucana utilizado, comprovando o revestimento das nanopartículas pela fucana;
- As nanopartículas obtidas por ambos os métodos e com diferente percentual de fucana permaneceram estáveis por pelo menos 6 meses, mantendo inalterado o tamanho das partículas e a carga da superfície;
- Nanopartículas preparadas por AEP apresentaram maior citotoxicidade em macrófagos J774 em relação aquelas obtidas por RREP;

- As análises em microscopia confocal de fluorescência não permitiram explicar o aumento da citotoxicidade das nanopartículas obtidas pelo método AEP nos macrófagos.
- Todos os tipos de nanopartículas foram encontrados nos dois tipos de células estudadas, demonstrando que elas podem ser capturadas independentemente pelos macrófagos e fibroblastos.
- A formulação AEP-Fuc-25% foi 3 vezes mais citotóxica em fibroblastos que a formulação correspondente obtida por RREP;
- As diferenças na aparência da fluorescência no interior das células, pontual ou difusa, sugerem que as nanopartículas penetraram nas células por mecanismos distintos. No entanto, nenhuma correlação pode ser estabelecida entre o tipo de nanopartículas (AEP ou RREP) e o teor de fucana.
- Com relação às propriedades de bioadesão nas células epiteliais do intestino, somente as nanopartículas revestidas com fucana obtidas pelo método RREP apresentaram uma redução na bioadesão comparada aos outros tipos de nanopartículas.
- A análise por microscopia de fluorescência revelou que as nanopartículas foram principalmente encontradas associadas ao muco residual. Apenas uma pequena quantidade de nanopartículas pôde ser visualizada na superfície das células epiteliais, do lado da luz intestinal do epitélio contendo as vilosidades e as placas de Peyer.
- Esses resultados sugerem que ocorreu uma retenção das nanopartículas no muco e que apenas uma pequena quantidade de nanopartículas chegaram à superfície do epitélio para ser eventualmente capturada pelos macrófagos.
- Embora o presente trabalho tenha permitido propor métodos para extração da fucana e de preparação de nanopartículas de PIBCA revestidas com a fucana, não foi capaz de demonstrar que as novas nanopartículas podem ser utilizadas para aumentar a especificidade e direcionamento para macrófagos.

- Experimentos complementares serão, portanto, necessários para melhor compreender a origem da citotoxicidade das nanopartículas obtidas pelo método AEP em macrófagos J774. Além do mais, para o direcionamento das nanopartículas para macrófagos presentes em mucosas, as propriedades bioadesivas devem ser ajustadas com vistas a reduzir a retenção pelo muco e de permitir um maior alcance na quantidade de nanopartículas a atingir a superfície das células epiteliais.