Universidade Federal de Pernambuco
Centro de Ciências Biológicas
Programa de Pós Graduação em Ciências Biológicas

RICARDO DE SOUZA SILVA

REVESTIMENTO DE PARTÍCULAS FERROMAGNÉTICAS COM HEPARINA E HEPARAN SULFATO E SEU USO COMO MATRIZ DE AFINIDADE PARA PURIFICAÇÃO DE PROTEÍNAS PLASMÁTICAS

Recife
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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas para obtenção do título de Doutor em Ciências Biológicas pela Universidade Federal de Pernambuco.

Orientador: Prof. Dr. Luiz Bezerra de Carvalho Júnior

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Para minha mãe, Maria,
pelo amor incondicional que sempre demonstrou por mim.

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“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes”.
(Marthin Luther King)

Resumo

Métodos de separação por afinidade têm se revelado eficazes e econômicos. Eles se baseiam na formação de um complexo estabelecido entre duas biomoléculas afins a um suporte insolúvel em água. Lavagens iniciais deste compósito removem impurezas de sorte que em uma segunda etapa o complexo é desfeito, liberando a biomolécula de interesse mediante seu rompimento (força iônica, pH, etc.). Dentre os diferentes métodos, aqueles baseados em suportes magnéticos têm as vantagens de fácil síntese, manuseio e o derivado ser recuperado por meio de um campo magnético. Esta tese se postulou a revestir partícula magnéticas com heparina (HEP) e heparan sulfato (HS) uma vez que esses glicosaminoglicanos são muito negativamente carregados e formam complexos com proteínas, por exemplo, proteínas plasmáticas. Três tipos de materiais foram sintetizados: polietilenotereftalato magnetizado (mPET), magnetita (MAG) e magnetita revestida com polianilina (mPANI). Inicialmente, filmes de PET sofreram hidrazinólise e o pó PET-hidrazida obtido foi magnetizado pelo método de co-precipitação em solução de cloretos férrico e ferroso. A MAG foi sintetizada conforme descrito acima, porém, sem adição do PET, e posteriormente foi revestida com PANI (mPANI). HEP e HS foram ativados com carbodiimida e N-hidroxissuccinimida e incubados com os suportes para que ocorressem o revestimento das partículas, produzindo os seguintes derivados magnéticos: mPET-HEP; mPANI-HEP; MAG-HEP; mPET-HS e mPANI-HS. As amostras de mPET-HEP foram investigadas por análise elementar e espectroscopia de infravermelho. A composição centesimal delas mostrou um aumento na razão carbono : nitrogênio, presumindo ser devido à imobilização de heparina. O espectro de infravermelho do derivado contendo heparina apresentou bandas em 1653 cm-1 e 1547 cm-1, característicos da ligação amida, formada pela conjugação. O derivado mPANI imobilizou cerca de 37% da heparina ofertada, embora a magnetita pura (MAG) também tenha imobilizado, porém em menor grau. Todas estas matrizes demonstraram uma boa estabilidade em 10 ciclos consecutivos de reutilização mesmo após 2 anos estocadas a 4°C. Quanto ao HS, este foi covalentemente fixado às partículas mPANI e mPET em torno de 35 µg e 38,6 µg por mg de suporte respectivamente, no entanto também foi adsorvido (18µg/mg) sobre as partículas controle de MAG. Indícios do sucesso da imobilização nestes compósitos foram a demonstração de que a heparina fixava o corante azul de metileno e retardava o tempo de coagulação do plasma recalcificado quando em contato com estes suportes. Finalmente, todos os derivados magnéticos foram incubados com plasma humano e lavados posteriormente com gradientes de NaCl para liberar as proteínas fixadas e detectá-las a 280nm. Eletroforese destas proteínas revelaram bandas referentes à antitrombina (58kDa) e as suas dosagens nos plasmas e eluatos confirmaram a eficiência do método de separação. Nos derivados mPET-HS e mPANI-HS, além da antitrombina, importantes fatores da coagulação também foram observados na eletroforese e comprovada a sua separação através de testes de coagulação específicos. Portanto, os resultados mostraram que estes compósitos produzidos são promissores na purificação da antitrombina e outros fatores da coagulação presentes no plasma humano.

Palavras-chaves: heparina; heparan sulfato; imobilização; polianilina; polietilenotereftalato; separação.

Abstract

Affinity separation methods have shown to be effective and economical. They are based on the formation of complex established between two related biomolecules to a water insoluble support. Initial washes of these composites remove impurities so that in a second step the complex is broken, releasing the biomolecule of interest through its breakup (ionic strength, pH, etc.). Among the different methods, those based on magnetic support have the advantages of easy synthesis, handling and the derivative to be retrieved by means of a magnetic field. This thesis has been postulated to coat magnetic particle with heparin (HEP) and heparan sulfate (HS) since these are very negatively charged glycosaminoglycans and form complexes with proteins, for example, plasma proteins. Three types of materials were synthesized: magnetized polyethylene terephthalate (mPET), magnetite (MAG) and magnetite coated with polyaniline (mPANI). Initially, PET films suffered hydrazinolysis and PET-hydrazide powder obtained was magnetized by co-precipitation method in a solution of ferric and ferrous chlorides. Initially, PET films suffered hydrazinolysis and PET-hydrazide powder obtained was magnetized by co-precipitation method in a solution of ferric and ferrous chlorides. The MAG was synthesized as described above but without the addition of PET and was subsequently coated with PANI. Heparin and heparan sulfate were activated with carbodiimide and N-hydroxysuccinimide and incubated with the supports for coating occurs and generate the following magnetic derivatives: mPET-HEP; mPANI-HEP; MAG-HEP; mPET-HS e mPANI-HS. The samples mPET-HEP were investigated by elemental analysis and infrared spectroscopy. The centesimal composition of them showed an increase in carbon : nitrogen ratio, presumed to be due to immobilization of heparin. The infrared spectrum of the heparin derivative showed bands at 1653 cm-1 and 1547 cm-1 which are characteristic of the amide linkage formed by conjugation. The mPANI derivative immobilized about 37% of heparin offered, although the pure magnetite (MAG) also has immobilized, but to a lesser degree. All these matrices showed good stability in 10 consecutive cycles of reuse even after 2 years stored at 4°C. As regards the heparan sulfate, it was covalently immobilized to particles mPANI and mPET about 35 μg and 38.6 μg respectively per mg of support, however it was also adsorbed (18μg/mg) on control particles (MAG). Evidence of the success of immobilization in these composites were the demonstration that heparin fixed the methylene blue dye and retarded the clotting time of plasma recalcified when in contact with these supports. Finally, all magnetic derivatives were incubated with human plasma and subsequently washed with NaCl gradients to release fixed proteins and detect them at 280 nm. Electrophoresis of these proteins revealed bands related to antithrombin (58kDa) and their dosages in plasma and eluates confirmed the efficiency of the separation method. In derivatives mPET-HS and mPANI-HS, beyond antithrombin, important coagulation factors were also observed in electrophoresis and confirmed their separation through specific coagulation tests. Therefore, the results showed that these composites produced are promising and important to separate antithrombin and others coagulation factors present in human plasma.

Keywords: heparin, heparan sulfate; immobilization; polyaniline, polyethylene terephthalate; separation.

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LISTA DE ABREVIATURAS E SIGLAS

Abs	absorbância
APS	persulfato de amônio
AT	antitrombina
bFGF	fator de crescimento de fibroblastos básico
EDAC	1-etil-3-(3-dimetilaminopropil) carbodiimida
Fe^{+2}	íon ferroso
Fe^{+3}	íon férrico
FN	fibronectina
GAG	glicosaminoglicano
GlcA	ácido glucurônico
GlcN	glucosamina
HCl	ácido clorídrico
IdoA	ácido idurônico
MAG	Partículas de magnetita pura
HEP	Heparina
HS	Heparan sulfato
PET	Polietilenotereftalato
PANI	Polianilina
mPET	Polietilenotereftalato magnetizado
mPET-HEP	Polietilenotereftalato magnetizado e heparina imobilizada
mPET-HS	Polietilenotereftalato magnetizado e heparan sulfato imobilizado
mPANI	Partículas de magnetita revestidas com polianilina
mPANI-HEP	Magnetita revestida com polianilina e heparina imobilizada
mPANI-HS	Magnetita revestidas com polianilina e heparan sulfato imobilizado
MAG-HEP	Magnetita com heparina imobilizada
SDS	Dodecil sulfato de sódio
PAGE	Gel de Poliacrilamida
nm	Nanômetro
µm	Micrômetro
°C	Grau celsius
pH	Potencial hidrogeniônico
Tris	Tris (hidroximetil) aminometano
IV	Infravermelho
FTIR	Fourier Transform Infrared
λ	Comprimento de onda
Oe	Oersted
cm^{-1}	número de ondas
mg	miligrama
g	grama
min	minuto
s	segundo
h	hora
M	molar
mM	milimolar
mL	mililitro
µL	microlitro
µmol	micromol

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1 Introdução

Imobilização de biomoléculas consiste em um método pelo qual elas são quimicamente ou fisicamente aprisionadas em um suporte insolúvel ou solúvel em água. A insolubilidade em água, conferida a estas biomoléculas após a imobilização, torna este material facilmente removível do meio de incubação, possibilitando a obtenção de produtos isentos de contaminação e com uma maior estabilidade mecânica. Além disso, a biomolécula imobilizada pode reter parte ou a totalidade de suas propriedades biológicas (TWEEDDALE e REDMOND, 1998; KORECKÁ et al., 2005).

A heparina e o heparan sulfato (HS) são polissacarídeos lineares sulfatados pertencentes à família dos glicosaminoglicanos (GAGs). A heparina tem seu uso bastante difundido na clínica devido à sua propriedade anticoagulante (VISKOV et al., 2013). Além disso, é bastante conhecida por apresentar afinidade com várias proteínas, as chamadas “proteínas de ligação à heparina”, tais como o fator de crescimento de fibroblastos básico (bFGF), fator de crescimento endotelial vascular (VEGF), a fibronectina (FN) e antitrombina (AT), além de desempenhar um importante papel na regulação da atividade e estabilidade destas proteínas (RODGERS et al., 2009; OLSON et al., 2010; ARISAKA et al, 2013). Outras proteínas biologicamente ativas interagem com a heparina, tais como, alguns fatores da coagulação sanguínea, lipases, etc. (KARLSSON e WINGE, 2004). Essas interações desempenham papéis importantes nos processos fisiológicos, bem como nos processos patológicos (OLAD e NABAVI, 2007). O heparan sulfato, por seu turno, devido à sua grande diversidade estrutural, também é capaz de vincular e interagir com uma grande variedade de proteínas: fatores de crescimento, quimiocinas, componentes da matriz extracelular, DNA e RNA polimerases, dentre outras. (FURUKAWA e BHAVANANDAN 1983; AVIEZER e YAYON 1994; KOOPMANN et al. 1999; SASISEKHARAN et al. 2002).

A mudança do microambiente ao redor das biomoléculas imobilizadas pode ocasionar mudanças nas suas propriedades, quando as mesmas são comparadas à sua forma nativa (ARAÚJO et al., 1997; GILL & BALLESTEROS, 2000). Assim, novas metodologias são constantemente propostas para a imobilização destes componentes biológicos, uma vez que nenhuma matriz é universal ou perfeita, ou seja, não há suporte que preserve totalmente a atividade da biomolécula (WEETALL & LEE, 1989; HERMANSON et al., 1992; BARBOSA et al., 2000; BUCUR et al., 2004).

A separação de biomoléculas de seus contaminantes ainda não é uma tarefa trivial. Existem vários métodos de purificação e aqueles baseados na afinidade entre uma biomolécula e seu ligante são os mais específicos. A adsorção por afinidade é uma ferramenta bastante útil na purificação de proteínas. Contudo, métodos preparatórios são normalmente utilizados para eliminar materiais particulados que podem obstruir as colunas clássicas de leito empacotado. Dessa forma, técnicas alternativas de separação por afinidade têm sido sugeridas para contornar esse problema e dentre essas se encontram as separações utilizando partículas magnéticas. Sendo a heparina um ligante já bem conhecido das cromatografias de afinidade, e a versatilidade de interações entre o heparan sulfato com diversas proteínas, o presente trabalho descreveu a síntese e caracterização de partículas ferromagnéticas e a imobilização destes dois glicosaminoglicanos no Dacron ferromagnético e em magnetita revestida com Polianilina (PANI) além de estudar o comportamento destes derivados para separação/purificação de proteínas usando o método de afinidade.

Capítulo 1

2 Revisão da Literatura

2.1 Imobilização de Biomoléculas

Imobilização é o método pelo qual moléculas (por exemplo, fármacos, carboidratos, ácidos nucléicos e proteínas) são quimicamente ou fisicamente aprisionadas em um suporte insolúvel ou solúvel em água. Dessa forma, a utilização da biomolécula torna-se economicamente viável e de grande interesse industrial. Além disso, a fixação a matrizes inorgânicas combina a alta seletividade das reações com as particularidades químicas e físicas do suporte (KONG e FUN HU, 2012).

Três aspectos da imobilização devem ser observados: método de imobilização (tendo em vista o objetivo da imobilização), natureza do suporte (apresentação do suporte tendo em vista suas características físico-químicas e mecânicas) e molécula a ser imobilizada (grupamentos funcionais disponíveis).

BICKERSTAFF (1997) cita os principais modos de interação entre as biomoléculas a serem imobilizadas com a respectiva matriz: formação de redes interpenetradas (enclausuramento), adsorção física, ligação cruzada, encapsulamento e ligação covalente (Figura 1) e considerações acerca do suporte e métodos de imobilização (Tabela 1). Dentre as técnicas de imobilização conhecidas pode-se citar: adsorção, onde há uma interação física não-específica entre a biomolécula e a superfície do suporte; oclusão ou aprisionamento em um suporte semipermeável ou no interior de polímeros; reações cruzadas ou cross-linking que consiste na ligação das próprias biomoléculas entre si formando uma malha que também pode ser insolúvel; e ligação covalente. Esta última apresenta como principais vantagens uma baixa lixiviação da biomolécula imobilizada e elevada estabilidade do derivado obtido. Diante disto, é muito importante escolher um método de imobilização que conserve grande parte das características da biomolécula imobilizada, pois uma simples alteração no microambiente destas biomoléculas imobilizadas pode levar a mudanças em suas propriedades (GUISAN, 2006; TWEEDDALE & REDMOND, 1998; KORECKÁ et al., 2005).

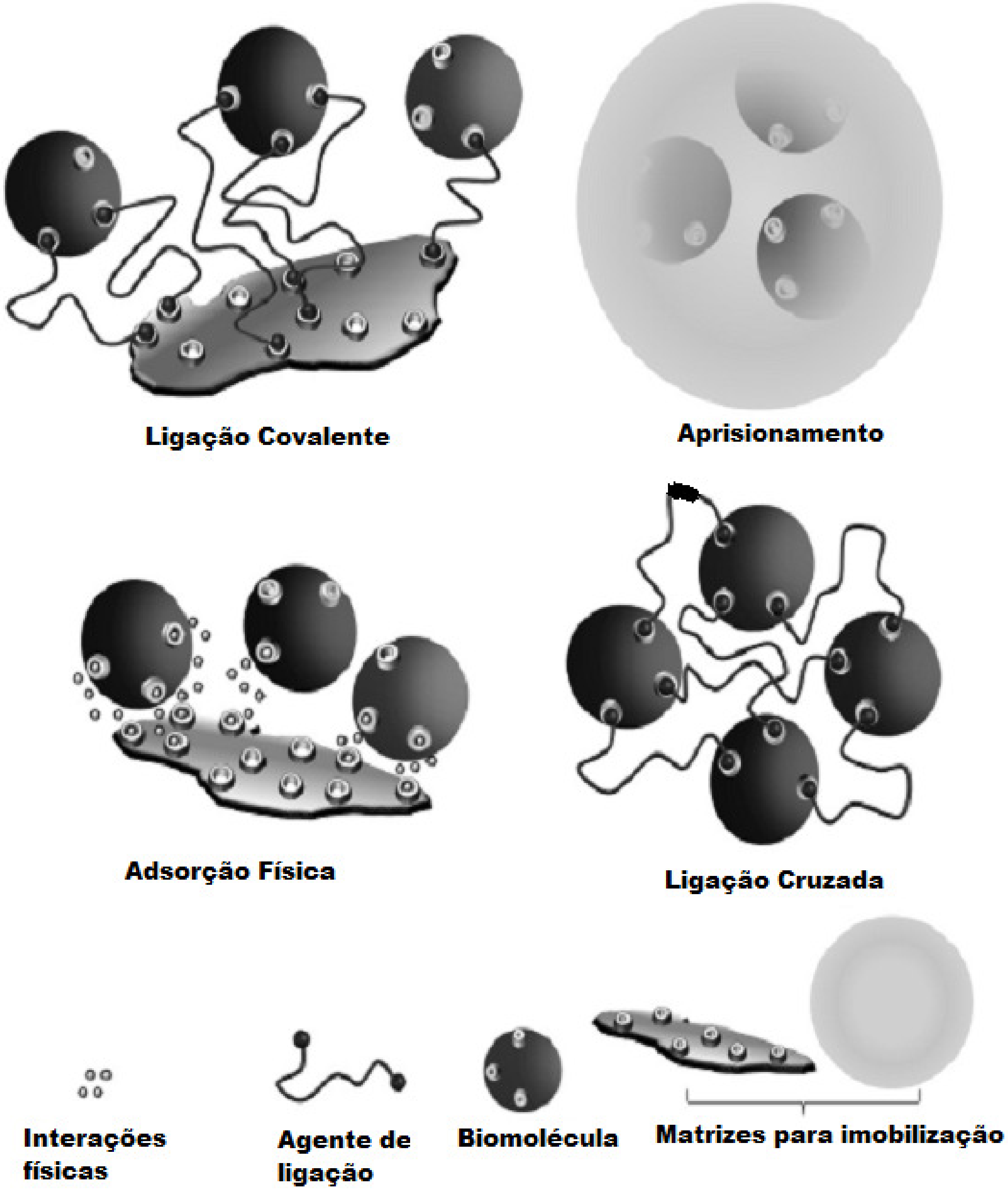

Figura 1. Métodos de imobilização de biomoléculas (adaptado a partir de Bickerstaff, 1997).

Tabela 1. Considerações fundamentais na seleção do suporte para imobilização (BICKERSTAFF, 1997).

Propriedades	Pontos para consideração
Física	Resistência, não compressão das partículas, área de superfície disponível, forma (partículas esféricas/lâminas/fibras), grau de porosidade, volume do poro, permeabilidade, densidade e vazão.
Química	Hidrofilicidade (adsorção de água pelo suporte), neutralidade em relação à biomolécula, disponibilidade de grupos funcionais para modificação e regeneração/reutilização do suporte.
Estabilidade	Estocagem, atividade residual da enzima, regeneração da atividade enzimática (reutilização), estabilidade mecânica do material do suporte.
Resistência	Ataque microbiano, rompimento por agentes químicos, pH, temperatura e solventes orgânicos.
Segurança	Biocompatibilidade, toxicidade dos componentes e reagentes, saúde e segurança dos operadores do processo e dos usuários do produto, especificações da preparação imobilizada para aplicações em alimentos, farmacêuticas e médicas.
Economia	Disponibilidade e custos do suporte, reagentes químicos e equipamentos, habilidade técnica requerida, impacto ambiental, preparação em escala industrial, possibilidade para aumentar a produção, processamento contínuo, reutilização do suporte, nível de risco calculado ou contaminação zero.
Reação	Vazão, carregamento do suporte com a enzima e produtividade catalítica, cinética da reação, reações laterais, sistema de múltiplas enzimas, tipo de reator, limitações de difusão e transferência de massa de cofatores, substratos e produtos.

Entre os vários processos citados na tabela 1 para imobilização de biomoléculas, o emprego da imobilização por ligação covalente é o que está associado a uma maior estabilidade. Nesse método ocorre uma reação química entre grupos funcionais da molécula e grupos reativos presentes no suporte.

A heparina e o heparan sulfato apresentam grupos funcionais (sulfatos e carboxilatos) reativos, o que proporciona a sua fácil imobilização a uma matriz, por isso o seu uso tem sido alvo de pesquisas na área de imobilização de biomoléculas (MURUGESAN et al., 2008).

Para que ocorra a ligação covalente da heparina e do heparan sulfato, primeiramente é necessário que seus grupos carboxílicos passem por um processo de ativação através da adição de EDAC (***1-etil-3-*(*3-dimetilaminopropilcarbodiimida***), Figura 2. O EDAC ativa a heparina levando à formação do *o*-acilureia, um composto intermediário, que apresenta um grupamento éster bastante reativo e fácil de sofrer hidrólise. Para resolver esse problema é adicionado o NHS (N-hidroxi-succinamida) que vai reagir com o grupo éster do composto intermediário deixando-o mais estável. Logo, em presença de um grupamento amino do suporte, este irá reagir com a carbonila do éster formando uma ligação amida (OLIVEIRA et al., 2003).

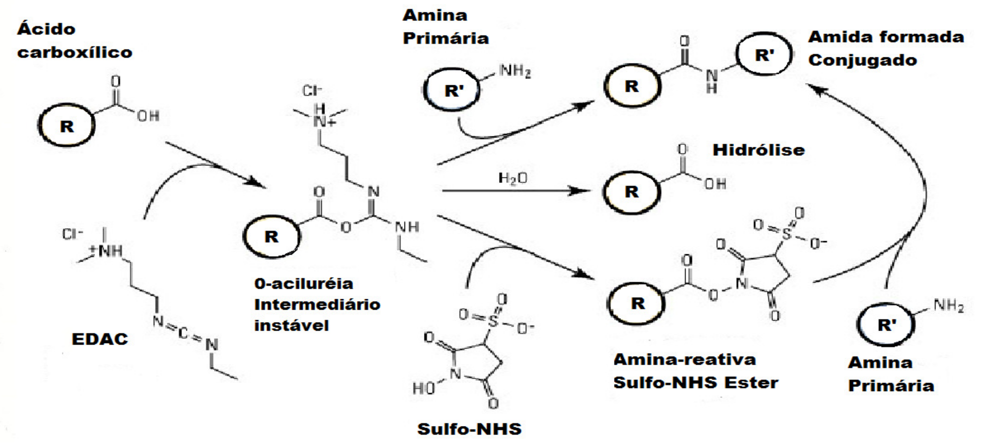

Figura 2. Esquema representativo da reação de ativação dos grupamentos carboxílicos através do tratamento com a carbodiimida (EDAC) e o N-hidroxi-succinamida (Sulfo-NHS); (adaptada do livro: Bioconjugate Techniques; 2ª Edição).

Quando imobilizada, a heparina pode interagir com fatores da coagulação, funcionando como um ligante de afinidade capaz de interagir com proteínas. Além disso, permitir a troca de cátions de alta capacidade devido à presença de grupos sulfatos aniônicos em sua estrutura (KRAPFENBAUER e FOUNTOULAKIS, 2009). Nesse contexto, o uso de heparina imobilizada a um suporte tem sido ferramenta para a produção de dispositivos biomédicos. Tais materiais são geralmente usados em cirurgias cardíacas para alcançar efeitos antitrombogênicos, baixo grau de ativação do complemento e redução da adesão bacteriana

(YE et al, 2013). Superfícies heparinizadas também mostram redução da adesão plaquetária, aumento do tempo de recalcificação do plasma e do tempo de tromboplastina parcialmente ativada (MURUGESAN et al, 2008).

2.2 Suporte Magnético

Um suporte ou matriz é qualquer material ao qual um ligante bioespecífico pode ser ligado. Escolher a melhor matriz para uma determinada aplicação, ocasionará um uso mais eficiente e otimizado da mesma. Ao escolher um suporte, duas premissas devem ser devidamente observadas: as suas propriedades físico-químicas e a possibilidade de regeneração do material (MACIEL, 2012). Os suportes utilizados na imobilização de biomoléculas tendem a possuir um bom perfil como resistência física, insolubilidade, resistência a ataques microbianos, além de estabilidade mecânica e térmica (CARAMORI e FERNANDES, 2004). E, quando magnetizados, a recuperação do compósito suporte-biomolécula pode facilmente ser obtida mediante a aplicação de um campo magnético.

As partículas magnéticas foram empregadas pela primeira vez na década de 1940 como uma nova tecnologia no tratamento de água poluída (ARIAS et al., 2001). Nos dias atuais, a síntese e aplicação das partículas magnéticas funcionalizadas vêm despertando grande interesse em diversas áreas (WU et al., 2008; HAO et al., 2010). No entanto, deve haver um cuidado ao escolher o suporte para determinada biomolécula, pois o suporte usado na imobilização pode afetar, significantemente, as características do material imobilizado (SRIVASTAVA et al., 2001), bem como a carga na superfície do carreador (GODJEVARGOVA et al., 2003). A imobilização na face externa do suporte é um dos mais eficientes meios de diminuir a resistência de deslocamento de massa. Um braço espaçador entre o suporte e a biomolécula pode diminuir as mudanças na estrutura desta, e torná-la mais flexível, levando, portanto, a um maior potencial de estabilidade de suas atividades (SRIVASTAVA et al., 2001).

Sendo as técnicas de imobilização de enzimas e carboidratos em diferentes suportes magnéticos uma das principais linhas de pesquisa desenvolvida no setor de Bioquímica do Laboratório de Imunopatologia Keizo Asami (LIKA), a tabela 2 apresenta alguns dos derivados bioativos, matrizes e respectivas biomoléculas imobilizadas desenvolvidos na UFPE nos últimos dez anos pelo grupo de pesquisa do LIKA.

Tabela 2. Principais derivados bioativos desenvolvidos na UFPE com suas respectivas biomoléculas imobilizadas.

Suporte	**Biomolécula Imobilizada**	**Referência Bibliográfica**
Dacron	Antígeno (SWAP) de *Schistosoma mansoni*	Montenegro et al., 1999
Papel de filtro	Albumina de Soro Humano	PINHEIRO et al., 1999
Plastificado com PVA*-glutaraldeído	Antígeno de *Yerssinia pestis* β-galactosidase	BARBOSA et al., 2000 NERI et al., 2009
Compósitos de polianilina e Dacron	Antígeno de *Toxocara canis* Tripsina	COELHO et al., 2001 MARCIEL et al., 2012
Compósito de polisiloxano e álcool polivinílico	Antígenos de *Trypanossoma cruzi* e *Schistosoma mansoni*	BARROS et al., 2002 COÊLHO et al., 2003 COELHO et al., 2002
Argila Magnética	Invertase	CABRERA et al., 2013
Diatomito Magnético	Invertase	CABRERA et al., 2013

* - PVA – álcool polivinílico

Dentre vários procedimentos sintéticos desenvolvidos para se obter partículas magnéticas, o procedimento mais barato, simples e até ecologicamente correto é o método de coprecipitação, o qual envolve a precipitação simultânea de íons Fe^{+3} e Fe^{+2} em meio básico e aquoso (KANG et al., 1996; QU et al., 1999).

Os materiais magnéticos modificados são constituídos por um núcleo de óxido de ferro revestido com um polímero. Esse revestimento possui grupos ativos que podem ser conjugados a biomoléculas tais como carboidratos, proteínas e enzimas (MA et. al, 2003; YAMAURA et al., 2004). As partículas de magnetita superparamagnéticas revestidas com polímeros são usualmente formadas por núcleos magnéticos responsáveis por uma resposta magnética forte e uma camada polimérica para fornecer grupos funcionalizáveis e característicos (WUNDERBALDINGER et al., 2002).

2.2.1 Dacron

Dacron, marca registrada para uma fibra de poliéster, é um polímero formado pela condensação do etilenoglicol (um diálcool) com o ácido tereftálico (ácido dicarboxílico). Esta condensação (esterificação) forma um longo poliéster chamado polietilenotereftalato ou PET (Figura 3). Utiliza-se principalmente na forma de fibras para tecelagem e de embalagens para bebidas, além de próteses e suturas (Figura 4). Pode ser encontrado nos mais diversos formatos, mais freqüentemente em pellet e folhas.

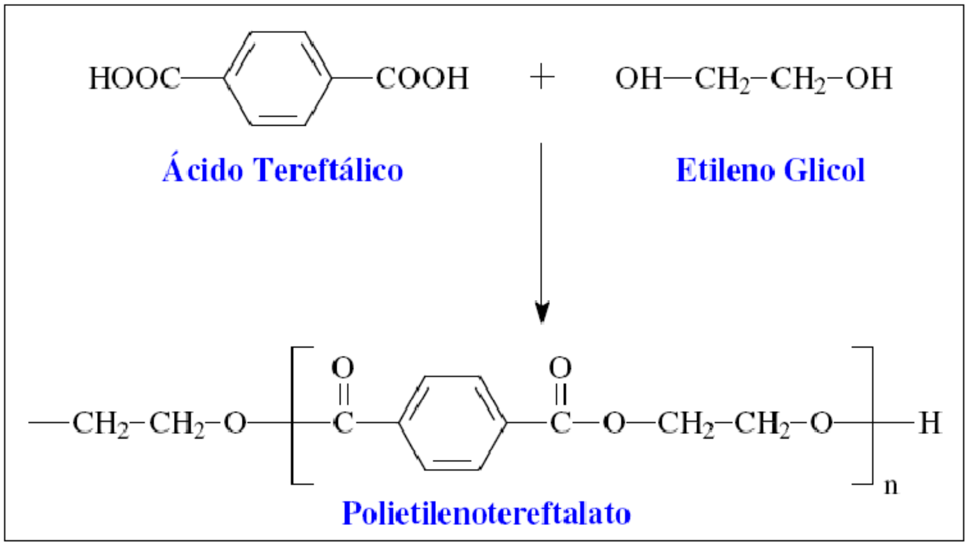

Figura 3. Síntese de Polietilenotereftalato a partir da reação de esterificação entre o ácido tereftálico e o etilenoglicol.

Figura 4. Aplicações do PET na tecelagem (A), embalagem (B), e prótese no tronco venoso braquiocefálico esquerdo (C). Fontes: (A) – www.asdivers.com.br; (B) – www.in.com.br; (C) – www.jornaldepneumologia.com.br. Sites acessados no dia 08/10/13.

Este poliéster também vem sendo empregado como matriz na imobilização de biomoléculas, pois possui um bom perfil como resistência física, insolubilidade, resistência a ataques microbianos além de estabilidade mecânica e térmica (CARAMORI & FERNANDES, 2004).

Para que ocorra a imobilização a este suporte é necessário que ele seja funcionalizado, ou seja, apresente grupos funcionais disponíveis para a imobilização. A primeira funcionalização do Dacron foi demonstrada por Weetall (1970) que propôs quatro reações para introduzir grupos aril-azidas para imobilização de L-asparaginase. A reação de Passerini também já foi empregada para imobilizar enzimas ao Dacron (GOLDSTEIN et al*.,* 1977). A reação de Passerini foi descrita em 1921 e é uma reação multicomponente clássica entre ácidos carboxílicos, aldeídos ou cetonas e isocianetos, conduzindo a interessantes compostos peptidomiméticos.

Melo (1984) descreveu uma metodologia para converter folhas de Dacron em um pó, num processo chamado hidrazinólise, onde o polímero é clivado e sofre uma redução em seu peso molecular sendo convertido em pó. E no intuito de facilitar a recuperação do suporte, Carneiro-Leão et al., (1991) propuseram a magnetização destas partículas, o que tornou este processo fácil e rápido com o auxílio de um campo magnético. (Figura 5 e 6).

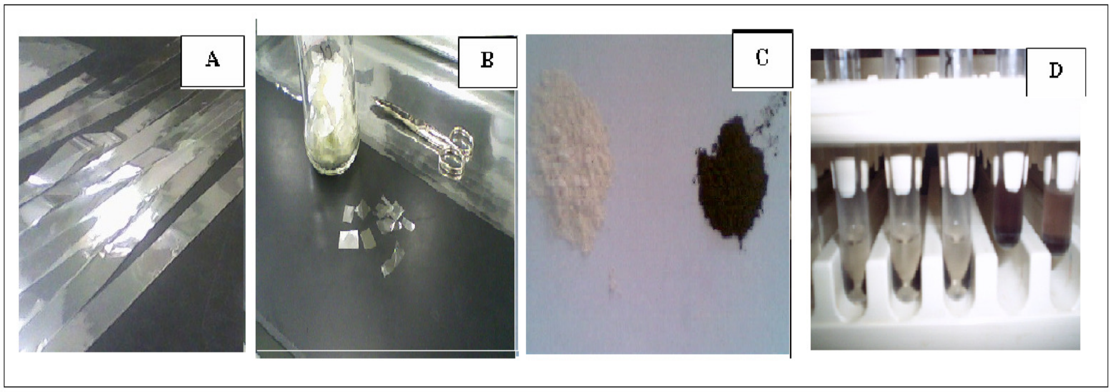

Figura 5. Conversão das folhas de Dacron em pó e sua posterior magnetização. (A) Dacron em forma de fitas; (B) Fitas de Dacron cortadas pedaços; (C) Pó de Dacron após o processo de hidrazinólise de coloração branco-amarelada (esquerda) e seu derivado magnetizado (direita) de coloração negra; (D) Recuperação do Dacron com o auxílio de campo magnético.

O pó obtido é mecanicamente resistente e não biodegradável. Essas propriedades o qualificam como um suporte adequado para imobilização. E quando magnetizado possibilita sua rápida separação de uma mistura reacional.

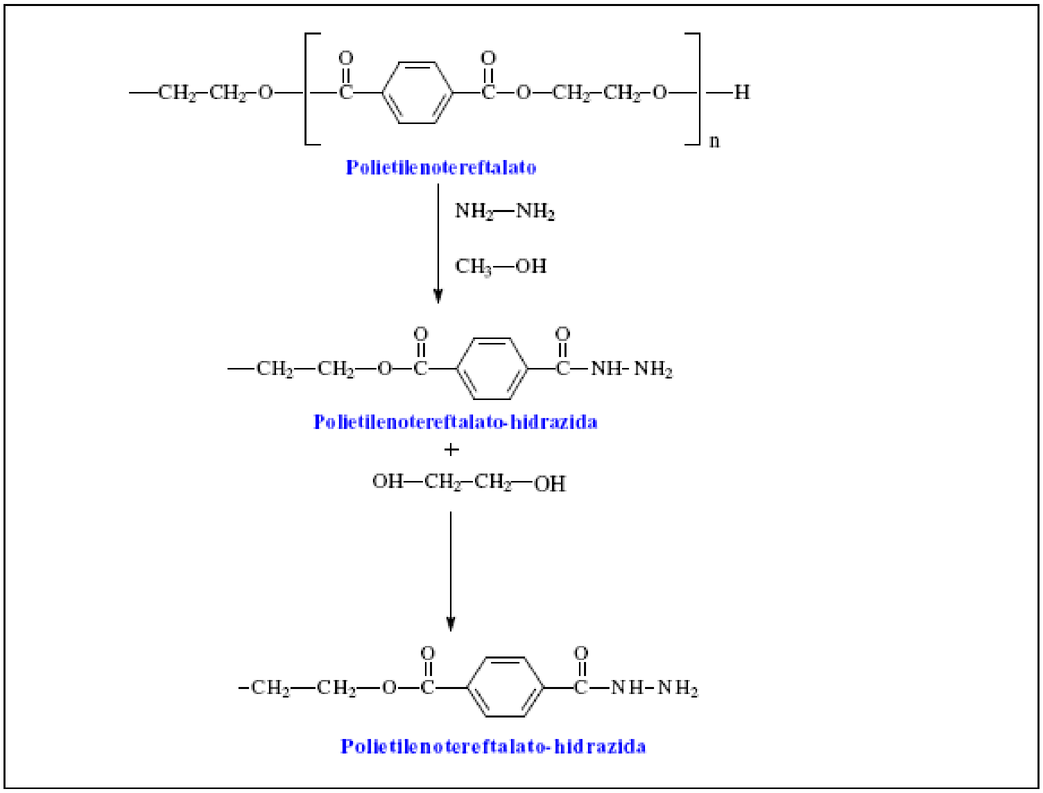

Figura 6. Funcionalização do Dacron com grupos hidrazida.

2.2.2 Polianilina

Polianilina (PANI) é um polímero condutor da família dos polímeros flexíveis. Apesar de ter sido descoberta há mais de 150 anos, só recentemente a polianilina recebeu a atenção da comunidade científica devido à descoberta de sua alta condutividade elétrica. Entre a família de polímeros condutores, polianilina é única devido à sua facilidade de síntese e estabilidade ambiental. Embora os métodos de síntese para produzir polianilina sejam bastante simples, o seu mecanismo de polimerização e a natureza exata de sua química de oxidação são bastante complexas. Devido à sua química rica, a polianilina tem sido um dos polímeros mais estudados nos últimos 20 anos. Dentre esses estudos, temos seu uso no revestimento de compósitos: imobilização de horseradish peroxidase (HRP) em magnetita revestida com PANI e ativada com glutaraldeído (BARBOSA et al, 2012); tripsina imobilizada em polivinil-alcool revestido também com PANI e tratado com gluteraldeído (CARAMORI et al, 2011).

A PANI é um polímero que apresenta maior estabilidade química na sua forma condutora, facilidade de polimerização e baixo custo do monômero (COELHO et al., 2001). O revestimento de uma matriz magnética com polianilina, por exemplo, devido a sua fácil síntese (eletroquimicamente ou mediante uso de agentes oxidantes), torna-a atrativa para aplicação como matriz de imobilização de moléculas (FERNANDES et al., 2003; NGAMNA et al., 2005; SINGH et al., 2006). A PANI pode ocorrer em diferentes estados de oxidação (Figura 7), dos quais a forma sal de esmeraldina, 50% oxidada, é a mais estável (MACIEL, 2012).

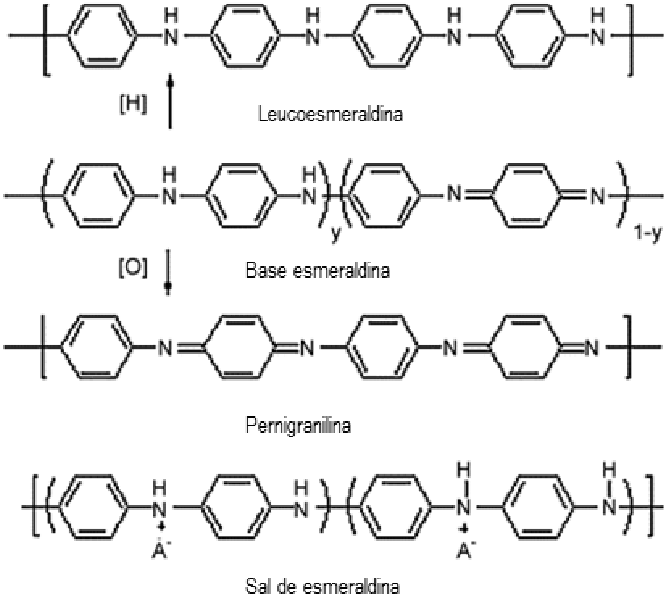

Figura 7. Diferentes estados de oxidação da polianilina. *Fonte: CHI, et al., 2009.*

2.3 Heparina

A heparina é um polissacarídeo com propriedades biológicas e químicas singulares, amplamente conhecido como uma droga anticoagulante. Ela foi descoberta em 1916 por Jay McLean, um estudante de medicina da Johns Hopkins University. Contudo, maiores detalhes sobre sua estrutura só foram desvendados em 1968. Assim, finalmente a heparina foi denominada como um polissacarídeo linear sulfatado, formado por unidades dissacarídicas contendo ácido idurônico e glicosamina. (NADER et al., 2001; CAPILA e LINHARDT, 2002; PETITOU et al., 2003).

Atualmente sabe-se que a heparina é um polímero linear, consistindo de unidades repetitivas de resíduos de ácido piranosilurônico e 2-amino-2-desoxiglicopiranose (glicosamina) ligados por ligações 1→4 (Figura 8). Os resíduos de ácido urônico consistem tipicamente de 90% de ácido L-idurônico e 10% de ácido D-glicurônico. A heparina possui a maior densidade de carga negativa que qualquer macromolécula biológica e isto é um resultado do alto conteúdo de grupos sulfonatos e carboxilatos. A estrutura mais comum que ocorre na heparina é o dissacarídeo trissulfatado. Entretanto, há uma variação estrutural deste dissacarídeo, o que leva a uma microheterogeneidade da heparina. O grupo amino do resíduo de glicosamina pode ser substituído com grupos acetil ou sulfonato ou não substituído. As posições 3 e 6 dos resíduos de glicosamina podem ser substituídas com um grupo O-sulfonato ou não substituídas. O ácido urônico pode conter também um grupo 2-O-sulfonato. Além disso, o glicosaminoglicano heparina tem uma faixa de peso molecular de 5-40 kDa, com um peso molecular médio de aproximadamente 15 kDa e uma carga negativa média de aproximadamente –75 (NADER et al., 2001; CAPILA & LINHARDT, 2002; PETITOU et al., 2003).

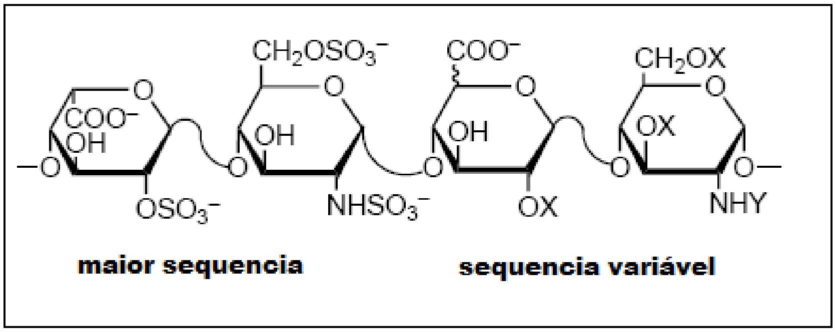

Figura 8. Repetição das unidades dissacarídicas na heparina (X = H ou SO_3^-, Y = Ac, SO_3^-, ou H).

A heparina é conhecida como sendo o anticoagulante mais comumente utilizado na clínica (MENG et al., 2009). Ao longo das últimas décadas, foi mostrado que a heparina está envolvida em muitos processos biológicos através da sua interação com um elevado número de proteínas (LEVER e PAGE, 2012). E que devido a sua estrutura altamente carregada, também está envolvida na regulação de eventos importantes como a sinalização celular e o controle de uma variedade de funções biológicas (HANSEN et al., 2013).

2.3 Heparan Sulfato

O heparan sulfato possui uma estrutura química semelhante à da heparina (Tabela 3). O heparan sulfato também tem seu grupo N-Glicosamina, N-acetilado ou N-sulfatado. Entretanto, na heparina, o grupo N-acetil corresponde a menos que 5%. Desta forma, a heparina demonstra possuir um maior grau de sulfatação (2,3 - 2,8 sulfato/dissacarídeo) quando comparado ao heparan sulfato (0,6 - 1,5 sulfato/dissacarídeo).

Diversos trabalhos na literatura demonstram claramente um alto número proteínas biologicamente ativas que podem interagir com o heparan sulfato. O heparan, devido à sua grande diversidade estrutural é capaz de vincular e interagir com uma grande variedade de proteínas: fatores de crescimento, quimiocinas, componentes da matriz extracelular, DNA e RNA polimerases, fatores da coagulação dentre outras. (AVIEZER e YAYON 1994; KOOPMANN et al., 1999; SASISEKHARAN et al., 2002).

Tabela 3. Diferenças estruturais entre a heparina e o heparan sulfato; (SAMPAIO et al, 2006).

GAG	**Dissacarídeos**		
	Ácido Urônico	**Ligação**	**Glicosamina**
Heparan Sulfato	D-GlcA	β1 → 4	D-GlcN-Ac
	D-GlcA	β1 → 4	D-GlcN-Ac (6S)
	D-GlcA	β1 → 4	D-GlcN (S)
	D-GlcA	β1 → 4	D-GlcN (S, 6S)
	L-IdoA	α1 → 4	D-GlcN (S)
	L-IdoA (2S)	α1 → 4	D-GlcN (S)
Heparina	D-GlcA	α1 → 4	D-GlcN (S)
	D-GlcA	α1 → 4	D-GlcN (S, 6S)
	L-IdoA (2S)	β1 → 4	D-GlcN (S)
	L-IdoA (2S)	β1 → 4	D-GlcN (S, 6S)

2S: 2-O-Sulfatado; 6S: 6-O-Sulfatado; S: N-Sulfatado

2.4 Proteínas de ligação aos Glicosaminoglicanos (GAGs)

Mais de 100 proteínas de ligação à glicosaminoglicanos (GAGs) foram descritas na literatura, alguns exemplos estão representados na Tabela 4. Uma maior extensão desses estudos está direcionada às interações entre essas proteínas e à heparina e ao heparan sulfato, os quais são altamente sulfatados (ESKO e LINHARDT, 2009).

Tabela 4. Exemplos de proteínas de ligação aos glicosaminoglicanos (GAGs) e a sua atividade biológica. *Fonte: adaptado de Esko e Linhardt, 2009.*

Classe	**Exemplos**	**Efeitos fisiológicos e/ou patológicos da ligação**
Enzimas	Enzimas de biossíntese de glicosaminoglicanos, trombina e fatores de coagulação (proteases), proteínas do complemento (esterases), superóxido dismutase extracelular, topoisomerase.	Múltiplos.
Inibidores enzimáticos	Antitrombina, cofator II da heparina, inibidor da protease dos leucócitos, inibidor de C1 esterase.	Coagulação, inflamação, regulação do complemento.
Proteínas de adesão celular	P-selectina, a L-selectina, algumas integrinas.	Adesão celular, inflamação, metástase.
Proteínas da matriz extracelular	Laminina, fibronectina, colágeno.	Adesão celular, organização de matriz.
Quimiocinas	Fator plaquetário IV, γ-interferon, interleucinas.	Quimiotaxia, sinalização, inflamação.
Fatores de crescimento	Fatores de crescimento de fibroblastos, fator de crescimento de hepatócitos, fator de crescimento endotelial vascular.	Mitogênese, migração celular.
Proteínas ligadoras de lipídeos	Apolipoproteínas E e B, lipase lipoprotéica, lipase hepática, anexinas	Metabolismo lipídico, funções da membrana celular.
Proteínas nucleares	Histonas, fatores de transcrição.	Desconhecido.
Proteínas de superfície do patógeno	Proteína da malária circunsporozoíto.	Infecções por patógenos.
Proteínas do envelope viral	Vírus herpes simplex, vírus da dengue, vírus da imunodeficiência humana, vírus da hepatite C.	Infecção viral.

As chamadas “proteínas de ligação à heparina”, como o próprio nome sugere, apresentam uma elevada afinidade em interagir com a heparina, tais como o fator de crescimento de fibroblastos básico (bFGF), fator de crescimento endotelial vascular (VEGF), a fibronectina (FN) e antitrombina (AT). Além disso, a heparina desempenha importante papel na regulação da atividade e estabilidade destas proteínas (ARISAKA et al., 2013). Outros exemplos de proteínas biologicamente ativas que interagem com a heparina, e também com heparan sulfato, são: alguns fatores da coagulação sanguínea, lipases, inibidores enzimáticos, etc. (KARLSSON e WINGE, 2004; GRUNERT et al., 2008). Essas interações desempenham papéis importantes nos processos fisiológicos, bem como nos patológicos (OLAD e NABAVI, 2007).

Os sítios de ligação nas proteínas sempre contêm aminoácidos básicos (Lys e Arg) cujas cargas positivas, provavelmente, interagem com os grupamentos sulfatos e carboxilatos (carregados negativamente) presentes nas cadeias dos GAGs (ESKO et al., 2009). A interação da heparina com a antitrombina foi o primeiro caso relatado de uma interação de significado fisiológico entre a heparina e uma proteína específica (CAPILA e LINHARDT, 2002). Sabe-se que a heparina liga-se à molécula da antitrombina (Figura 9), aumentando em mais de 1.000 a 2.500 vezes sua função de inibir a cascata da coagulação, através da inibição da formação da trombina. A AT possui uma região central que consiste em 3 folhas-β, α-hélices e ainda uma alça reativa em sua superfície (r*eactivesite loop)*. Este local, por sua vez, é onde a antitrombina liga-se à protease-alvo de acordo com o mecanismo de ação das serpinas: resíduos de arginina liga-se covalentemente à serina ou cisteína da protease resultando na formação do complexo intermediário acila, que irá aumentar sua susceptibilidade à proteólise por parte da enzima, facilitando sua depuração (OLSON et al, 2010). Em sua forma latente inativa, a AT circula com seu loop reativo não exposto. Uma vez ligado à heparina, a AT sofre uma mudança conformacional e se transforma em uma forma ativada permitindo a exposição do sítio reativo e assim aumentando sua reatividade (ASMAL et al, 2012).

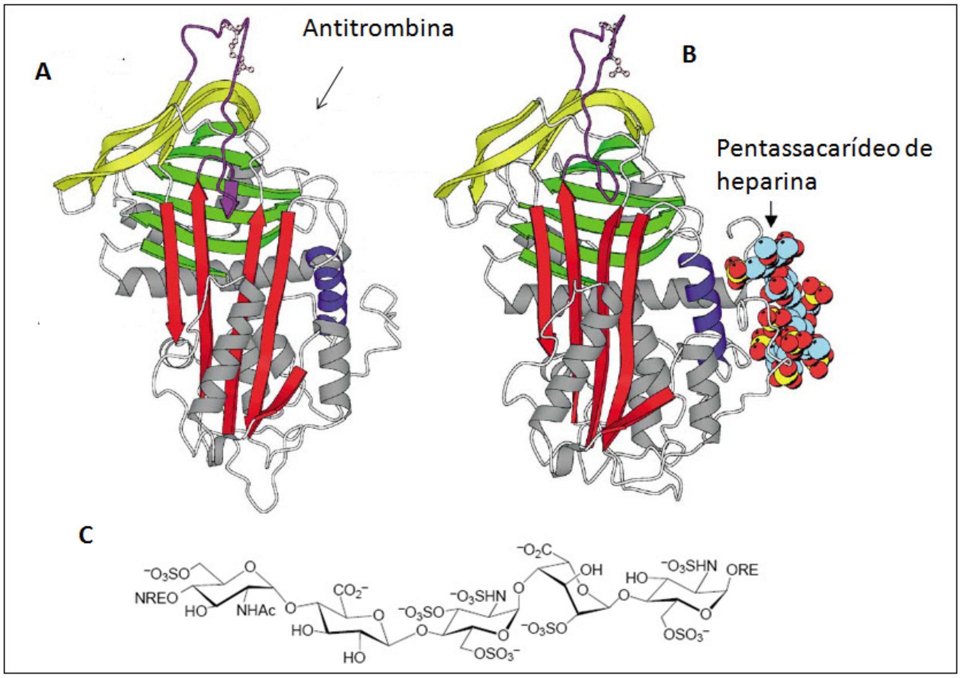

Figura 9. A – Estrutura da antitrombina; B – Antitrombina complexada com uma sequência pentassacarídica de heparina; C – Sequência pentassacarídica da heparina essencial para ocorrer a interação heparina-AT. *Fonte: http://en.academic.ru/pictures/enwiki/65/Antithrom%2Bheparin.jpeg*

Quando imobilizados, a heparina e o heparan sulfato, em decorrência da semelhança estrutural, podem interagir com fatores da coagulação, funcionando como um ligante de afinidade capaz de interagir com proteínas. Além disso, permitir a troca de cátions de alta capacidade devido à presença de grupos sulfatos aniônicos em sua estrutura (KRAPFENBAUER e FOUNTOULAKIS, 2009).

2.5 Adsorção por afinidade à heparina e ao heparan Sulfato (HS)

Existe uma gama de materiais heparinizados segundo a literatura com diversas aplicações no ramo da biotecnologia. Podemos citar algumas destas publicações: em polidimetilsiloxano (THORSLUND et al, 2005); em enxertos de politetrafluoretileno para cirúrgia vascular (HEYLIGERS et al, 2006); em superfície de polietileno (KONDO et al, 2008); superfície de titânio com heparina revestida (HUH et al, 2011); em dacron e polietileno tereftalato (CHANDY et al, 2000); em matriz de celulose (MURUGESAN et al, 2008); imobilização de heparina em nanopartículas (nanotubos de carbono, óxidos de ferro, sílica, fosfato de cálcio e pontos quânticos) (MURUGESAN et al, 2008; XING et al, 2010). Um outro emprego é verificar a afinidade de ligação entre heparina e proteínas. A heparina imobilizada em suportes sólidos é amplamente utilizada para a purificação de proteínas de ligação a glicosaminoglicanos (MURUGESAN et al., 2008).

Posto isto, a adsorção por afinidade à heparina e ao heparan sulfato é um método bastante utilizado para fracionar ou purificar proteínas e outras substâncias biológicas mediante a interação (afinidade) entre as moléculas de interesse (Figura 10) e esses GAGs imobilizados ao suporte insolúvel (XIONG et al., 2008).

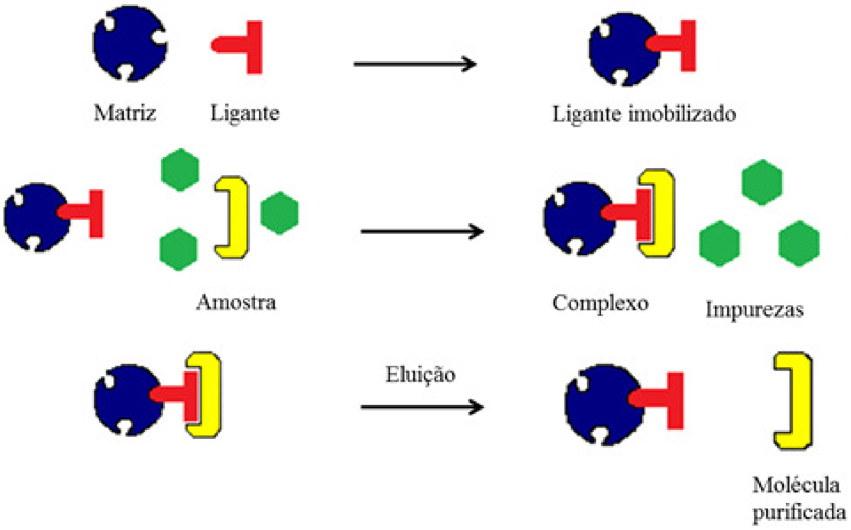

Figura 10. Princípios da adsorção por afinidade.

A heparina já vem sendo utilizada para o isolamento de proteínas plasmáticas e de membrana, sendo a utilização mais frequente o isolamento da antitrombina a partir de plasma humano (JOSIC, 1993). A alta afinidade de ligação da heparina e do heparan sulfato a várias proteínas, incluindo fatores de crescimento e enzimas da coagulação sanguínea, tem sido relacionada com a sua carga líquida negativa em pH neutro (FENG et al., 2004; KAWAKAMI et al., 2006; JOHNSON et al., 2010). Além disso, o uso da adsorção por afinidade à heparina pode ser aplicada como uma estratégia para remover seletivamente algumas proteínas de grande abundância, facilitando a análise de proteínas de baixa concentração no plasma. Já é demonstrado que a albumina pode ser removida, por exemplo, através de técnicas de colunas de imunoafinidade, aprisionamento isoelétrico, adsorção por afinidade, etc. (LEI et al., 2008).

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4 Objetivos

4.1 Objetivo Geral

Propor derivados magnéticos de heparina e heparan sulfato com vistas à obtenção de uma matriz de afinidade para purificação de proteínas do plasma.

4.1 Objetivos específicos

- Sintetizar as partículas magnéticas;
- Imobilizar heparina às partículas de PET ferromagnético e magnetita revestida com PANI;
- Determinar a quantidade de heparina e heparan sulfato fixadas às matrizes;
- Caracterizar as partículas magnéticas por análises biológicas, e físico-químicas;
- Utilizar o derivado para separar/purificar proteínas do plasma.
- Identificar as proteínas purificadas.
- Realizar testes in vitro com as proteínas purificadas mediante suas funções biológicas específicas.

Capítulo 2

5 Artigo a ser submetido à revista internacional "Biomaterials"

Título: Ferromagnetic Polyethyleneterephthalate (PET) covered with heparin for affinity separation of antithrombin

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Ferromagnetic Polyethyleneterephthalate (PET) covered with heparin for affinity separation of antithrombin

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Abstract

This work describes the use of ferromagnetic Dacron (polyethyleneterephthalate; PET) coated with heparin for antithrombin purification (affinity binding). PET film was converted to PET-hydrazide powder by methanolic hydrazine hydrate and magnetized by thermal co-precipitation of ferric and ferrous chlorides. Heparin was activated by carbodiimide and N-hydroxysuccinimide and coupled to the magnetic support. The magnetic derivatives remained stable for 24 months demonstrating its stability even after 10 reuses. Elemental and Infrared spectra analyses presented evidences of heparin immobilization. Electrophoresis revealed bands with molecular mass 58 kDa corresponding to that referred to antithrombin. Moreover, laboratory tests detected a low activity of antithrombin in plasmas that were tested by affinity assay to heparin and delay clotting time of plasmas that came into contact with the eluted fractions containing antithrombin. Therefore, the results presented confirm the success of immobilization and affinity separation process.

Keywords: antithrombin, Dacron, heparin, immobilization, polyethyleneterephthalate

1. Introduction

Affinity separation is one of the most powerful techniques in selective purification and isolation of a great number of compounds [1]. This technique has the purification power to eliminate steps, increase yields and improve process economics [2]. Heparin chromatography is an adsorption chromatography in which molecules to be purified are specifically and reversibly adsorbed by heparin immobilized on an insoluble support [3]. In general, heparin chromatography is a method of affinity chromatography used to purify or fractionate biological substances that can interact with heparin. This method has been extensively developed following the progress of chromatographic supports and technologies.

In our laboratory, Dacron or polyethyleneterephthalate, also known as PET and widely used as plastic vessel, has been proposed as a support for biomolecules immobilization [4, 5].

Heparin is a biologically important and chemically unique polysaccharide, widely recognized as an anticoagulant drug. Many studies have demonstrated that heparin has the ability to bind a wide range of biomolecules, including serine protease inhibitors (especially of antithrombin) [6].

In the present study, heparin was immobilized on ferromagnetic PET and used as affinity matrix for purification of antithrombin.

2. Material and methods

2.1. *Materials*

The Dacron (polyethyleneterephthalate) were donated by Terphane Ltda (Cabo de Santo Agostinho, PE, Brazil). Heparin was purchased from Cristália Chemicals & Pharmaceuticals Ltda. Carbodiimide(1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EDAC), N-hydroxysuccinimide (NHS) and hydrazine hydrate were purchased from Sigma-Aldrich Ltda, whereas ferric and ferrous chlorides were from Merck SA. The activated partial thromboplastin time (aPTT) kit was acquired from Labtest Diagnóstica.

2.2. *Ferromagnetic PET-hydrazide synthesis*

Films of PET (4g) were cut in strips and incubated in methanol (100 mL) containing hydrazine hydrate (10 mL) at 40^{o} C for 16 h with stirring. Afterwards the PET-hydrazide (white powder) was washed twice with methanol. The suspension of PET-hydrazide (2 g in 100 mL of water) was allowed to sedimentfor 10 min and the supernatant was decanted to remove fines (five times). Finally, the material was filtered and oven-dried (40^oC) homogenized and sieved (250μm).

PET-hydrazide (2g) was stirred in deionized water (100 mL) and an aqueous solution (10mL) containing $FeCl_3.6H_2O$ (300mg) and $FeCl_2.4H_2O$ (121mg) was added dropwise. The pH and temperature of this mixture were raised up to 11 (with 28%, v/v, NH_4OH) and 100^oC, respectively, and kept for 30 min with stirring. Lastly, ferromagnetic PET-hydrazide (mPET) also was filtered, oven-dried (40^oC), homogenized and sieved (250μm).

2.3. *Heparin immobilization on ferromagnetic PET-hydrazide (mPET).*

N-hydroxysuccinimide (60 mg) and carbodiimide (100 mg) were added to a heparin solution (36 mg in 12 mL) and the solution pH was maintained between 4.5 and 5.0 for 30 min. An aliquot of this mixture (1 mL) was incubated with the mPET (30 mg) for 16 h with mild stirring to form the derivative mPET-HEP (heparin immobilized on ferromagnetic PET). Particles were recovered under a magnetic field (6,000Oe) and washed ten times with deionized water to remove non-immobilized heparin. The supernatants were washed and reserved for heparin determination. Magnetic products were stored in water for 2 years at 4°C for use in two stages: start (time zero) and final (24th month) to study the stability of the support in the purification of antithrombin.

2.4. *Heparin determination.*

Heparin was determined in accordance with the methodology proposed by Oliveira et al. [7]. This method is based on the property of heparin to form complexes with basic dyes such as methylene blue (MB). The dye was prepared with 30 mg of MB in 1 L 0.01N HCl containing 0.2% NaCl. The sample (1 mL) was added to the MB solution (2.5 mL) and the volume was completed to 17.5 mL with water. The mixture was stirred and left to stand for 5 min. Absorbance was measured at 664 nm. The calibration curve was constructed using heparin concentrations from 10 to 100 mg/mL. The immobilized heparin was established by adding MB solution (1 mL) to the mPET-HEP (30 mg) and kept for 5 min under mild stirring. The magnetic particles were collected and the absorbance of the supernatant read at 664 nm. The mPET (30 mg) was used as control. These procedures were repeated four times to evaluate the MB adsorption by support.

2.5. *Physical-chemical analysis*

Infrared spectra and elemental composition of the magnetite, PET-hydrazide, mPET and mPET-HEP, all after 24 months of use, were carried out by using, respectively, a BRUKER instrument model IFS 66 and CHNS-O Carlo Erba model EA 1110. These analyses were made in the Central Analytical of the Departamento de Química Fundamental, Universidade Federal de Pernambuco, Brazil.

2.6. *Antithrombin purification.*

To 30 mg of mPET-HEP matrix was added 1 mL of citrated human plasma. This mixture was kept under mild stirring for 45 minutes at 25^{o}C. The magnetic particles were collected by the magnetic field and the supernatant was discarded. The magnetic particles were washed with 0.01 M phosphate buffer, pH 7.4, until no absorbance at 280 nm was detected. Subsequently the magnetic particles were incubated with the buffer containingincreasing concentrations of NaCl (0.25M; 0.5M, and 1.0 M). The incubation and elution procedures were repeated five times to increase the total mass of proteins to enable the polyacrylamide gel electrophoresis analysis according to Laemmli [8]. For these tests, were used magnetic products prepared at time zero and those stored at 2 years.

2.7. *Anticoagulant activity*

Fifty milligrams of the mPET-HEP and mPET (control) were incubated under mild stirring with 400 µL of 0.15 M NaCl and 500 µL of plasma at 37° C. After 5 min, 100 µL of 1 %, w/v $CaCl_2$ was addedand the time to onset of fibrin registered.

The antithrombotic activity of eluted fractions was performed consisting of the first pre-incubation of plasma in the magnetic particles with and without heparin, after the thrombin with its inhibitor (fresh plasma added antithrombin eluted with 1.0 M NaCl). The residual activity of antithrombin was measured by automated chromogenic method (action amidolytic on the substrate Tos-Gly-Pro-Arg-ANBA-IPA), reading the absorbance at 405nm. Activated partial thromboplastin time (aPTT) was performed by adding 20 µL of eluate obtained with the gradient 1.0 M to the reaction system kit. All of these coagulation tests were repeated ten times intercalating each successive use with buffer washing (10 mM PBS pH 7.2) fifty times. Magnetic derivatives synthesized at time zero and after 2 years were also analyzed for these tests ten times.

3. Results

3.1. *Yield of immobilization of heparin*

Approximately 30 µg of heparin were immobilized in each mg of support for a period of 16h of hydrazinolysis. This corresponds to a rate of approximately 52% of heparin was incubated for support was immobilized.

3.2. *Adsorption of heparin with methylene blue (M.B.)*

Table 1 presents the results of adsorption of methylene blue dye for support and its derivative containing heparin. The adsorption of the dye was greater for the derivative of detention, which supports the presence of heparin. It is observed that even after four reuses, there is still a relatively large adsorption compared to the control.

Table 1. Methylene blue (MB) adsorption on derivatives*

Derivatives	**Reuse Number (Abs. at 664 nm)**			
	1st	**2nd**	**3rd**	**4th**
mPET	**0.818**	**0.900**	**1.075**	**1.107**
mPET-HEP	**0.195**	**0.247**	**0.319**	**0.324**
MB (19.1 µM)	**1.331**			

*The derivatives were incubated with 1mL of 19.1µM MB for 5 minutes under stirring, afterwards the supernatants absorbance were read at 664 nm.

3.3. Assignment of infrared absorption bands

In Figure 1, can observe that the infrared spectrum of the derivative containing heparin showed a pattern of bands in the region between 1700 and 1500 cm^{-1}, and this may indicate that the immobilization procedure was successful. In this region of the spectrum we can point to the amide I bands (1650 cm^{-1}) and amide II (1544 cm^{-1}) (arrows), which may represent the amide group formed by linking the carboxyl group of heparin with the hydrazide group support. The hydrazides of the support groups also have bands of amide I and amide II, however, at different location and intensities. Furthermore, it should be noted that the band seen around 3300 cm^{-1} in the spectrum of support was not viewed in the derivative of immobilization. This band is produced by the NH_2 group of the hydrazide and its absence can demonstrate that these groups were consumed in the reaction of immobilization.

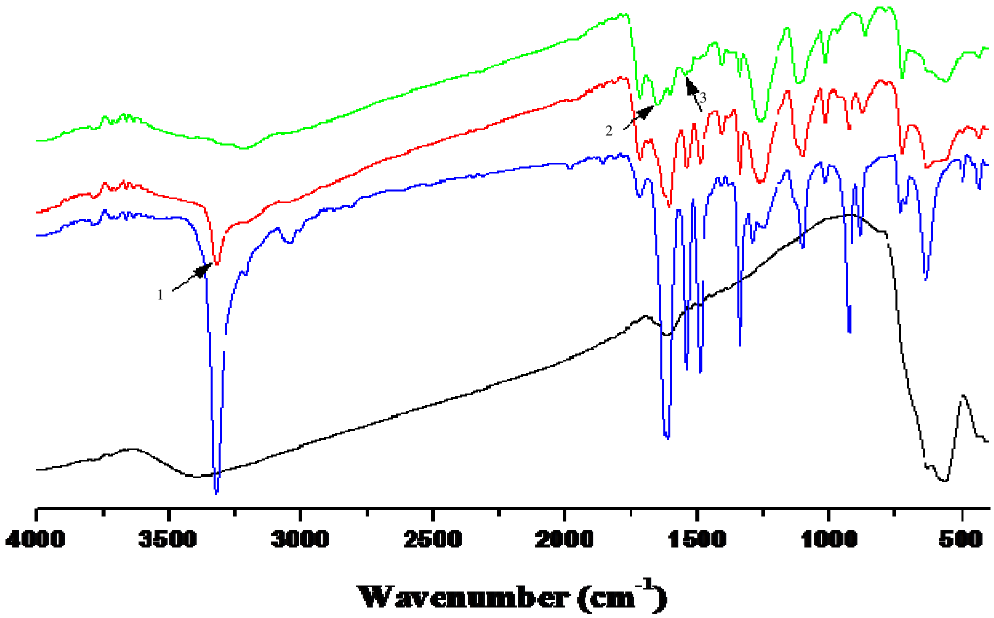

Figure 1: Infrared spectra of derivatives. In ascending order: magnetite; PET-hydrazide; mPET and mPET-HEP. 1: Band of NH_2 group of the hydrazide; 2: Band of amide I; 3: Band of amide II.

3.4. *Elemental composition of the derivatives*

The elemental composition of the derivatives obtained can be seen in Table 2. In both derivatives, hydrazide-Dacron and this support with immobilized heparin were found the elements C, H and N. It was also observed a 2% difference between the nitrogen and the support that the derivative of detention. There was also a reduction in the content of carbon and nitrogen.

Table 2. Elemental composition of derivatives

Element	Derivatives	
	mPET	mPET-HEP
C	36.37%	35.85%
H	3.65%	3.74%
N	7.99%	6.03%

3.5. *Electrophoresis*

Figure 2 shows two electrophoresis carried out from the magnetic derivatives mPET-HEP and mPET (control). Columns 1.0 M and 1.0 M* (derivative eluted with 1.0 M NaCl at time zero and stored for 24 months respectively), it is possible to observe the presence of a band corresponding to antithrombin 58 kDa (arrows). This fact is not observed in support that does not contain immobilized heparin. Furthermore, the bands in the fractions eluted with 0.5 M NaCl (at time zero and 24 months stored) of mPET-HEP support are more emphasized than in the control indicating a larger amount of proteins adsorbed on the support containing heparin.

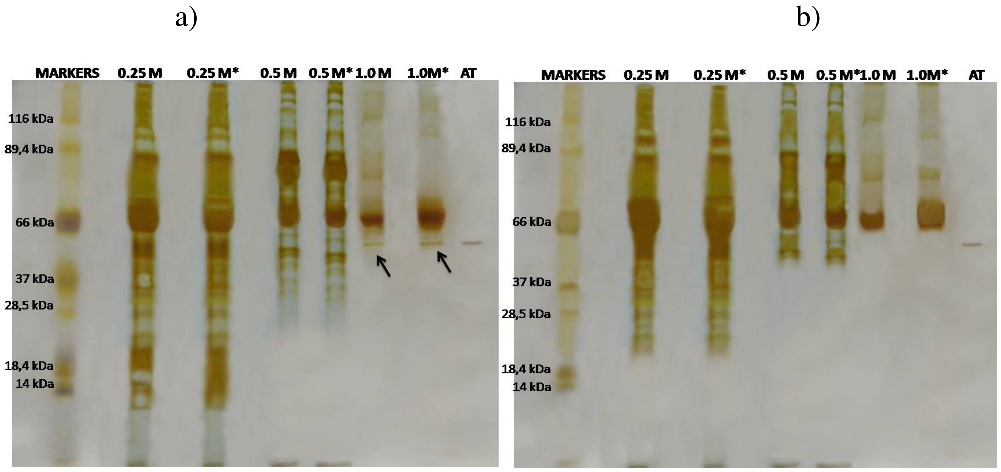

Figure 2: Electrophoresis of protein fractions of plasma obtained with the use of magnetic particles. Elutions were carried out using increasing NaCl concentrations (0.25; 0.5 and 1.0 M) in the magnetic derivatives: a) mPET-HEP and b) mPET (control). AT: Standard Antithrombin. *Elutions performed in supports stored for 24 months. Arrows: Antithrombin (58 kDa).

3.6. *Anticoagulant/antithrombotic activity*

The Table 3 shows the anticoagulant/antithrombotic activity of the magnetic derivatives. The plasma added to the magnetic particles with immobilized heparin did not coagulate. However the plasmas that were incubated with support without heparin were clotted in less than 4 min.

Table 3. Anticoagulant/antithrombotic activity of magnetic particles*.

Sample	Clotting Time (min)
Plasma control	4,0 ± 0
mPET	3,0 ± 1
mPET-HEP	n.c.

*30 mg of particles were incubated with 400 μL of saline (NaCl 0.15 M) added to 500 μL of plasma in a water bath at 37°C. After temperature stabilization, 100 μL of calcium chloride 1% was added and the time to onset of fibrin registered. * Independent samples / triplicate. N.C. - Not coagulated during the time observed (>5 h).

Moreover, was detected a low activity of antithrombin in plasmas that were assayed by test of affinity to heparin at time zero and after 24 months of storage. As presented in Figure 3, this activity is still present after ten reuses of magnetic derivative.

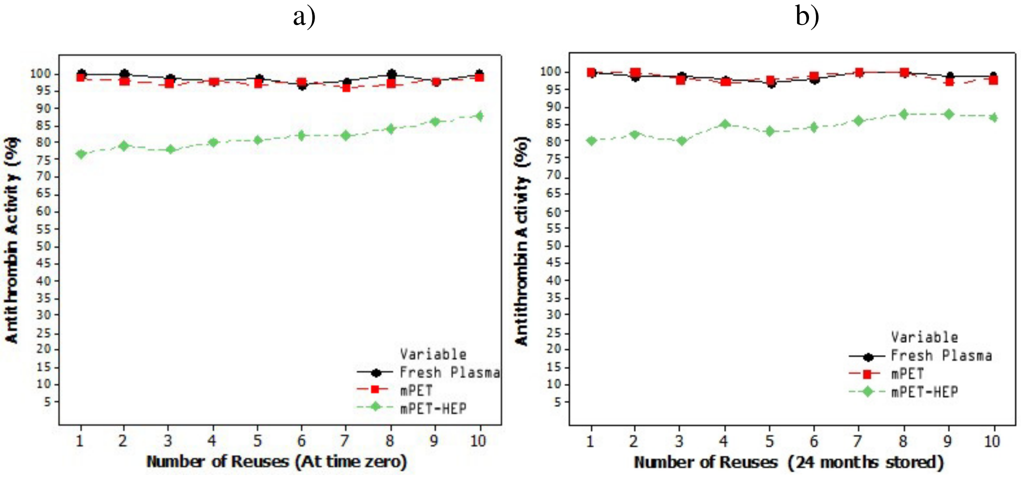
a) b)

Figure 3: Activity of antithrombin present in fresh plasma and after direct contact with the support: a) immediately synthesized (time zero) and b) 24 months stored at 4°C.

The Figure 4 shows a higher value of aPTT in fresh plasmas which have received a portion of eluate 1.0 M when compared to the control. Wherefore, the influence of the purified antithrombin is also observed when the same support (at time zero and after 24 months of storage) is reused ten times.

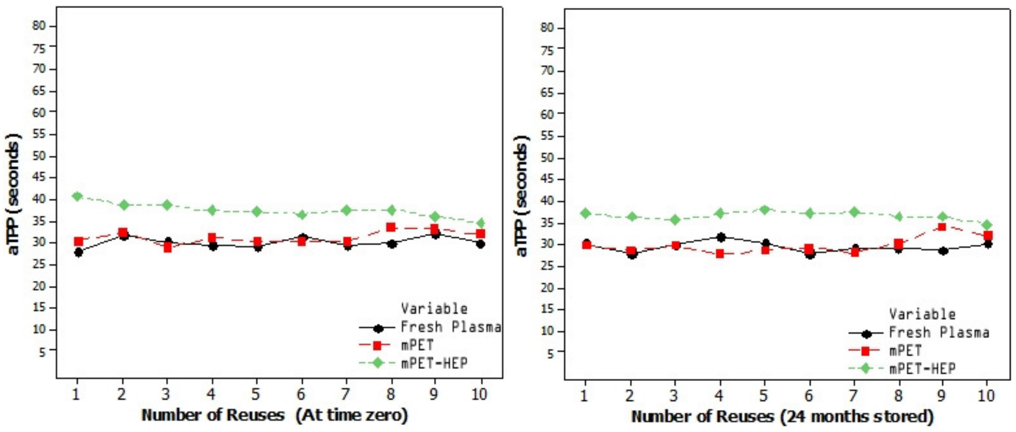
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Figura 4: Activated partial thromboplastin time (aPTT) of fresh plasma performed by adding small aliquot of the eluate (1.0 M) of magnetic derivatives: a) Immediately synthesized (time zero) and b) 2 years stored at 4°C.

4. Discussion

Heparin chromatography is a powerful technology for separating various target biomolecules and has been widely used to fractionate proteins from the extract of prokaryotic organism or eukaryotic cells [9, 10].

In this study heparin was immobilized on the proposed matrix to purify plasma proteins, especially antithrombin. These quantities of immobilized heparin are comparable to those described in the literature, disregarding the differences between the methods of restraint that were used [11, 12, 13]. However, Funahashi et al. (1982) [14] obtained a level of 16 µg of heparin per mg of wet gel (Sepharose-4B modified to contain amine groups) also using as a coupling agent EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide). A sign of immobilization can be observed by the interaction between heparin and methylene blue dye as it is known that basic dyes such as methylene blue, form complexes with this polymer [7]. Even after successive reuses was still a significant adsorption. This feature can be important for applications in the treatment of effluents from textile industries. Magnetic nanoparticles were coated with polyacrylic acid in order to adsorb methylene blue [15] and the authors observed that these particles were quick and efficient in the adsorption of dye from aqueous solutions.

Infrared spectra of the three constituent particle magnetite, Dacron-hydrazide and heparin have been described in the literature. Yamaura et al. (2004) [16], observed strong bands around 632 cm^{-1} and 585 cm^{-1} in their magnetic nanoparticles and attributed to the presence of magnetite. Similar spectra were published by Ma et al. (2003) [17] and Peng et al. (2004) [18]. Obtaining terephthalehydrazide from polyethylene terephthalate (PET) as a method of recycling of plastic was described by Yamaya et al. (2002) [19]. These authors described the band in 3330, 1630 and 1540 cm^{-1} and characteristics of dihydrazide obtained from the PET. The infrared spectra shown in Figure 1 does not denote any band characteristic of heparin, probably because they were overlapped by bands of PET-hydrazide. Infrared heparin should present bands around 3400, 1624, 1425 and 1236 cm^{-1} [7].

In all derivatives were found the elements C, H and N as expected, because both the mPET as well as this support with immobilized heparin have these elements. An indication that the immobilization occurred, is the C: N ratio of support (4.55) which is smaller than that for heparin (10.0) [20, 21, 22] and the relationship found for the derivative containing heparin was higher (5.94) than the support. The reduction in the content of carbon and nitrogen and this may be due to immobilization of heparin, which has a lower amount of carbon relative to its support. And this is due to the presence of a large content of oxygen and sulfur in this polysaccharide.

Electrophoresis in polyacrylamide gel with sodium dodecyl sulfate was performed in an attempt to identify possible proteins adsorbed by the mPET-HEP. The main focus was antithrombin, because as we know, it specifically binds heparin with a Kd approximately 20 nM [23] and has many of its features fully described in the literature [24], which would facilitate the study. It is known that antithrombin has a molecular mass of 58 kDa and that in Figure 2 it is represented as a band just below the albumin (66kDa) represented by this band.Thesedata canbe seenin both thederivativesusedimmediately(time zero) and those thatwere just usedtwo yearsafterstocked. Thus, these experiments showed that protein adsorption occurs and that these were eluted with 1.0 M NaCl even after 2 years of storage.

The Table 3 showed the capacity of the magnetic support containing heparin to prevent clots formation. The literature is replete with efforts to improve blood compatibility of surfaces after immobilization of heparin [25. 26]. This should acquire biocompatibility to the anticoagulant properties of heparin, its interaction with antithrombin and the consequent inhibition of proteases of the cascade of blood coagulation.

Thus, the PET with heparin immobilized may be an important tool for the purification of antithrombin and have an excelente stability even kept for 2 years as shown in Figure 3 and 4, in which there is a significant reduction in activity of this inhibitor of serine protease in the plasma after its incubation to support. It was also noted that the fresh plasma samples that received a small portion of the purified antithrombin by Dacron with immobilized heparin presented a high aPTT compared to control (Figure 4), result of delay the clotting time by inhibiting thrombin. Furthermore, the antithrombin that was separated from plasma remaining active, when it was subjected to affinity chromatography to heparin.

5. Conclusion

The data presented confirm the presence of heparin in Dacron magnetic particle obtained, and the presence of the hydrazide group, essential for the covalent immobilization proposed in this paper. Therefore, these results, coupled with an efficient absorption of methylene blue, confirm successful immobilization of heparin. Finally, the results, even after 2 years stored, demonstrate the efficiency of magnetic separation of antithrombin.

Acknowledgements

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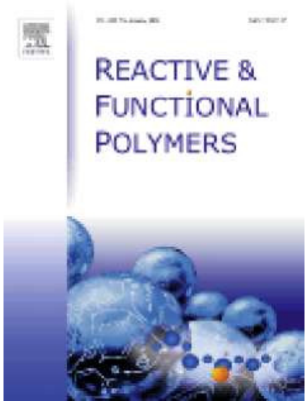
Capítulo 3

6 Artigo a ser submetido à revista internacional “Reactive and Functional Polymers”



Título: Antithrombin purification by affinity matrix using magnetite coated with polyaniline

Autores: Ricardo Souza Silva; Aurenice Arruda das Merces; Givanildo Bezerra Oliveira e Luiz Bezerra Carvalho Jr.

Antithrombin purification by affinity matrix using magnetite coated with polyaniline

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Abstract

Heparin is widely used as a matrix for affinity purification of proteins, mainly of antithrombin. For this reason, the magnetite (MAG) was coated with PANI. Heparin activated with carbodiimide and N-hydroxysuccinimide was incubated for support to occur immobilization. 37% of heparin was retained to support and this matrix magnetic remained stable for 2 years even after 10 reuses. In the process of leaching of plasma proteins, when added 1.0 M NaCl, there is a protein peak to 280 nm in the two forms of magnetite: treated with heparin (MAG-HEP) and coated with PANI (mPANI-HEP). Electrophoresis of these proteins revealed a band corresponding to that referred to the antithrombin (58kDa). This occurred using these two forms of magnetite, but with lower intensity in preparations using HEP-MAG than that coated with PANI. Moreover, alow activity of antithrombin and delay clotting time of plasmas that were exposed to the eluted fractions containing antithrombin was reported especially insupport mPANI-HEP although the MAG-HEP was also noted. Thus these results show the need of PANI for the immobilization of heparin and use this as a support for the purification of antithrombin.

Keywords: antithrombin; heparin; immobilization; polyaniline; separation

1. Introduction

Heparin has been used for some time in the purification of plasma proteins. However, its use is more frequent in the isolation of antithrombin in human plasma by affinity chromatography to heparin [1] which is a valuable technique for the purification of many proteins from human plasma.

Magnetic fields have been utilized in support systems for the study of different immobilized biomolecules [2, 3]. The advantages of small magnetic particles for immobilization in bioprocesses are: improvement of the mass transfer properties of immobilized enzymes suspended in a viscous solution, in which the promotion of mass transfer by agitation is difficult [2]; reuse and easy separated from reaction medium by applying a magnetic field [4] and reduction of the capital and operation costs [5].

The polyaniline (PANI) is a class of polymers highly promising, due to its low cost of synthesis, low grade difficulty of handling and also to present the property to conduct electrons [6]. Depending on the conditions of synthesis, the polymer can act as a semiconductor [7]. Due to the versatility of this polymer, the PANI has been proposed different applications: construction of large capacitors [8], sensors for detection of ammonia [9], remediation for reduction of heavy metals [10], and as a matrix for immobilization of enzymes [11, 12. 13].

In this paper a support is investigated to obtain an array of magnetic based heparin aiming at the purification of antithrombin: magnetic particles coated with polyaniline (PANI) so that heparin was covalently immobilized to the organic polymer.

2. Material and methods

2.1. Production of magnetite and coating with PANI

A magnetic matrix synthesis was conducted as follows: 10 ml of a solution of ferric chloride (1.1 M) and ferrous chloride (0.6 M), both from Sigma Chemical Company, in a 2:1 ratio, was combined with 50 ml of water and adjusted to pH to 11 with ammonium hydroxide [14]. The mixture was heated at 100°C under vigorous stirring for 30 min. The precipitated black (magnetite: MAG) obtained was washed thoroughly with water to remove excess of ammonium hydroxide. The material was then filtered and oven dried, screened and homogenized in sieveopening 250μm.

The magnetic particles (30 mg) were treated with 1.0 mL of ammonium persulfate 0.61 M (APS; Sigma) prepared in 2.0 M HCl, which was under gentle shaking for 30 minutes at 25°C and after successive washes with 10 Mm PBS pH 7.2 were made. In some magnetic support treated with ammonium persulfate (APS) was subsequently incubated with 1.0 ml 0.44 M aniline (Sigma; 99%) for 60 minutes at 4°C to obtain magnetite coated with polyaniline support (mPANI).

2.2. Immobilization of heparin on the magnetic particles

To a solution of heparin (Cristália) 3mg/mL was added N-hydroxysuccinimide (NHS; Sigma) and carbodiimide (EDAC; Sigma). Kept the solution pH between 4.5 and 5.0 for 30 minutes or until there was no increase in pH. Some supporters were not treated to see the importance of activation with EDAC/NHS. These mixtures (1.0 mL) was incubated for 72 hours with 30 mg: a) magnetite (MAG) as control to obtain heparin adsorbed (MAG-HEP); and b) magnetite coated with PANI (mPANI) with a view to obtaining the magnetic particles coated with polyaniline containing heparin covalently attached (mPANI-HEP). These magnetic products were stored in the water for 24 months at 4°C for use in two stages: start (time zero) and final (24th month) to study the stability of the support in the purification of antithrombin.

2.3. Determination of heparin

Heparin and washed was determined in accordance with the methodology used by Oliveira et al. [15]. The method is based on the property of heparin to form complexes with basic dyes such as methylene blue proceeding to reading at 664nm.

2.4. Separation/purification of plasma proteins

To 30 mg of matrix with PANI containing heparin (mPANI-HEP) was added 1.0 mL of citrated human plasma. This mixture was kept under stirring for 1 h at 4°C. Then, with the help of a magnetic field of 6000 Oe, the supernatants were collected and washed out with phosphate buffer 0.01 M pH 7.4, subsequently, the matrix was eluted with buffer containing an increasing gradient of NaCl (0.25 M; 0,5 M; 1.0 M and 2.0 M). These eluates were analysed by polyacrylamide gel electrophoresis according to Laemmli [16]. For these assays, were used magnetic products prepared at time zero and those stored at 24 months.

2.5. Measurement of antithrombin activity

The activity of antithrombin was performed using three forms of magnetite: mPANI-HEP; MAG-HEP and pure magnetite (MAG). Fifty milligrams of these magnetic derivatives were incubated under mild stirring with 400 μL of 0.15 M NaCl and 500 μL of plasma at 37° C. After 5 min, 100 μL of 1 %, w/v $CaCl_2$ was added and the time to onset of fibrin registered.

The activity of antithrombin was studied in two ways: the first held in plasmas that were in direct contact with the magnetic support to evaluate the residual activity of antithrombin by automated chromogenic method. The second observed the activated partial thromboplastin time (aPTT Labtest Diagnóstica) in plasma by adding 20 μL of eluate obtained with the gradient 1.0 M NaCl to the reaction system kit.

3. Results and discussion

3.1. Characterization of magnetic derivative

Conventional chemical synthesis of PANI has great advantage of producing a polymer of high molecular weight and high purity, which can be obtained in large amount in the form of a green powder [17]. In this work, the particles had an excellent magnetic activity by their conduct through a magnetic field. Moreover, these particles were properly coated with PANI, which was obtained through oxidation of aniline by ammonium persulfate (APS) and the reaction showed a "emerald" color (greenish), indicating the formation of polyaniline in its most stable form (Fig. 1) [18].

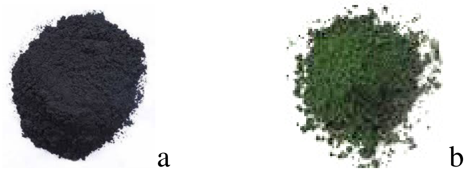


Fig 1: Magnetic matrix: (a) pure magnetite, (b) magnetite coated with PANI

3.2. Immobilization of heparin on magnetic matrix

Heparin chromatograph is a method of affinity chromatography used to purify or fractionate biological substances that can interact with heparin immobilized on insoluble support. The immobilization can occur by covalent bonding or physical adsorption [19]. Tab. 1 shows the amount of heparin (3 mg) recovered during the washing of the magnetic particles, those that are not immobilized to the support. With this, it can be seen that approximately

37% of the activated heparin was supplied covalently immobilized on supports coated with PANI. However, this is also heparin binding to particles even without PANI - around 15% - although at a lower rate, probably by nonspecific physical interactions. Further studies should be conducted to see if the pure magnetite is effectively capable of immobilizing heparin. Another important finding in the table is clear that the carboxylic groups of heparin, when activated with EDAC / NHS, are more reactive to promote a more efficient covalent interaction with the support coated with PANI. This is proved by observing a lower degree of immobilization to supports coated with PANI when heparin was not activated (22.7%). This shows that the polyaniline available chemical groups for the occurrence of covalent immobilization. These quantities of immobilized heparin are comparable to those described in the literature, disregarding the differences between the methods of restraint that were used [20, 21, 22].

Tab 1: Recovery of heparin (3mg) not fixed on magnetic particles

	mPANI-HEP	mPANI-HEP*	MAG-HEP	MAG-HEP*
Supernatant	1,75 ± 0,01	2,21 ± 0,02	1,91 ± 0,70	2,60 ± 0,21**
1st wash	0,11 ± 0,02	0,10 ± 0,02	0,53 ± 0,04	0,05 ± 0,03
2nd wash	0,04 ± 0,01	0,01 ± 0,01	0,11 ± 0,01	004 ± 0,01
3rd wash	0,00	0,00	0,00	0,00
Total	1,89 ± 0,04	2,32 ± 0,05	2,55 ± 0,75	2,69 ± 0,25

*Heparin has not previously treated with EDAC/NHS

** Average ± SD

3.3. Separation/purification of plasma proteins

Heparin is not only an affinity ligand with biological specificity, but also an ion exchanger with high-charge density and distribution [23]. In this kind of chromatography, heparin covalently immobilized on matrix has two modes of interaction with proteins in sample solutions: a) acting as a specific affinity ligand, and b) serving as a cation exchanger through its high content of anionic sulphate groups. In 1973, Rosenberg and Damus suggested that heparin binds to antithrombin, causing a conformational change within antithrombin leading to a greatly accelerated reaction with thrombin and the formation of an inactive complex of the two proteins [24]. The interaction of heparin with antithrombin is a highly specific interaction. For any separation process could use ionic strength [25]. In the process of affinity purification of proteins from human plasma (Fig.2) using ionic strength (1.0 M NaCl), shows that the two forms of magnetite (just treated with heparin: MAG-HEP and that coated with polyaniline and immobilized heparin: mPANI-HEP) were able to purify a protein in fraction 45 when it was measured by 280 nm, while pure magnetite (MAG) does not serve to purify. However, adds to this other behavior that is the possibility of their own antithrombin or other proteins binding to the particles without treatment of any kind (pure magnetite), as shown in the same Fig. 2.

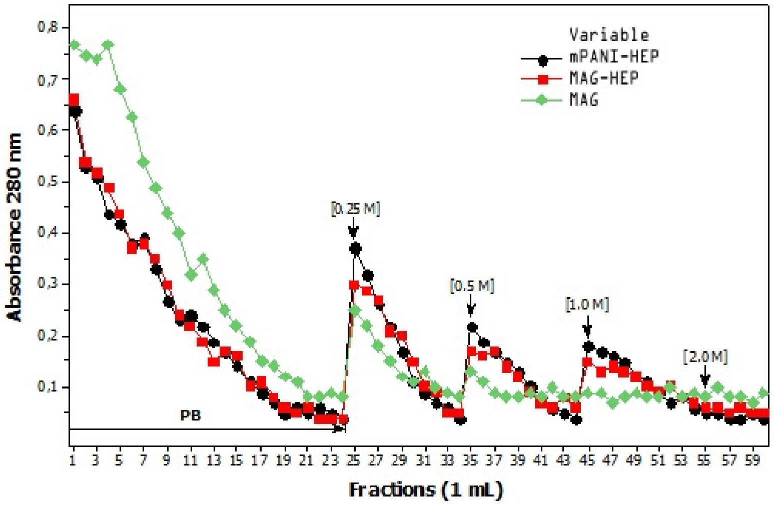
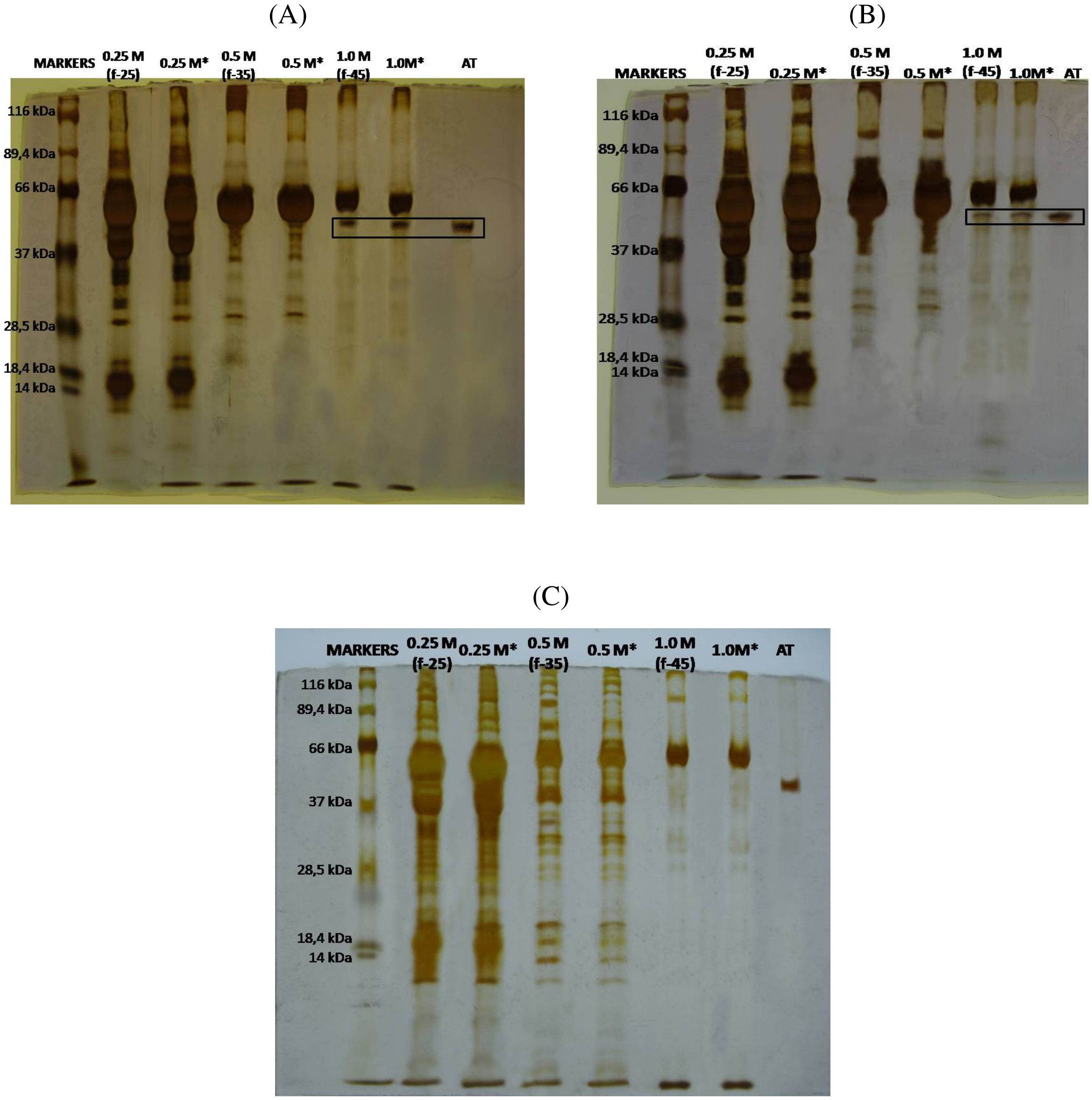


Fig.2: Affinity chromatography of human plasma protein using magnetic particle washed with phosphate buffer (PB) and increasing NaCl concentrations (0.25; 0.5; 1.0 M and 2.0 M).

3.4 Electrophoresis

Electrophoresis in polyacrylamide gel with sodium dodecyl sulfate was performed in an attempt to identify possible proteins adsorbed by different forms of magnetite. The electrophoresis (Fig.3A and 3B) shows protein with molecular weight 58 kDa (rectangle), corresponding to that referred to pure antithrombin with a greater amount of protein in fraction obtained from the preparation mPANI-HEP and MAG-HEP respectively. The main focus was antithrombin, because as you know, it specifically binds heparin with a Kd approximately 20 nM [26] and has many of its features fully described in the literature [27], which would facilitate the study. In Fig.3C there is a gel that no band was observed. Thus, these experiments showed that protein adsorption occurs and that these were eluted with up to 1.0 M NaCl in the magnetic derivatives containing immobilized heparin, as shown in Fig. 2.


(A)

(B)

(C)

Fig. 3: *Electrophoresis*. The figures indicate the number of the fractions, according to the Fig. 2, collected by increasing NaCl concentrations (0,25; 0.5 and 1.0 M) from the plasma protein adsorbed onto the: a) mPANI-HEP; b) MAG-HEP and c) MAG. The rectangle shows the fractions that presented higher aPTT antithrombotic activity values. AT: Standard Antithrombin

* Elutions performed in supports stored for 2 years.

3.5. In vitro anticoagulant assay

The aPTT test showed that the plasma that came into direct contact with the magnetic derivatives containing heparin activated or not with EDAC/NHS not coagulated, thus proving that heparin was indeed linked to support (Tab 2).

Tab 2: Activity anticoagulant / antithrombotic directlyon magnetic particles

Essay	Coagulation Time (min: s)
Plasma	00:50
MAG	01:31
MAG-HEP*	Not coagulated
MAG-HEP	Not coagulated
mPANI-HEP*	Not coagulated
mPANI-HEP	Not coagulated

*heparin not previously treated with EDAC/NHS

To assess how much of antithrombin was retained in every form of magnetite, we measured the activity of plasma antithrombin remainder who underwent each type of magnetic support (Fig. 4). There is a decreased activity of antithrombin in plasmas that came into contact with the magnetite coated with PANI and heparin immobilized, although there has also been a rapid decline in support control without PANI and with immobilized heparin. Furthermore, the fraction 45 with purified antithrombin when incubated with the fresh plasma slows thromboplastin time by means of inhibition of thrombin as can be seen in eluates coming from the support containing heparin (Fig. 5). This is probably due to the support with heparin to retain other factors of blood coagulation [28]. Both Fig. (4 and 5) clearly demonstrate these antithrombotic activities are enhanced in support containing heparin, especially derivative which is coated with PANI.

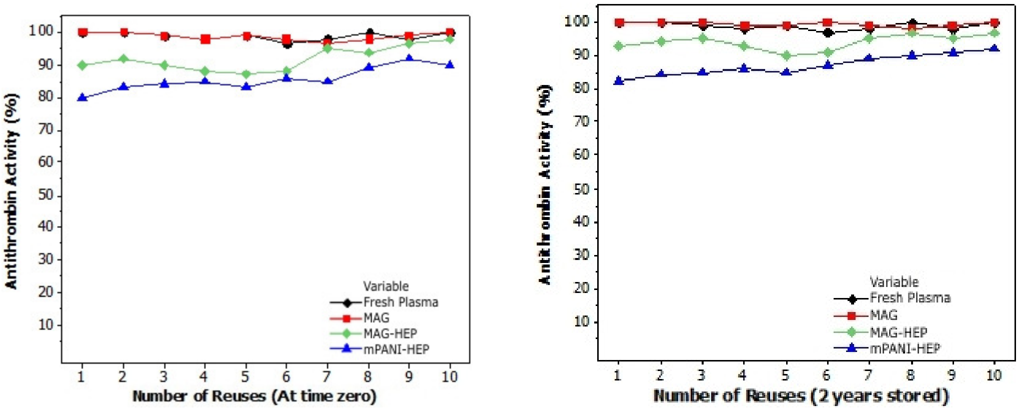

a) b)

Fig. 4: Activity of antithrombin present in fresh plasma and after direct contact with the support: a) Immediately synthesized (time zero) and b) 24 months stored at 4°C.

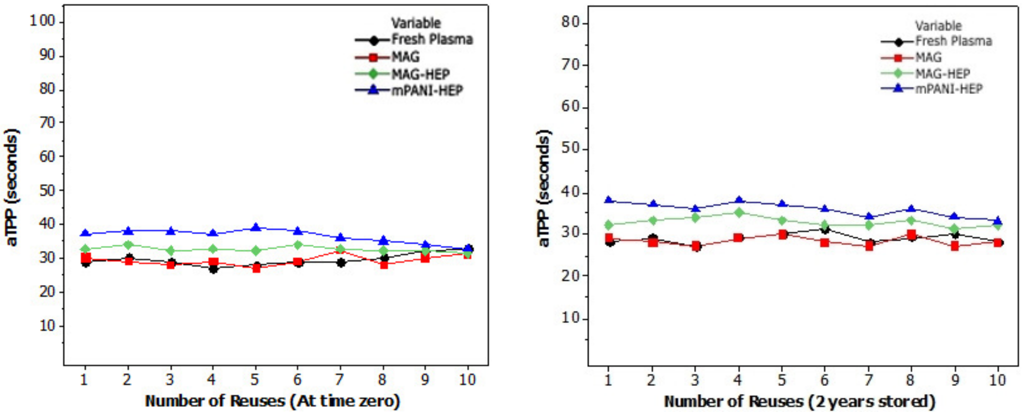

a) b)

Fig. 5: Activated partial thromboplastin time (aPTT) of fresh plasma performed by adding small aliquot of the eluate (1.0 M) of derivatives magnetic: a) Immediately synthesized (time zero) and b) 2 years stored at 4°C.

4. Conclusion

It follows from the results that the magnetic particles coated with PANI and immobilized heparin (activated with EDAC/NHS) can be used to purification of proteins from human plasma, but to a lesser extent the pure particles and treated with heparin also can be used to the same purpose. Furthermore, both supports remained stable after 10 reuses although stored for 2 years.

5.Acknowledgements

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6. References

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Capítulo 4

7 Artigo a ser submetido à revista internacional “Composites Science and Technology

Título: Affinity separation of atithrombin using heparan sulfate immobilized on magnetic iron oxide particles coated with polyethylene terephthalate or polyaniline

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Affinity separation of antithrombin using heparan sulfate immobilized on magnetic iron oxide particles coated with polyethylene terephthalate or polyaniline

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Abstract

The present study aimed to develop magnetic matrices for the affinity separation of proteins using the heparan sulfate (HS) as a ligand. For this reason, ferromagnetic polyethylene terephthalate and magnetite coated with polyaniline were used for immobilization of HS. Initially, PET suffered hydrazinolysis and then was magnetized (mPET), and the magnetite (MAG) was synthesized and coated with PANI (mPANI). The HS was covalently fixed to the particles mPANI and mPET retaining 35 µg e 38.6 µg per mg of support respectively. Magnetic derivatives containing heparan sulfate immobilized (mPANI-HS and mPET-HS) were incubated with plasma and washed with NaCl gradients to release the fixed proteins and detect protein peaks at 280 nm. Electrophoresis of this eluate revealed bands corresponding to factors II, IIa, III, VII, XI, protein S and antithrombin, important proteins involved in the clotting process. Furthermore, activated partial thromboplastin time (aPTT) and prothrombin time (PT) of plasmas were prolonged when contacted with these eluates (1.5 and 2.0 M NaCl). The antithrombin activity in these eluates also demonstrated the effectiveness of the separation of this process using HS in both magnetic derivatives suggesting that these preparations are able to separate affinity components of human plasma as the factors of coagulation.

Keywords: antithrombin, heparansulfate; immobilization; polyaniline, polyethylene terephthalate.

1. Introduction

Glycoaminoglycans (GAGs) is widely used in biotechnology and one of its applications is in protein purification method using affinity chromatography [1 - 7]. This technique consists to fractionate or purify proteins and other biological substances through interaction (affinity) between molecules of interest fixed to the insoluble support [8]. The support used need to have a good profile as physical strength, insolubility, resistance to microbial attack as well as mechanical and thermal stability [9]. And when magnetized, facilitates the recovery of the carrier molecule to the immobilized affinity chromatographic processes, making this process easier and faster with the aid of a magnetic field.

Previous studies revealed that ferromagnetic particles PANI (polyaniline) or PET - Polyethylene terephthalate (polyester derived from the condensation of terephthalic acid with ethylene glycol), are useful to serve as supports and immobilize proteins [10, 11] or GAG [12]. In this study we investigated the use of heparan sulfate (HS) for the purpose of obtaining a magnetic matrix with this GAG covalently immobilized on solid supports ferromagnetic (PET and magnetite coated with PANI) with a view to the separation of plasma proteins.

The HS has group N-Glucosamine N-acetylated or N-sulfated. Several studies in the literature clearly show a high number biologically active proteins that can interact with heparan sulfate. The heparan, due to its great structural diversity, is able to interact with a variety of proteins: growth factors, chemokines, extracellular matrix components, DNA and RNA polymerases, coagulation factors, among others [13 - 17]. Thus, the focus of this paper was the separation of proteins that play an important homeostatic role in human plasma.

2. Material and methods

2.1. Materials

Films of Dacron (polyethyleneterephthalate) were donated by Terphane Ltda (Cabo de Santo Agostinho, PE, Brazil). The heparan sulfate was extracted from bovine lung [18] and was donated by the Clinical Laboratory (ANALAB) through the courtesy of Dr Adeilton Oliveira in partnership with Dr Leandro Fernandes Machado of UNB. Carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EDAC), N-hydroxysuccinimide (NHS), hydrazine hydrate, ammonium persulfate (APS) and aniline (99%) were purchased from Sigma-Aldrich Ltda, whereas ferric and ferrous chlorides were from Merck SA. The kits of ***activated partial thromboplastin time*** (aPTT) and prothrombin time (PT) was acquired from Wiener Lab and Inlab Hemostasis respectively.

2.2 Synthesis of magnetite particles (MAG).

A magnetic matrix synthesis was conducted as follows: 25 ml of a solution of ferric chloride (1.1 M) and ferrous chloride (0.6 M) in a 2:1 ratio was combined with 125mL of water and adjusted to pH solution to 11 with ammonium hydroxide. Then the mixture was heated in a water bath at 100°C under vigorous stirring for 30 min. The black precipitate obtained, which are magnetite particles (MAG), washed thoroughly with distilled water to remove excess ammonia and to achieve a neutral pH. The material was then filtered and oven dried, screened and homogenized in sieve opening 250µm.

2.3. Coating the magnetic matrix with polyaniline.

The magnetic particles (<250µm) were treated with ammonium persulfate 0.61 M (APS; Sigma) prepared in 2.0 M HCl, which was under gentle shaking for 30 minutes at 25°C and after successive washes with 10 mM PBS pH 7.2 were made. The support treated with ammonium persulfate (APS) was subsequently incubated with 1.0 ml 0.44 M aniline (Sigma; 99%) for 60 minutes at 4°C to obtain coated magnetite support polyaniline (mPANI).

2.4. Hydrazinolysis of PET

The hydrazinolysis of the PET was conducted as follows: 10 g of PET (sheets) were added to a 250 ml erlenmeyer flask, and this material was added 100 ml of methanol and 25 mL of hydrazine hydrate. This mixture was subjected to agitation at 150 rpm at 40 ° C for 24 hours [19]. After this process, we obtained a whitish liquid filtrate which was then dried and sieved in sieve opening 250μm. The final product is obtained is a fine white powder (PET-hydrazide) that will be used later in step magnetization.

2.5. Magnetization of PET

The magnetization was conducted by preparing a suspension according to the following proportions: 2g PET-hydrazide in water, followed by addition of 10 ml of a solution of ferric and ferrous chlorides in a 1:1 ratio, the pH was raised to 11 by adding ammonium hydroxide, and finally, this mixture was kept under vigorous stirring at 100°C. Finally, the black precipitate was obtained which was washed thoroughly with water to remove excess ammonium hydroxide, reaching a neutral pH. Shortly afterwards the material was filtered and dried at 50°C, then homogenized and sieved in sieve opening 250μm. The particles of ferromagnetic PET (mPET) were then synthesized.

2.6. Immobilization of heparan sulfate (HS) covalently on magnetic support: mPET and mPANI.

For activation of heparan sulfate (1 ml containing 3mg/mL) was used in an amount carbodiimide (EDAC) in proportion to the Heparan 1:1 w/w and an amount of sulfate N-hydroxysuccinimide (NHS) in proportion of EDAC 1:1 M/M. The resulting solution was subjected to 1 hour of agitation at a pH of around 4.5 to 4.8 at 25°C. At the end of the reaction, the pH was raised to 8 with NaOH. The activated heparin solution was incubated for 72 hours at room temperature with 30 mg of supports magnetic particles coated with PANI (mPANI) and magnetic PET (mPET) obtaining the magnetic derivatives: mPANI-HS and mPET-HS.

2.7. Heparan sulfate determination

To determine the amount of heparan sulfate that was immobilized to the supports was necessary to make a standard curve for measurement of heparan sulfate with the use of methylene blue dye [20]. After the incubation period, the samples were withdrawn from the supernatant and of washed 1 and 2. The dye methylene blue (0.005% 0.01% NaCl 0.2 N) was used to determine the amount of heparan sulfate which was contained in supernatant, wash 1 and Wash 2, this measurement was made in the spectrophotometer wavelength of 664 nm and yield immobilization was calculated as the difference between the quantity supplied and that found in supernatants and washed.

2.8. Separation of plasma protein

In each of 30 mg of matrix containing heparan sulfate, add 1 ml of citrated human plasma. This mixture was stirred for 45 minutes at room temperature. Then, with the aid of a magnetic field of 6000 Oe, supernatants were collected and washings performed with phosphate buffer pH 7.2 to obtain low concentrations of protein in the eluate ($\lambda = 280$ nm). Subsequently, the matrix was eluted with the same buffer containing increasing concentrations of 0.5 M, 1.0 M, 1.5 M and 2.0 M sodium chloride. The process of incubation and elution were repeated about five times, to increase the total protein mass, dialyzed, lyophilized and finally, analyzed on electrophoresis SDS/PAGE gel to 10.0% and stained with silver nitrate.

2.9. Prothrombin time (PT), activated partial thromboplastin time (aPTT) and antithrombin activity.

The quantitative determination of antithrombin activity in the fractions was established using a chromogenic (SAR-PRO-ARG p-Nitroanilide) assay (TriniCHROM^{TM} Antithrombin IIa, Trinity Biotech, Ireland). Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were made with the eluates (fractions) obtained from the increasing concentrations of NaCl. An aliquot of 20 µL was added to the reaction system kit according to the standards required by manufacturer instructions.

3. Results and discussion

3.1. Synthesisof magnetic particles: magnetite (MAG), magnetite coated with PANI (mPANI), and ferromagneticPET (mPET)

The particles had an excellent magnetic activity by their conduct facing a magnetic field of 6000 Oe. Magnetite (Fe3O4) was indeed obtained by co-precipitation of ferric ions (Fe^{3+}) and ferrous (Fe^{2+}). The magnetic particles were actually coated with polyaniline. This can be seen by the reaction showed a color between green and blue being indicative of polyaniline in its most stable form – state esmeraldine. Although a small portion of the material was lost during the washes, mPET support also obtained excellent magnetic activity.

3.2. Hydrazinolysis reaction

The PET had hydrazinolysis total time of 24 h. Therefore, this was the time needed to react all of Dacron sheets (10g) with hydrazine hydrate. Thus, the hydrazide chemical groups that are important for the covalent immobilization of heparan sulfate were added.

3.3. Immobilization of heparan sulfate on magnetic derivatives: mPET and mPANI

Table 1 shows the amount of heparan sulfate (3mg) recovered during the washing of the magnetic particles, in other words, those that are not immobilized to the support. With this, it can be seen that the amount of heparan sulfate (HS) immobilized was 35µg/mg on mPANI support (mPANI-HS). However, there is also heparan sulfate binding (18µg/mg), although at a lower rate, on pure magnetite (MAG). The table also shows that 38.6 µg of HS was immobilized in each mg of ferromagnetic PET (mPET). This is explained due to the covalent interaction between the hydrazide groups present in support mPET forming a specific amide bond with the most reactive carboxyl groups of heparan sulfate because of its activation by EDAC and NHS.

Table 1: Recovery heparan sulfate (3mg) not fixed tomagnetic support mPANI and mPET

	mPANI-HS	MAG (Control)	mPET-HS
Supernatant	1,69 ± 0,23	2,31 ± 0,31	1,52 ± 0,44*
1st wash	0,19 ± 0,09	0,12 ± 0,03	0,23 ± 0,02
2nd wash	0,07 ± 0,04	0,03 ± 0,07	0,09 ± 0,02
3rd wash	0,00	0,00	0,00
Total	1,95 ± 0,36	2,46 ± 0,41	1,84 ± 0,48

* Average ± SD

3.4. Separation of plasmaprotein.

Affinity chromatography is recognized as a simple and effective method for separating various molecules contained in a mixture using for this physicochemical property of the distribution from the components of the mixture into two phases, one stationary and the other movable, which are in intimate contact. The specificity between the elements of these phases allows you to remove the mobile component of the mixture. In this work, the stationary phase is represented by the supports mPANI and mPET containing immobilized heparan (mPANI-HS and mPET-HS respectively).

Fig. 1 and 2 show the leaching of plasma proteins attached to the stationary phases (mPANI-HS and mPET-HS respectively). First, it is observed that the absorbance at 280 nm is indicative of the presence of protein in the laundered. Before elution process repeated, washing with phosphate buffer pH 7.2 were performed. When increasing the added solutions of 0.5 M, 1.0 M, 1.5 M and 2.0 M NaCl in the same buffer (shown in the figures) protein peaks were eluted in each fraction are observed. It is noted that this behavior was observed in eluates of plasma proteins employing the two magnetic support which have the covalently immobilized heparan sulfate: mPANI-HS and mPET-HS. The fractions corresponding to each peak were subjected to electrophoresis. These eluted proteins are, among others, mainly serine proteases of coagulation cascade, contain large numbers of the basic amino acids (lysine, arginine and in some cases histidine). These basic residues can be found in linear arrangements or in spatial folded clusters. Nevertheless, links can also involve basic amino acids which are distant in the linear sequence of the protein are pooled and protein in the folded state [21 - 25]. However, they can be separated from the solid phase by ionic strength as shown in Fig. 1 and Fig. 2.

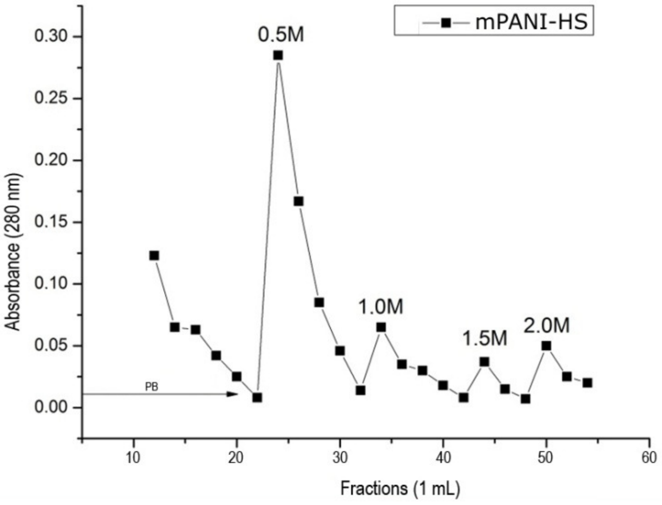

Figure 1: Affinity chromatography of human plasma proteins using magnetite coated with polyaniline and heparan immobilized: mPANI-HS was incubated with human plasma and washed with phosphate buffer ph 7.2, to balance the absorbance (280 nm) – 20th fraction: the particles were then washed with buffer containing increasing concentrations of NaCl (0.5M; 1.0M; 1.5M and 2.0M).

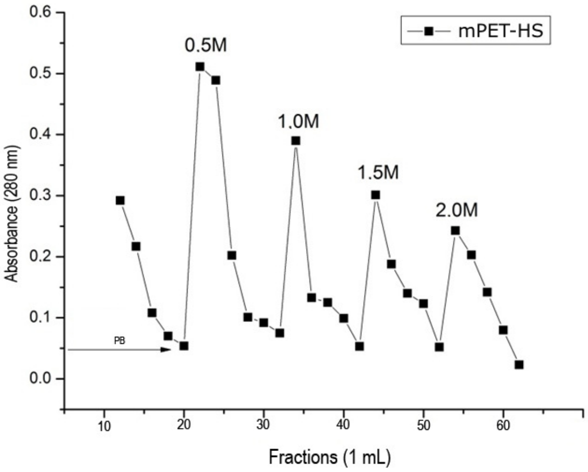

Figure 2: Affinity chromatography of human plasma proteins using ferromagnetic PET with heparan sulfate immobilized: mPET-HS was incubated with human plasma and washed with phosphate buffer ph 7.2, to balance the absorbance (280 nm) – 20th fraction: the particles were then washed with buffer containing increasing concentrations of NaCl (0.5M; 1.0M; 1.5M and 2.0M).

3.5. Influence of the eluates on coagulation tests

Table 2a and 2b shows the prothrombin time (PT), activated partial thromboplastin time (aPTT) and antithrombotic activity, clinical test commonly used to diagnosing the absence of coagulation factors. Proteins presenting and increasing the aPTT and PT were collected from the mPANI-HS and mPET-HS that are equivalents to NaCl concentrations of 1.5 and 2.0 M, this is due to the fact these proteins have been held in the magnetic derivatives. The proteins eluted with lower NaCl concentrations did not present antithrombotic activity and relevant aPTT and PT values. This finding is a strong indication of the effectiveness of magnetic supports (mPANI-HS and mPET-HS) in the separation of antithrombin and other coagulation factors, since it is known that there is an intense physiological interaction between this glycoprotein with heparin, which has structural similarity with heparan sulfate [26].

Table 2 – The activated partial thromboplastin time (aPTT), prothrombin time (PT) andantithrombin activity of the fractions collected from the elution of the plasma proteins adsorbed onto the: a) mPANI-HS and b) mPET-HS.

a)

Fractions (Number)	[NaCl] (M)	aPTT[1] (min: s)	PT[2] (min: s)	AT activity (%)
20	PB	00:38	00:13	0
25	0.5	00:40	00:12	0
30	0.5	00:39	00:12	0
35	1.0	00:44	00:15	0
40	1.0	00:40	00:14	0
45	1.5	02:41	00:39	3.11
48	1.5	02:23	00:30	2.27
50	2.0	01:21	00:35	2.44
55	2.0	01:03	00:27	0.20

b)

Fractions (Number)	[NaCl] (M)	aPTT[1] (min: s)	PT[2] (min: s)	AT activity (%)
20	PB	00:36	00:12	0
25	0.5	00:38	00:11	0
30	0.5	00:37	00:12	0
35	1.0	00:43	00:14	0
40	1.0	00:39	00:12	0
45	1.5	02:36	00:36	2.97
50	1.5	02:18	00:27	2.12
55	2.0	01:27	00:31	1.97
60	2.0	01:11	00:20	0.23

[1]Reference values: 30 – 42 seconds; [2] Reference values: 12 – 15 seconds. PB: 10 mM phosphate buffer, pH 7.2.

3.6. Electrophoresis

The findings presented in Table 2 were corroborated with the electrophoresis results (Fig. 3). Suggestive bands of protein significant in the coagulation cascade can be visualized for the elutions with 1.5 and 2.0 M NaCl that revealed higher aPTT and antithrombotic activity values: a) factor XI (160 kDa); b) protein S (75 kDa); c) factor II (72kDa); d) antithrombin (58 kDa), e) factor VII (55 kDa); f) factor III (37 kDa) and g) factor IIa (36,7 kDa) [27,28]. These are molecules which can interact physiologically with the heparan sulfate and depends on the overall organization of the glycosaminoglycan chain rather than on the fine structure of the individual sequences to achieve its functional role [29-32]. This result are clearly shown in both support containing heparan sulfate covalently immobilized: mPANI-HS (Fig. 3A) and mPET-HS (Fig. 3B).

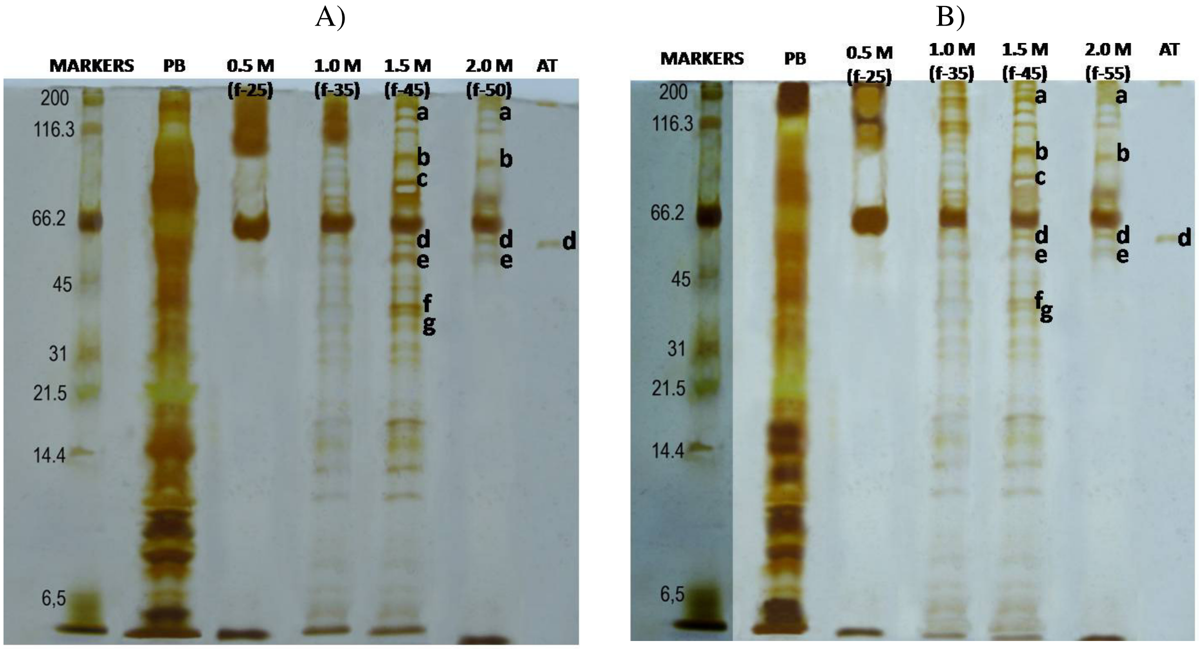

Figure 3: SDS-PAGE of the Fractions collected from the plasma protein adsorbed onto: A) mPANI-HS and B) mPET-HS. Elutions were carried out using phosphate buffer (PB) and increasing NaCl concentrations (0.5; 1.0; 1.5 and 2.0 M). The figures indicate the number of the fractions (f) collected according to the Fig.1 and Fig.2. Bands: a) factor XI; b) protein S; c) factor II; d) AT: antithrombin; e) factor VII; f) factor IIIand g) factor IIa.

4. Conclusion

The data presented confirm the presence of heparan sulfate bound covalently to magnetic derivatives mPANI and mPET. Therefore, these magnetic derivatives can be a useful tool in purification / separation not only of antithrombin process, but also of other proteins of interest to study the coagulation cascade.

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8 Conclusões

- As partículas magnéticas sintetizadas se mostraram adequadas quando submetidas a um campo magnético de 6000 Oe, facilitando seu manuseio em métodos de separação por afinidade.

- A heparina foi imobilizada com sucesso aos suportes magnéticos de PET, bem como à magnetita revestida com polianilina. Em um menor percentual, a heparina também ficou retida à magnetita sem o revestimento com PANI. Sendo assim, o revestimendo das partículas magnéticas com a polianilina (PANI) foram importantes para a efetivação do processo de imobilização covalente da heparina, podendo esta ser ativada ou não com EDAC/NHS. Porém, a heparina funcionalizada com EDAC/NHS imobilizada na matriz magnética revestida com PANI, apresentou uma atividade melhor no processo de fracionamento do plasma, onde houve uma maior ligação das proteínas a heparina presente neste suporte.

- Todas as matrizes magnéticas com heparina imobilizada se demonstraram estáveis em seu uso mesmo após 2 anos estocadas a 4 °C. A estabilidade também foi mantida após 10 ciclos consecutivos de reutilização no processo de separação por afinidade da antitrombina. Assim sendo, esses derivados tendem a ser uma alternativa interessante quanto ao apelo industrial.

- O dobro de quantidade do heparan sulfato foi covalentemente fixado às partículas de magnetita revestidas com PANI e também no PET magnetizado se comparado à matriz controle. Isso demonstrou que também é possível imobilizar o heparan sulfato quando devidamente funcionalizado com EDAC/NHS.

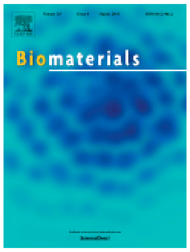
- As eletroforeses mostraram claramente a purificação da antitrombina em todos os suportes sintetizados. Este resultado foi corroborado com o teste amidolítico que avaliou a presença da antitrombina nos eluatos bem como sua atividade no retardo no tempo de coagulação plasmático.

- Os derivados contendo heparan sulfato, além da antitrombina, podem separar importantes proteínas da coagulação. Isso foi comprovado mediante os testes de TTPa e TP, os quais avaliam a via intrínsceca e extrínseca da coagulação das quais essas proteínas fazem parte.

- Os derivados magnéticos assim obtidos prestam-se à purificação de proteínas do plasma humano, especialmente a antitrombina, através da técnica de adsorção por afinidade à heparina e ao heparan sulfato.

9 Anexos

9.1 Instruções para Autores



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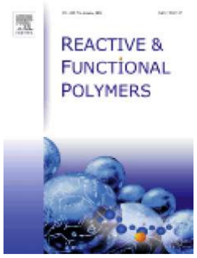

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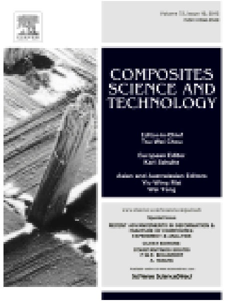

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9.2 Trabalhos apresentados em Congressos

- Antithrombin purification by heparin affinity chromatography using magnetic particles coated with polyaniline.

  Autores: MERCES, A. A. D. ; **SILVA, R. S.** ; CARVALHO JUNIOR, L. B.

  Reunião Regional Nordeste da Sociedade Brasileira de Bioquímica e Biologia Molecular SBBq e 4th International Symposium in Biochemistry of Macromolecules and Biotechnology, Recife, PE, 2012.

- Imobilização de heparina em partículas ferromagnéticas e seu uso na purificação de proteínas plasmáticas.

  Autores: MERCES, A. A. D. ; **SILVA, R. S.** ; CARVALHO JUNIOR, L. B.

  XI Curso de Inverno Bioquímica e Biologia Molecular.Faculdade de Medicina de Ribeirão Preto FMRP/USP, Ribeirão Preto, SP, 2012.

- Purificação de antitrombina via adsorção por afinidade à heparina imobilizada em dacron ferromagnético. Autores: MERCES, A. A. D. ; **SILVA, R. S.** ; CARVALHO JUNIOR, L. B.

  VII Reunião Regional da Federação de Sociedades de Biologia Experimental-FeSBE. Maceió, AL, 2012.

- Purificação de Proteínas Plasmáticas via Cromatografia de Afinidade à Heparina.

  Autores: MERCES, A. A. D. ; **SILVA, R. S.** ; CARVALHO JUNIOR, L. B.

  XVIII Semana de Biomedicina - Inovação em Saúde e Difusão do Conhecimento Científico, Recife, PE, 2011.

9.3 Premiações

- Antithrombin purification by heparin affinity chromatography using magnetic particles coated with polyaniline.

  MELHOR CARTAZ CIENTÍFICO, Prêmio “Marciolino Lins" apresentado na XI Reunião Regional Nordeste da SBBq.

- Imobilização de heparina em partículas ferromagnéticas e seu uso na purificação de proteínas plasmáticas.

  TERCEIRO MELHOR trabalho apresentado no XI Curso de Inverno em Bioquímica e Biologia Molecular da FMRP/USP.

- Imobilização de Heparina em partículas Ferromagnéticas e seu uso na Purificação de Proteínas Plasmáticas.

  SEGUNDO MELHOR trabalho apresentado no XIX CONIC da UFPE, Recife, PE.

9.4 Orientações e Colaborações

Co-orientação de **Aurenice Arruda das Merces.** Iniciação Científica – Curso de Biomedicina

- Imobilização de Heparina em partículas Ferromagnéticas e seu uso na Purificação de Proteínas Plasmáticas. 2011, Recife, PE.

- Heparinização de Partículas de Magnetita revestidas com Polianilina e seu uso na Separação por afinidade de proteínas plasmáticas. 2012, Recife, PE.

- Imobilização de Heparan Sulfato Em Partículas Ferromagnéticas e seu uso na Separação/Purificação de proteínas plasmáticas. 2013, Recife, PE.