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CENTRO DE BIOCIÊNCIAS
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AURENICE ARRUDA DUTRA DAS MERCES

IDENTIFICAÇÃO E PURIFICAÇÃO DAS PROTEÍNAS PLASMÁTICAS MEDIANTE O EMPREGO DE COMPÓSITOS MAGNÉTICOS COM HEPARINA

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Biologia Aplicada à Saúde, da Universidade Federal de Pernambuco, para obtenção do título de Doutora em Biologia Aplicada à Saúde.

Área de concentração: Ciências Biológicas 1

Orientador: Prof. Dr. Luiz Bezerra de Carvalho Júnior.

Coorientadora: Profa. Dra. Jackeline da Costa Maciel.

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O que sabemos é uma gota, o que ignoramos é um oceano (NEWTON, Isaac).

RESUMO

Heparina é um glicosaminoglicano de elevada carga negativa que pode ser utilizado como ligante de afinidade ou trocador iônico para purificação, identificação e depleção de proteínas. Por apresentar grupos funcionais reativos, a heparina, se torna bastante atrativa para os estudos em imobilização de biomoléculas. No presente estudo, foram sintetizadas e caracterizadas três diferentes partículas magnéticas: Dacron(polietilenotereftalato)-hidrazida magnético (mDAC), quitosana magnética (QS-MAG) e magnetita revestida com polianilina (MAG-PANI). Essas partículas foram magnetizadas a partir do método da co-precipitação de cloreto férrico e ferroso em meio aquoso e alcalino. Esses materiais serviram como suporte para imobilização covalente da heparina mediante a formação de uma ligação amida entre os grupamentos amina presentes nas partículas magnéticas com os grupos carboxílicos da heparina funcionalizados com carbodiimida (EDAC) e N-hidroxissuccinimida (NHS). A morfologia e distribuição das partículas foram analisadas por microscopia eletrônica (varredura e transmissão) e indicaram que mDAC, QS-MAG e MAG-PANI apresentaram um tamanho de 1-2 μm, 100-300 μm e 12 nm, respectivamente. Foram realizadas análises por difração de raio-X para evidenciar a presença da magnetita (Fe_3O_4) e dos polímeros (polietilenotereftalato, quitosana de baixo peso molecular e polianilina) nas diferentes partículas sintetizadas. Para finalizar a caracterização física dos materiais, medidas de magnetização foram realizadas, e todas as partículas sintetizadas demonstraram um comportamento superparamagnético. A saturação magnética (M*s*) para mDAC, QS-MAG e MAG-PANI heparina foram 23, 15 e 68 emu/g, respectivamente, nas temperaturas de 293 K, 300 K e 313 K. A quantidade de heparina imobilizada em mDAC, QS-MAG e MAG-PANI foi, respectivamente, 51 ± 0,1; 93,8 ± 1,93 e 43 ± 6,2 μg de heparina por mg de cada suporte. Na etapa de purificação/separação das proteínas plasmáticas, diferentes volumes de plasma humano total e diluído foram incubados com as partículas de mDAC e QS-MAG com heparina imobilizada covalentemente. A eluição das proteínas foi realizada com concentrações crescentes de NaCl entre 0,15 e 2,0 M em tampão fosfato 10 mM pH 7,4 ou tampão fosfato 10 mM pH 5,5 ou tampão Tris-HCL 50 mM pH 8,5. As nanopartículas de MAG-PANI com heparina imobilizada covalentemente foram utilizadas para avaliar a interação/afinidade com enzimas plasmáticas humanas específicas: trombina e Fator Xa. As proteínas que foram eluídas após incubação do plasma humano com a heparina imobilizada nas partículas magnéticas foram investigadas por eletroforese SDS/PAGE e/ou separadas por gel-filtração (Superdex G75 - AKTA Purifier) e/ou sequenciadas por LC/MS. Ensaios de atividade

biológica das proteínas purificadas foram analisadas pelo TP, TTPa e teste de inibição da trombina (cromogênico) e revelaram a presença de inibidores da família das serpinas, como por exemplo a antitrombina, que foram separados/purificados. Além disso, proteínas como albumina humana, protrombina, trombina e Fator Xa apresentaram afinidade à heparina imobilizada nas partículas magnéticas, o que garante a utilização dessa ferramenta como uma alternativa fácil e de baixo custo para a purificação e identificação dessas proteínas.

Palavras-chave: Afinidade. Heparina. Partículas magnéticas. Proteínas da coagulação. Purificação.

ABSTRACT

Heparin is a high negative charge glycosaminoglycan that can be used as an affinity ligand or ion exchange for protein purification, identification and depletion. Heparin contains has reactive functional groups that are very attractive for studies in immobilization of biomolecules. In the present study, three different magnetic particles were synthesized and characterized: Dacron (polyethylene terephthalate)-hydrazide (mDAC), magnetic chitosan (MAG-CH) and magnetite coated with polyaniline (MAG-PANI). These particles were magnetized from the co-precipitation method of ferric and ferrous chloride in aqueous and alkaline media. These materials served as a support for immobilization of heparin, which was covalently attached through by the amine groups present in the composites and the carboxylic groups of heparin functionalized with carbodiimide (EDC) and N-hydroxysuccinimide (NHS). The morphology and distribution of the particles were analyzed by electron microscopy (sccaning and transmition), so mDAC, MAG-CH and MAG-PANI presented sizes of 1-2 μm, 100-300 μm and 12 nm, respectively. X-ray diffraction analysis was performed to show the presence of magnetite (Fe_3O_4) and polymers (polyethylene terephthalate, low molecular weight chitosan and polyaniline) in the different synthesized particles. All magnetization measurements were performed, and the synthesized particles showed a superparamagnetic behavior. Magnetic saturation (Ms) for mDAC, MAG-CH and MAG-PANI was 23, 15 and 68 emu/g, respectively, at 293 K, 300 K and 313 K. The amount of immobilized heparin in mDAC, MAG-CH and MAG-PANI was, respectively, 51 ± 0.1; 93.8 ± 1.93 and 43 ± 6.2 μg of heparin per mg of each support. The plasma protein purification/separation steps, different volumes of total and diluted human plasma were incubated with mDAC and MAG-CH particles containing covalently immobilized heparin. Elution of the proteins were performed with increasing concentrations of NaCl (0.15-2.0 M) in 10 mM phosphate buffered saline pH 7.4 or 10 mM phosphate buffer pH 5.5 or 50 mM Tris-HCL buffer pH 8.5. MAG-PANI nanoparticles with covalently immobilized heparin were used to evaluate the interaction/affinity with specific human plasma enzymes: thrombin and Factor Xa. Proteins that were eluted after incubation of the human plasma with the heparin immobilized on the magnetic particles were investigated by SDS/PAGE electrophoresis or gel-filtration separated (Superdex G75 - AKTA Purifier) or sequenced by LC/MS. Biological activity assays of the purified proteins were analyzed by TP, TTPa and thrombin inhibition test (chromogenic) and revealed the presence of inhibitors of the serpin family, such as antithrombin, which were separated/purified. In addition, proteins such human

albumin, prothrombin, thrombin and Factor Xa showed affinity for heparin immobilized on magnetic particles. Therefore these materials can be used as an easy and inexpensive alternative tool for the purification and identification of these proteins.

Key-words: Affinity. Coagulation proteins. Heparin. Magnetic particles. Purification.

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LISTA DE ABREVIATURAS E SIGLAS

AT: antitrombina

BSA: albumina do soro bovina

CBB: corante azul brilhante de coomassie (R250)

DRX: difração de raios X

EDC: 1-etil-3-(3-dimetilaminopropil) carbodiimida

HSA: albumina sérica humana

K: kelvin

kDa: kilodalton

LC/MS: cromatografia líquida/espectrometria de massa

MAG: magnetita

MAG-PANI: partículas de magnetita revestidas com polianilina

mDAC: dacron hidrazida magnético

mDAC-hep: dacron hidrazida magnético com heparina imobilizada covalentemente

MET: microscopia eletrônica de transmissão

MEV: microscopia eletrônica de varredura

mM: milimolar

M_s: saturação magnética

NHS: N-Hydroxysuccinimide

Oe: Oersted

PBS: tampão fosfato salino 10 mM pH 7,4 150 mM NaCl

PET: polietileno tereftalato

QS: quitosana de baixo peso molecular

QS-MAG: partícula de quitosana magnética

QS-MAG-hep: partícula de quitosana magnética com heparina imobilizada covalentemente

SDS/PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TP: tempo de protrombina

TTPa: tempo de tromboplastina parcial ativado

SUMÁRIO

1 INTRODUÇÃO

As partículas magnéticas de óxidos de ferro têm despertado bastante interesse nas últimas décadas devido às suas excelentes propriedades físicas e químicas, além das diversas possibilidades de aplicações tecnológicas que variam desde dispositivos de memória magnética até aplicações biomédicas, como por exemplo agentes de contraste para ressonância magnética, *drug delivery* e hipertermia magnética (WU et al., 2015). A magnetita (Fe_3O_4) é uma das partículas de óxido de ferro mais estudadas e tecnologicamente mais usadas; além de ser um material magnético estável à temperatura ambiente é adequado para muitas aplicações biotecnológicas (MORALES et al., 2019). O revestimento das partículas de Fe_3O_4 com polímeros sintéticos, biocompatíveis ou biodegradáveis as tornam ainda mais atraentes. Essas partículas de Fe_3O_4 revestidas 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). A modificação da superfície das partículas magnéticas com grupos amina ou carboxílico, por exemplo, fornecem locais para imobilizar biomoléculas específicas e o núcleo magnético desses materiais modificados são responsáveis por uma rápida e fácil separação desses compósitos no meio reacional (ZHAO et al., 2019).

A heparina é um polissacarídeo de cadeia linear altamente sulfatado, formado por unidades repetidas de dissacarídeos, constituídos por um ácido urônico (glucurônico ou idurônico) e uma glucosamina (MOURIER et al., 2015) o que confere a presença de uma elevada carga negativa em sua estrutura e isto possibilita a interação da heparina com diferentes proteínas. Isso é possível devido à presença de sítios de ligação nas proteínas que contêm aminoácidos básicos (Lys e Arg) cujas cargas positivas, provavelmente, interagem com os grupamentos sulfatos e carboxilatos (carregados negativamente) presentes nas cadeias da heparina (ESKO et al., 2009). Mais de 400 proteínas, por exemplo, proteínas extracelulares, fatores de crescimento, quimiocinas, citocinas, enzimas, lipoproteínas e outras moléculas que estão envolvidas numa variedade de processos biológicos são capazes de interagir com a heparina (PEYSSELON e RICARD-BLUM, 2014).

A heparina imobilizada em suportes sólidos é amplamente utilizada na cromatografia de afinidade para a purificação e identificação de proteínas, as chamadas “proteínas de ligação à heparina”. Por apresentar grupos funcionais (sulfatos e carboxilatos) reativos, a heparina é

facilmente imobilizada a uma matriz, por isso o seu uso tem sido alvo de pesquisas na área de imobilização de biomoléculas (MURUGESAN et al., 2008).

Com base no exposto, a presente tese de doutorado teve como objetivo produzir partículas magnéticas e incorporar diferentes polímeros em sua estrutura para conseguir imobilizar a heparina e então utilizar esses compósitos magnéticos na separação e/ou purificação de proteínas do plasma sanguíneo humano. A heparina imobilizada em partículas magnéticas pode se tornar uma ferramenta bastante útil para obtenção de hemoderivados na indústria farmacêutica, ser aplicada como ferramenta de depleção de proteínas e em estudos de biomarcadores tumorais devido à sua ampla capacidade de ligação às proteínas plasmáticas.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Produzir e funcionalizar partículas magnéticas, imobilizar heparina e utilizar esses compósitos magnéticos na separação e identificação de proteínas do plasma sanguíneo humano.

1.1.2 Objetivos específicos

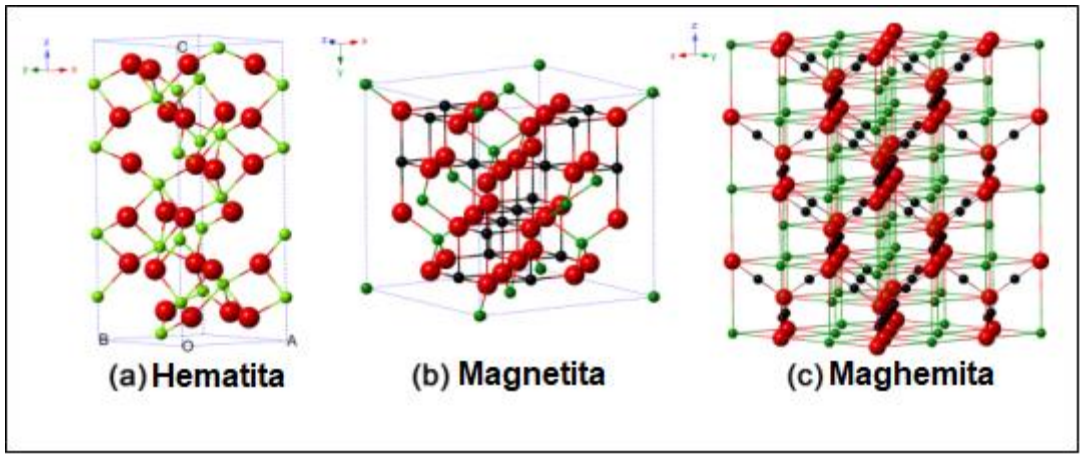
a) Produzir as partículas magnéticas pelo método de co-precipitação de sais de ferro II e III e modificá-las com polímeros orgânicos e inorgânicos;
b) Realizar a caracterização físico-química das partículas magnéticas sintetizadas;
c) Imobilizar e quantificar a heparina nas partículas magnéticas modificadas: mDAC (Dacron-hidrazida magnético), MAG-PANI (magnetita revestida com polianilina) e QS-MAG (quitosana magnética);
d) Utilizar a heparina imobilizada nas partículas magnéticas como um ligante de afinidade e trocador iônico;
e) Utilizar os compósitos na separação e purificação das proteínas plasmáticas.

2 REVISÃO DA LITERATURA

2.1 Partículas magnéticas: síntese e tipos de revestimentos

As partículas magnéticas à base de óxido de ferro como a magnetita (Fe_3O_4) e hematita (α-Fe_2O_3) pertencem a uma classe de materiais que oferecem várias possibilidades de aplicação e estão em constante crescimento na área biomédica, principalmente pelo fato de apresentarem propriedades únicas, incluindo o superparamagnetismo e a baixa toxicidade (PAYSEN et al., 2019). As estruturas cristalinas da α-Fe_2O_3 e Fe_3O_4 e são apresentadas na Figura 1a e 1 b, respectivamente. Estas partículas têm sido utilizadas como suporte para a imobilização de enzimas devido à várias vantagens, como fácil separação e reutilização do material através da aplicação de um campo magnético externo (WANG et al., 2015). Além disso, as nanopartículas de óxido de ferro estão sendo desenvolvidas como novos sistemas promissores com diferentes aplicações, tais como: agentes de imagem por ressonância magnética, mediadores de calor no tratamento do câncer por hipertermia, suportes sólidos em catálise heterogênea, biossensores, adsorventes para a remoção de corantes, metais e em sistema de liberação de drogas (DEBRASSI et al., 2011).

Figura 1 - Estruturas cristalinas das principais partículas magnéticas à base de óxido de ferro.



Fonte: Adaptado de WU et al. (2015).

As partículas magnéticas à base de óxido de ferro podem ser sintetizadas e obtidas por diferentes métodos tais como: co-precipitação dos íons Fe^{+2}/Fe^{+3} em uma solução aquosa utilizando uma base como agente de precipitação (WU et al., 2007; FRIED et al., 2001), técnicas de sol-gel (XU et al., 2007), métodos coloidais (MARTÍNEZ et al., 2007), reação de

pirólise (CHIU et al., 2007) entre outros. O procedimento mais barato e simples é o método de co-precipitação pois dispensa o uso de reagentes e equipamentos de alto custo (KANG et al., 1996; QU et al., 1999).

De acordo com Laurent et al. (2008), a técnica de co-precipitação é provavelmente a via mais eficiente para obter partículas magnéticas. Os óxidos de ferro tais como Fe_3O_4 ou γ-Fe_2O_3 são geralmente preparados por uma mistura estequiométrica de sais férricos e ferrosos em meio aquoso. A reação química de formação de Fe_3O_4 pode ser escrita de acordo com a equação abaixo:

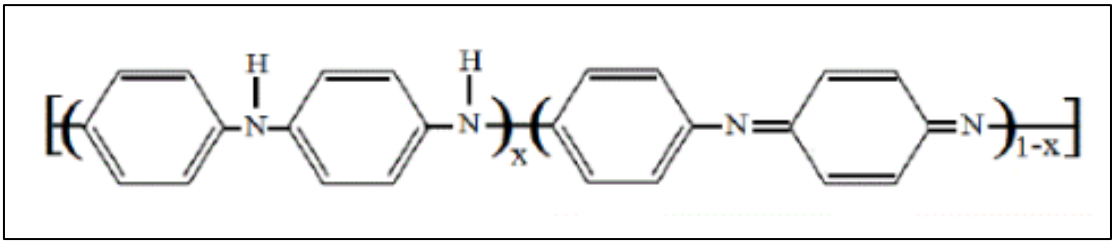
$$\mathbf{Fe^{+2} + 2Fe^{+3} + 8OH^{-} \rightarrow Fe_3O_4 + 4H_2O}$$

Ainda de acordo com os mesmos autores, a precipitação completa de Fe_3O_4 deve acontecer a um pH entre 8 e 14, com uma relação estequiométrica de 2:1 (Fe^{3+}/Fe^{2+}) em um ambiente não oxidante sem oxigênio.

Modificações das partículas de Fe_3O_4 usando polímeros sintéticos, biocompatíveis ou biodegradáveis com grupos funcionais específicos as tornam mais atraentes, pois as partículas de magnetita superparamagnéticas revestidas 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). Além disso, o revestimento é utilizado para evitar agregação, sendo possível revestir as partículas com uma camada de material geralmente diamagnético. As estratégias utilizadas para o revestimento podem ser divididas em dois grupos: (i) revestimentos com compostos orgânicos (polímeros, por exemplo) e (ii) revestimentos com materiais inorgânicos (sílica, carbono, ouro, etc.) (MORALES et al., 2019).

O revestimento de uma matriz magnética com a polianilina ou PANI apresenta vantagens, tais como a sua fácil síntese (eletroquimicamente ou mediante uso de agentes oxidantes) (SINGH et al., 2006), baixo custo e boa estabilidade ambiental (BALINT et al., 2014). A sua estrutura é composta por dois segmentos: uma estrutura plana de dois grupos imina e um anel quinóide e segmentos tetraédricos de dois grupos amina que separam três anéis benzênicos (Figura 2).

Figura 2 - Estrutura química da polianilina (PANI).

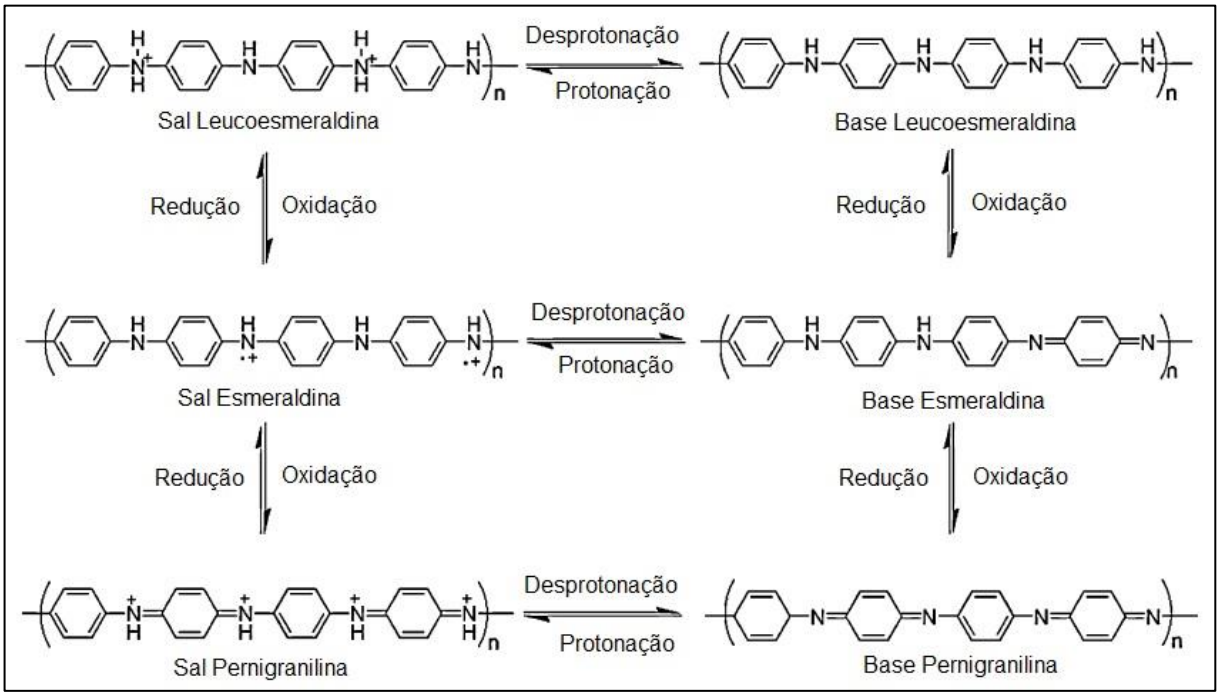


Fonte: Adaptado de HASOON (2014).

A PANI é sintetizada através da polimerização da anilina, que pode ser obtida por três métodos de polimerização: química, eletroquímica e fotoeletroquímica. O mais utilizado e vantajoso é o de síntese química, que possibilita uma reação a baixo custo. A síntese química envolve a oxidação direta do monômero de anilina por oxidantes químicos, sendo realizada na presença de um ácido forte em meio aquoso (MOLAPO et al., 2012).

A estrutura química da PANI pode ser facilmente modificada mediante protonação. A Figura 3 mostra as diferentes formas da PANI e a sua transformação por qualquer ácido/base ou por reações eletroquímicas (JARAMILLO-TABARES et al., 2012). Porém entre essas formas, a PANI esmeraldina, é a mais estável e condutora (BALINT et al., 2014).

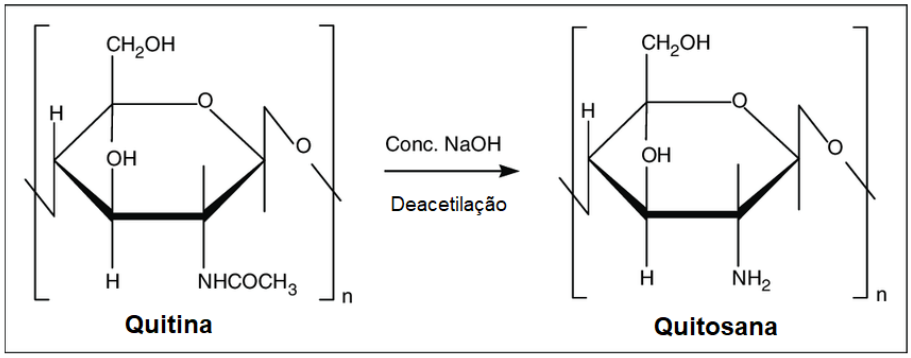
Figura 3 - Estruturas químicas da polianilina (PANI) em diferentes estados de oxidação.



Fonte: Adaptado de JARAMILLO-TABARES et al. (2012).

Outro exemplo de revestimento de uma matriz magnética é a quitosana (QS), um derivado alcalino N-desacetilado de quitina (Figura 4). A QS é o segundo biopolímero mais abundante encontrado na natureza depois da celulose (HO et al., 2016). Recentemente, em uma revisão feita por Ahsan et al. (2018), é demonstrado que devido às suas propriedades farmacológicas, a QS consiste em um importante biomaterial que pode ser utilizado em sistemas de liberação de drogas e, também, na engenharia de tecidos.

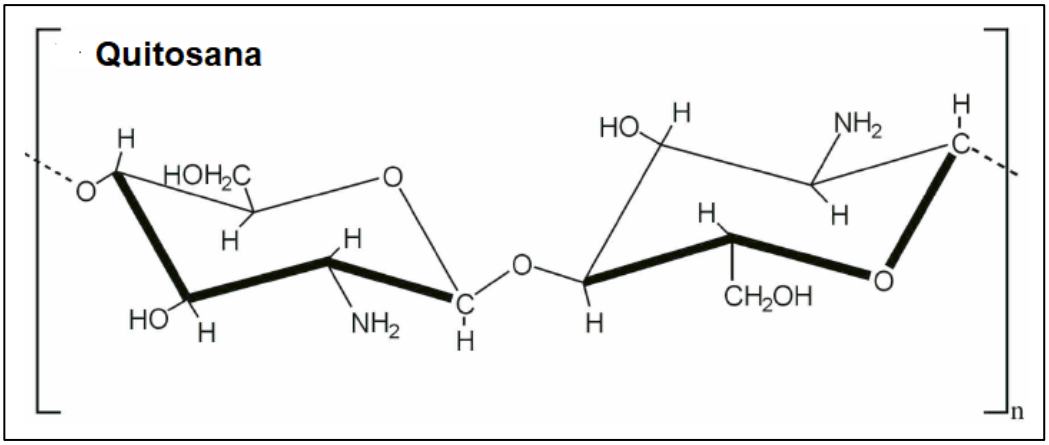
Figura 4 – Quitosana (QS) obtida a partir da deacetilação alcalina da quitina.



Fonte: Adaptado de DUTTA; TRIPATHI; DUTTA (2011).

QS também tem sido amplamente aplicada como suporte para biomoléculas devido à sua funcionalidade, não toxicidade, biocompatibilidade e baixo preço (HOU et al., 2016). As partículas de magnetita revestidas com QS podem ser um bom suporte para imobilização, pois possuem vários grupos funcionais, tais como grupos amino, hidroxila e hidroximetila (Figura 5) para ligação de drogas, proteínas, enzimas e outras moléculas biológicas (SAHIN; OZMEN, 2016). Além disso, a presença dos grupos amina permite usar esse material para imobilizar heparina (YANG; LIN, 2002), o que a torna bastante atraente para as aplicações biomédicas. Devido ao magnetismo e biocompatibilidade das nanopartículas magnéticas de QS, as enzimas imobilizadas podem ter alta atividade específica, fácil recuperação e maior estabilidade (WANG et al., 2015).

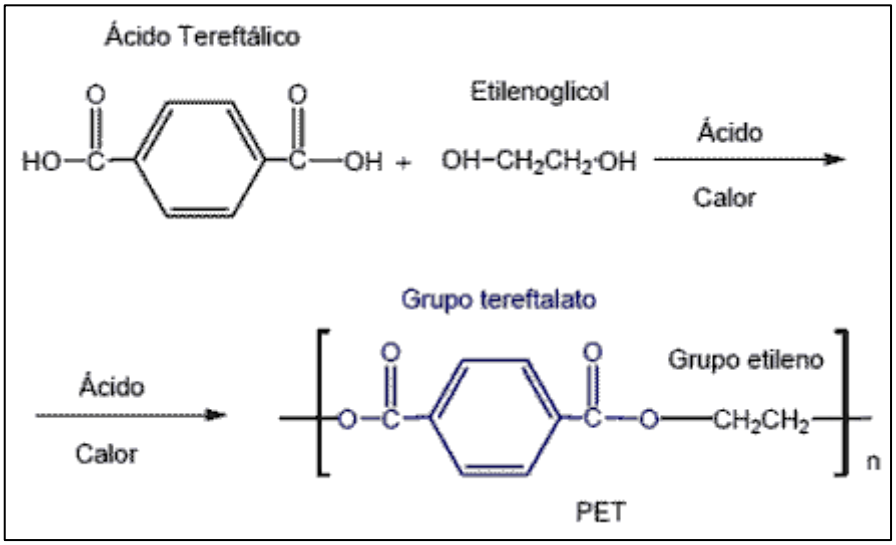
Figura 5 - Estrutura química da quitosana e seus grupos funcionais.



Fonte: Adaptado de LÓPEZ-GARCÍA et al. (2014).

Modificações de partículas magnéticas com o polietileno tereftalato (PET), ou Dacron (nome comercial) tem sido descrito na literatura (MERCÊS et al., 2016; AMARAL et al., 2006). O PET é um poliéster insolúvel em água, obtido comercialmente pela condensação do etileno glicol com o ácido tereftálico (Figura 6).

Figura 6 - Estrutura do polietileno tereftalato (PET) formado pela condensação do ácido tereftálico e etileno glicol.



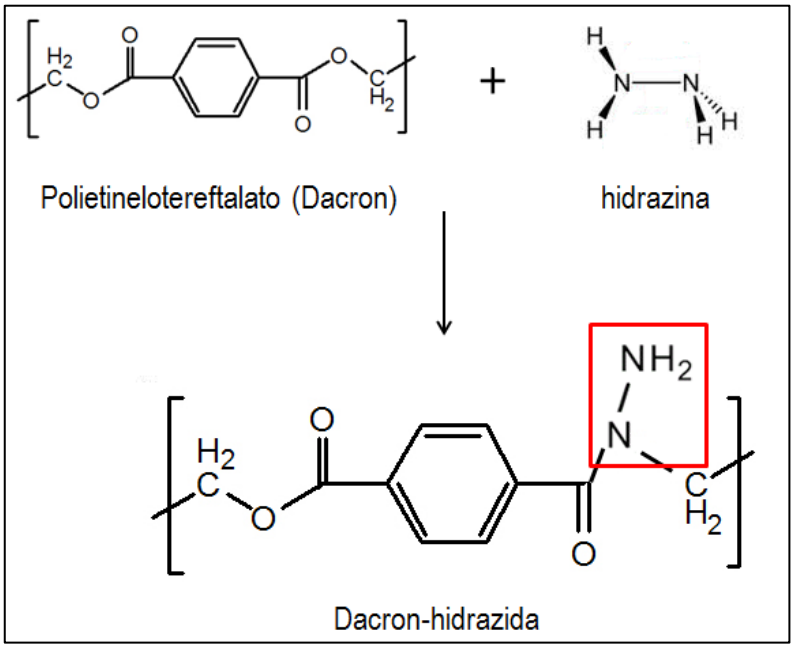
Fonte: MERCES (2016).

Como a maioria dos polímeros sintéticos, o PET é relativamente inerte e hidrofóbico sem grupos funcionais úteis para processos de imobilização. Para resolver este inconveniente, algumas modificações químicas são realizadas para alterar as propriedades de superfície desse

material. Um dos métodos inclui a reação do polímero com substâncias de baixo peso molecular contendo grupos hidroxila, carboxila ou amina, que após a reação esses grupos funcionais se incorporam na superfície do polímero. Os grupos funcionais criados durante os processos de modificação podem servir para imobilização covalente de várias biomoléculas como, por exemplo, carboidratos, peptídeos e proteínas (IRENA et al., 2009).

A Figura 7 demonstra um tipo de reação para modificação do PET a partir da adição de grupos amina com hidrato de hidrazina, descrito pela primeira vez por Carneiro Leão et al. (1991). Este suporte depois de magnetizado possibilita sua rápida separação de uma mistura reacional.

Figura 7 - Formação de Dacron-hidrazida a partir da reação de hidrazinólise do Dacron. Em destaque (vermelho) o grupo hidrazida formado.



Fonte: MERCÊS (2016).

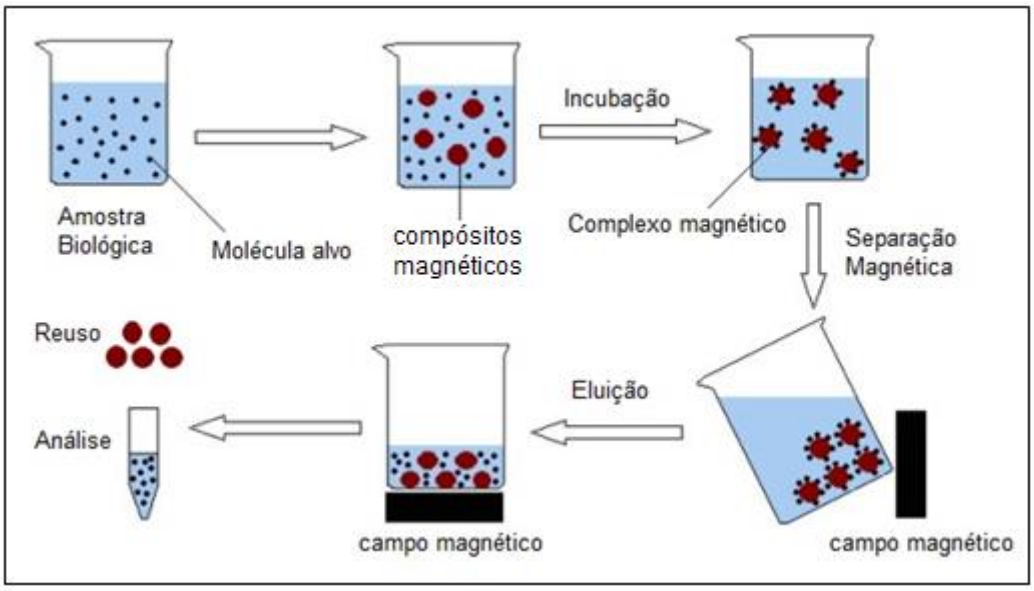
2.2 Separação magnética por bioafinidade

O processo de separação por afinidade é um poderoso método utilizado na purificação de proteínas e se baseia na formação de complexos específicos e reversíveis entre um ligante imobilizado e suas moléculas alvo a serem purificados. O uso da cromatografia de afinidade

magnética possui algumas vantagens, como ser realizada de forma mais rápida e utilizar processos de separação baseados em campo magnético (LAN et al., 2015).

A modificação da superfície das nanopartículas magnéticas com grupos tiol, amina ou carboxílico fornecem locais para imobilizar ligantes específicos e o núcleo magnético dessas partículas são responsáveis por uma rápida e fácil separação das substâncias adsorvidas nesses materiais (ZHAO et al., 2019). Os materiais magnéticos funcionalizados e com ligantes imobilizados exibem afinidade por uma molécula alvo quando são misturados com uma amostra contendo essas moléculas alvo e dentro de um período de incubação, as moléculas alvo ligam-se às partículas magnéticas. A separação do material magnético funcionalizado da amostra ocorre por meio da aplicação de um campo magnético externo e, após lavagens, as moléculas alvo são isoladas (HE et al., 2014). O método de separação por bioafinidade magnética está representado na Figura 8.

Figura 8 - Procedimento realizado na técnica de separação magnética por afinidade.



Fonte: Adaptado de CHEN et al. (2011).

Devido à facilidade e eficiência desse método, a separação magnética por bioafinidade, cada vez mais se destaca na área biotecnológica como uma ferramenta para obtenção e purificação de vários tipos de moléculas orgânicas. Ao utilizar essa técnica não há necessidade de diluir amostra e, comparando com outros métodos semelhantes, a perda de material durante as lavagens pode ser considerada mínima ou insignificante. Além dessas vantagens, é um método que garante uma separação rápida e eficaz das partículas magnéticas da mistura

reacional sem precisar utilizar métodos como filtração ou centrifugação (HORÁK et al., 2007), reduzindo o tempo e custo do processo.

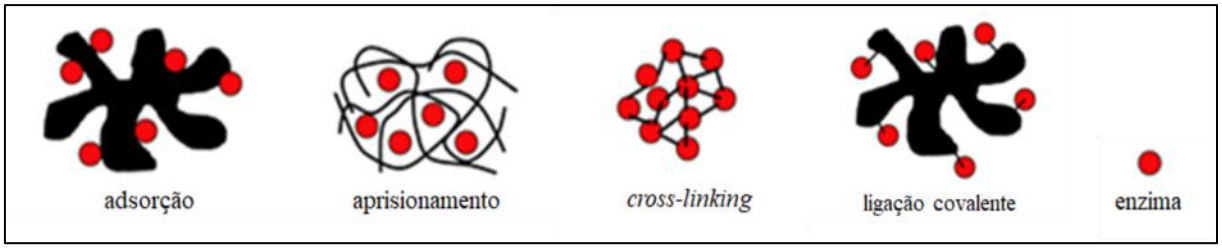
Recentemente Wang et al. (2020), imobilizaram um anticorpo (anti-apoAI) em partículas magnéticas para capturar o HDL do soro dos pacientes através do processo de biosseparação magnética, esta ferramenta foi utilizada como método para mensurar o colesterol não HDL no soro dos pacientes e monitorar o desenvolvimento do risco para doenças cardiovasculares. Lee et al. (2018) desenvolveram um método simples de purificação da proteína fetuína do soro bovino e da albumina do ovo por bioseparação magnética usando nanopartículas de Fe_3O_4 revestidas com sílica. Zhang et al. (2016) estudaram o uso de micropartículas hidrofóbicas com propriedades magnéticas para serem utilizadas na remoção eficaz do óleo na água em diferentes vias e Gao et al. (2016) utilizaram nanopartículas magnéticas "*imprinted*" de alta eficiência para separação e identificação de 17-β-Estradiol por afinidade magnética a partir do leite. Lan et al. (2015) realizaram um estudo sobre a purificação da enzima conversora de angiotensina através do processo de separação por afinidade magnética utilizando microesfera de agarose magnética.

O uso da separação por bioafinidade magnética também foi investigado para purificação de proteínas do plasma sanguíneo humano, como foi demonstrado por Mercês et al. (2016) que descreveram o uso de heparina imobilizada em partículas de Dacron magnético como matriz de afinidade para purificação de antitrombina a partir do plasma humano. E Sennikov et al. (2013) utilizaram cromatografia de afinidade e separação magnética para purificação de anticorpos para o fator de necrose tumoral a partir do soro humano.

2.3 Imobilização de biomoléculas

Imobilização é um processo pelo qual biomoléculas, como enzimas e proteínas, são aprisionadas em suportes insolúveis em água. Em especial, o uso de enzimas imobilizadas consiste em um processo de rotina aplicado na obtenção de produtos nas indústrias farmacêutica, química e de alimentos. Existem muitos métodos diferentes usados para imobilização de biomoléculas, mas a indústria e a maioria dos grupos de pesquisa sempre preferem métodos simples e econômicos. Os métodos mais utilizados (Figura 9) são baseados na imobilização física (adsorção ou aprisionamento físico) e imobilização química (ligação covalente e *cross-linking*) (BASSO; SERBAN, 2019).

Figura 9 - Métodos de imobilização de biomoléculas.



Fonte*:* Adaptado de MATHUR (2016).

De acordo com Liu, Ma e Shi (2019) a adsorção física é simples porque não há requisito para a funcionalização do suporte. Além disso, a conformação da enzima, por exemplo, pode ser mantida e a atividade catalítica da enzima imobilizada é relativamente alta. No entanto, na adsorção física, a lixiviação enzimática é um problema crítico que limita o uso da enzima imobilizada nas diferentes condições de reação. Para resolver esse problema, o aprisionamento é empregado para restringir a enzima nas estruturas poliméricas.

Ainda de acordo com os autores supracitados, nos métodos químicos, a enzima é firmemente imobilizada no suporte quimicamente modificado por meio de ligação covalente e *cross-linking*. O suporte modificado fornece, por exemplo, multipontos para a enzima, melhorando a estabilidade operacional da enzima imobilizada. O *cross-linking* é uma melhoria na ligação covalente porque a enzima é reticulada ao suporte com a ajuda de um reticulador.

De acordo com Mathur (2016), a ligação covalente é o método mais utilizado e como o nome sugere, este método utiliza grupos químicos presentes na biomolécula e no suporte para imobilização. Portanto, uma ligação covalente é formada entre grupos químicos da biomolécula e do suporte. Alguns métodos de ligação covalente são: *diazoation* e ligação peptídica. Na *diazoation* ocorre entre o grupo amino do suporte e os resíduos dos aminoácidos tirosina e histidina da enzima, por exemplo. Na ligação peptídica a ligação mais comumente formada ocorre entre aminoácidos da proteína e os grupos funcionais do suporte. Ocorre entre um grupo amina do suporte e um grupo carboxila de uma biomolécula. Ainda de acordo com o mesmo autor, as vantagens de utilizar a imobilização covalente é devido a obtenção de uma ligação muito forte entre suporte e biomolécula, ampla aplicabilidade e flexibilidade com os grupos funcionais. Algumas desvantagens como inativação ou eficiência reduzida da biomolécula é possível caso a conformação da biomolécula for alterada.

O suporte é um fator chave no processo de imobilização enzimática e a sua escolha atrai muita atenção. De uma forma geral, os suportes ideais para imobilização de biomoléculas devem apresentar excelente biocompatibilidade, propriedades físicas e químicas estáveis e abundantes locais de ligação para as biomoléculas específicas (HARTMANN; KOSTROV, 2013). Alguns suportes comumente empregados incluem nanofibras (LI; CHEN; WU, 2007), polímeros monolíticos (HASAN et al., 2013), materiais porosos como sílicas (HUDSON; COONEY; MAGNER, 2008), nanomateriais como nanotubos (GUPTA; MURTHY; PRABHA, 2018) e nanopartículas magnéticas (XU et al., 2014), entre outros.

Durante o processo reacional, é necessária uma recuperação fácil do ligante imobilizado, com a finalidade de separar os reagentes dos produtos finais. A fim de tornar o processo mais simples e barato, utilizar suportes magnéticos para essa finalidade tem sido alvo de estudos de muitos trabalhos na literatura. Por exemplo, diversos tipos de enzimas e outras biomoléculas têm sido imobilizadas covalentemente em diferentes suportes magnéticos há mais de três décadas pelo grupo IMOBIO (LIKA/UFPE). As moléculas e suportes magnéticos utilizados pelo grupo de pesquisa desde o início até os dias atuais são apresentadas no Quadro 1. As biomoléculas imobilizadas em suportes magnéticos são rapidamente e facilmente separadas do meio reacional a partir da aplicação de um campo magnético externo, além do baixo custo outra grande vantagem é a reutilização do material, além da excelente estabilidade físico-química (LIU; CHEN; SHI, 2018).

Quadro 1 - Diferentes biomoléculas e suportes magnéticos produzidos e estudados nas últimas décadas pelo grupo IMOBIO (LIKA/UFPE).

Suporte Magnético	**Biomolécula imobilizada**	**Referência**
Terra de diatomáceas magnética revestida com polianilina (mDE@PANI)	Tanase (enzima)	Lima et al. (2018)
Terra de diatomáceas magnética revestida com polianilina (mDE@PANI)	Invertase (enzima) β-galactosidase (enzima) Tripsina (enzima)	Cabrera et al. (2018)
Magnetita revestida com polianilina (mPANI)	Protease de *Penicillium aurantiogriseum* (enzima)	Duarte Neto et al. (2017)
Magnetita revestida com polianilina (mPANI)	Tripsina (enzima)	Maciel et al. (2016)
Dacron-hidrazida magnético (mDAC)	Heparina (carboidrato)	Mercês et al. (2016)
Sementes magnéticas de *Parkia pendula*	Lectina Concanavalina A (proteína)	Rêgo et al. (2014)
Argila magnética (mMMT) Terra de diatomáceas magnética (mDE)	Invertase (enzima)	Cabrera et al. (2014)
Levana magnética	Tripsina (enzima)	Maciel et al. (2012)
Dacron-hidrazida, POS/PVA magnético Quitosana magnética	α-l-Rhamnosidase de *Aspergillus terréus* (enzima)	Soria et al. (2012)
Dacron-hidrazida magnético	β-galactosidase (enzima)	Neri et al. (2011)
Magnetita revestida com polianilina (MAG-PANI)	β-galactosidase (enzima)	Neri et al. (2011)

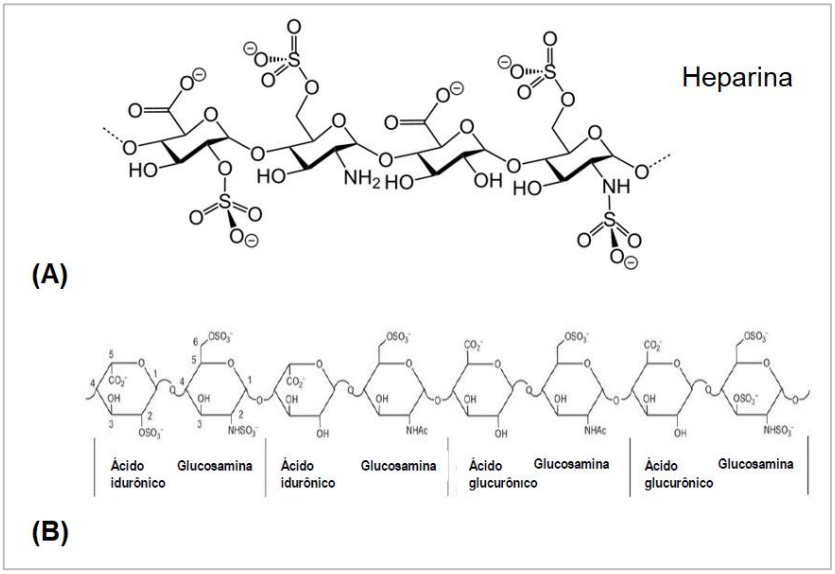
Polissiloxano magnetizado revestido com polianilina (mPOS – PANI)	β-galactosidase (enzima)	Neri et al. (2009)
Polissiloxano - álcool polivinílico magnético (mPOS-PVA)	β-galactosidase (enzima)	Neri et al. (2009)
Polissiloxano - álcool polivinílico magnético (mPOS-PVA)	β-galactosidase de *Kluyveromyces lactis* (enzima)	Neri et al. (2008)
Dacron-hidrazida magnético	Tripsina de *Oreochromis niloticus* (enzima)	Amaral et al. (2006)
Polissiloxano - álcool polivinílico magnético	Antígeno	Coelho et al. (2002)
Dacron ferromagnético	Albumina humana (proteína)	Leão et al. (1994)
Dacron ferromagnético	Proteínas	Leão et al. (1991)

Fonte: Autoria própria (2020).

2.4 Interação entre heparina e proteínas

A heparina consiste em um polissacarídeo aniônico formado por uma estrutura linear complexa com um peso molecular médio de 16 kDa e é amplamente utilizada na clínica como anticoagulante (LIU et al., 2017). A análise da sua estrutura química (figura 10) revela que a heparina consiste em unidades repetidas de dissacarídeos formados por →4) D-GlcA β(1→4) D-GlcN α(1→ e →4) L-IdoA α(1→4)D-GlcN α(1→, onde D-GlcA representa o ácido D-glucurônico, L-IdoA representa o ácido L-idurônico e D-GlcN representa D-glucosamina. Cada resíduo de açúcar pode conter grupos O-sulfo e GlcN ou também pode conter grupos N-sulfo ou N-acetil, resultando em uma mistura de moléculas sulfatadas com elevado comprimento de cadeia e peso molecular de 8000 a 40.000 Da (SOMMERS et al., 2017).

Figura 10 - Estrutura química da heparina (A) e distribuição dos resíduos formados por unidades repetidas de dissacarídeos (B).

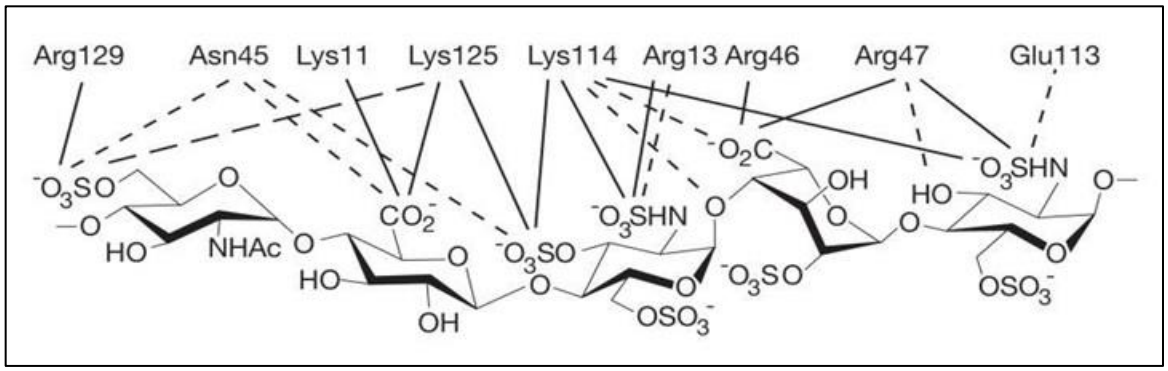


Fonte: Autoria própria (2018)

Mais de 400 proteínas, por exemplo, proteínas extracelulares, fatores de crescimento, quimiocinas, citocinas, enzimas, lipoproteínas que estão envolvidas numa variedade de processos biológicos, tais como formação da matriz extracelular, desenvolvimento, sinalização e câncer são capazes de interagir com a heparina (PEYSSELON; RICARD-BLUM, 2014).

A heparina é o glicosaminoglicano que possui a maior carga negativa distribuída em sua estrutura polianiônica poli-sulfatada. Análises críticas nos sítios de ligação entre heparina e proteínas revelaram uma distribuição específica de aminoácidos de cargas positivas que primordialmente estão envolvidos na interação eletrostática com a heparina carregada negativamente (Figura 11). Estudos iniciais realizados por Cardim e Weintraub (1989) mostraram que as proteínas de ligação à heparina contêm sequências conhecidas que são responsáveis pela interação e especificidade com a heparina. Alguns dos segmentos de ligação à heparina eram XBBXBX, XBBBXXBX e XBBXXBBBXXBBX, onde B é um dos três aminoácidos básicos (arginina, lisina ou histidina) e X é qualquer um dos outros 17 aminoácidos naturais (MORRIS et al., 2016)

Figura 11 - Interação entre os resíduos negativos da heparina com os resíduos de aminoácidos básicos das proteínas.



Fonte: Adaptado de ESKO et al. (2009).

A interação da heparina com a AT foi o primeiro caso relatado de uma interação de significado fisiológico entre a heparina e uma proteína específica (CAPILA; LINHARDT, 2002). Uma regulação eficiente da atividade da antitrombina requer a presença da heparina que possibilita o aumento da atividade de inibição da AT em milhares de vezes (MARIE et al., 2015). De acordo com Arisaka et al. (2013), além da AT, outras proteínas são capazes de se ligar à heparina, tais como fator de crescimento de fibroblastos básico, fator de crescimento endotelial vascular, fibronectina e fatores da coagulação e são descritas na literatura como "proteínas de ligação à heparina", pois apresentam uma elevada afinidade em interagir com esse glicosaminoglicano. Além disso, a heparina desempenha um importante papel na regulação da atividade e estabilidade destas proteínas.

2.5 Cromatografia de afinidade à heparina

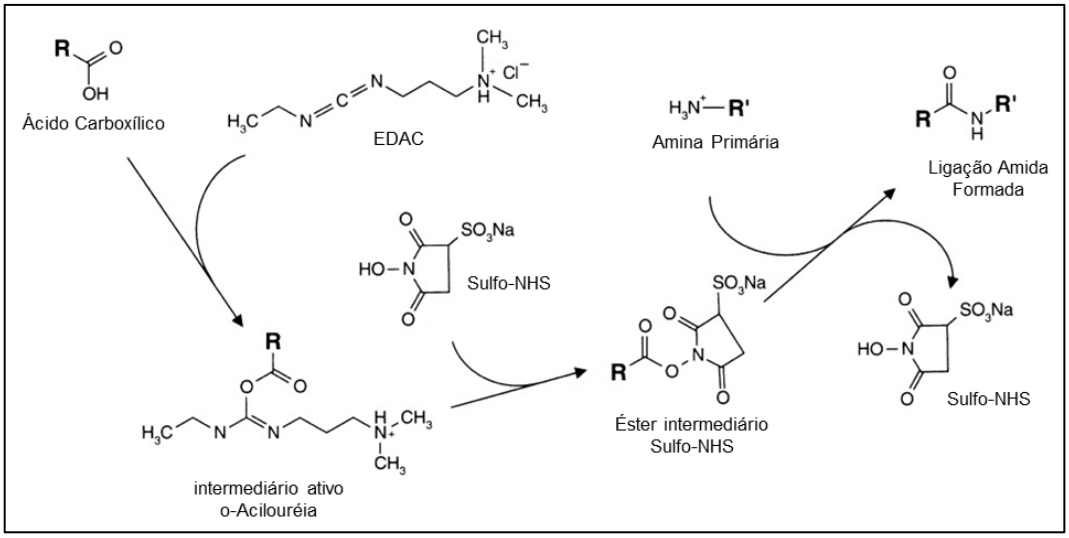
A imobilização do ligante é o fator mais importante em uma cromatografia de afinidade e pode determinar o sucesso do método, juntamente com o tipo de suporte ao qual o ligante é imobilizado (TEMPORINI et al., 2018). A heparina imobilizada em suportes sólidos é amplamente utilizada na cromatografia de afinidade para a purificação e identificação das chamadas "proteínas de ligação à heparina". Por apresentar grupos funcionais (sulfatos e carboxilatos) reativos, a heparina é facilmente imobilizada a uma matriz, por isso o seu uso tem sido alvo de pesquisas na área de imobilização de biomoléculas (MURUGESAN et al., 2008). Além disso, alguns trabalhos sobre imobilização de heparina são demonstrados na literatura com algumas aplicações relevantes, por exemplo Dang et al. (2019) imobilizaram a heparina

em microesferas para melhorar a compatibilidade sanguínea na hemoperfusão. Além disso, biomacromoléculas miméticas de heparina foram imobilizadas em nanopartículas de Fe_3O_4 por Li et al. (2020) para obtenção de um anticoagulante imobilizado em partículas magnéticas para ser reutilizado. Em um estudo realizado por Fellows et al. (2018), a heparina foi imobilizada em nanopartículas de óxido de ferro modificadas com um revestimento à base de poli (óxido de etileno) formando um compósito para ser utilizado no tratamento da hiperplasia neointimal. Mercês et al. (2016) sintetizaram compósitos magnéticos de Dacron com heparina imobilizada para usar como uma ferramenta na purificação de antitrombina humana.

Para que ocorra a ligação covalente da heparina, primeiramente é necessário que seus grupos carboxílicos passem por um processo de ativação através da adição de (1-etil-3-**(**3-dimetilaminopropil carbodiimide)**.** O EDAC ativa a os grupos carboxílicos da 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 (HERMANSON, 2008).

A reação de ativação dos grupos carboxílicos da heparina por EDAC/NHS está ilustrada na Figura 12. Estudos realizados por Oliveira et al. (2003) avaliaram as propriedades da heparina modificada com EDAC/NHS e relataram essa ativação como importante processo para imobilização da heparina em materiais.

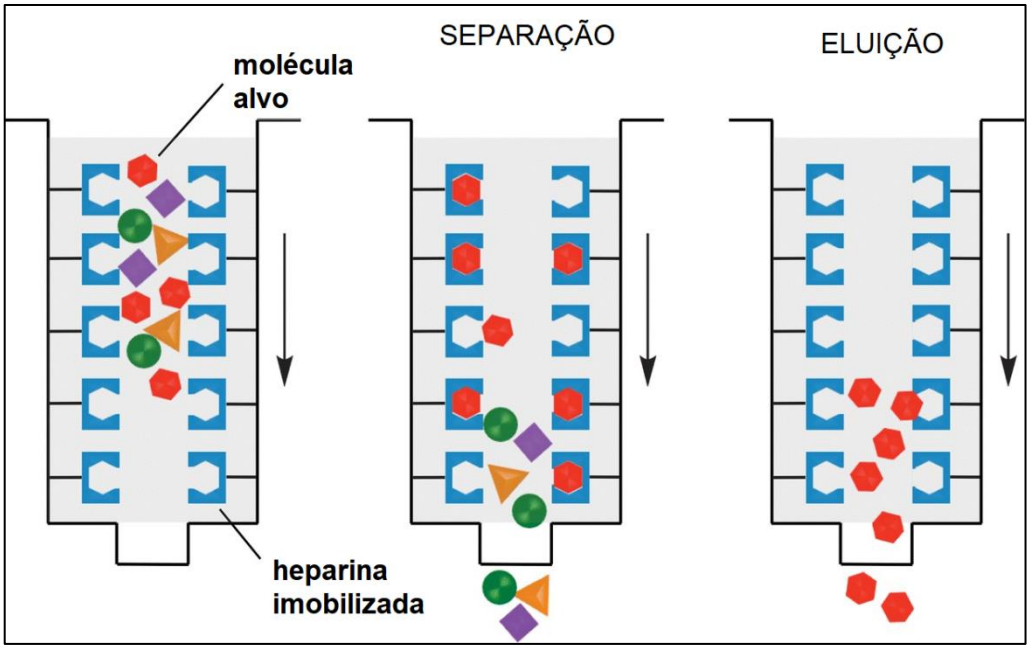
Figura 12 - Esquema da reação de ativação dos grupos carboxílicos por EDAC/NHS.



Fonte: Adaptado de HERMANSON (2008).

Devido as possíveis interações específicas entre a heparina com várias proteínas pode-se purificá-las mediante a chamada cromatografia de afinidade à heparina. Neste caso, a heparina é covalentemente imobilizada em um suporte ou esfera porosa e atua como um ligante de afinidade específico (Figura 13).

Figura 13 - Cromatografia de afinidade à heparina.



Fonte: Adaptado de: https://www.creative-biostructure.com/images/Custom-Affinity-Chromatography-Service.jpg. Acesso em 15/08/2019.

Com base em sua alta quantidade de grupos sulfatos aniônicos, ela também funciona como um trocador de cátions e com essa metodologia as misturas de proteínas podem ser separadas. Além disso, a aplicação de colunas de cromatografia por afinidade à heparina resulta frequentemente na concentração de proteínas de ligação a heparina a partir de lisados celulares, mesmo que as proteínas estejam presentes apenas em baixa concentração (BOLTEN et al., 2018).

Recentemente, Minkner et al. (2018) utilizaram a cromatografia de afinidade à heparina para concentrar as proteínas alvo semelhantes ao vírus do papiloma humano (HPV-LPs) durante a separação das proteínas contaminantes na amostra.

A elevada afinidade de ligação da AT com a heparina tem sido utilizada em sistemas de cromatografia de afinidade para o isolamento dessa proteína a partir do plasma (HEGER et al., 2002). Embora a cromatografia de afinidade seja uma boa abordagem para estudar essa interação entre a AT e a heparina, até agora essa metodologia não foi explorada para determinar constantes de ligação entre estes dois ligantes (MARIE et al., 2015).

A cromatografia de afinidade à heparina também foi utilizada para purificar lactoferrina a partir de leite de cabra, bovino e humano. Além de seu uso na purificação de alguns fatores de crescimento (fator de betacelulina e fator de crescimento de fibroblastos básico) a partir do soro de leite bovino (OUNIS et al., 2008).

Bjarnadóttir e Flensrud (2014) utilizaram cromatografia de afinidade à heparina para detectar proteínas do plasma humano e foi observado que entre essas proteínas muitas eram biomarcadores já descritos na literatura. Hu et al. (2010) utilizaram a cromatografia de afinidade à heparina para purificação do vírus da síndrome reprodutiva e respiratória dos suínos em cultura de célula, chegando a remover das células cerca de 96% desse vírus. A separação de pequenos componentes proteicos presentes no soro do leite foi realizada por Ounis et al. (2008) utilizando a cromatografia de afinidade à heparina.

A cromatografia de afinidade à heparina também 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 foi demonstrado que a albumina pode ser removida, por exemplo, através de técnicas de colunas de imunoafinidade, aprisionamento isoelétrico e cromatografia de afinidade (LEI et al., 2008).

2.6 Proteínas plasmáticas

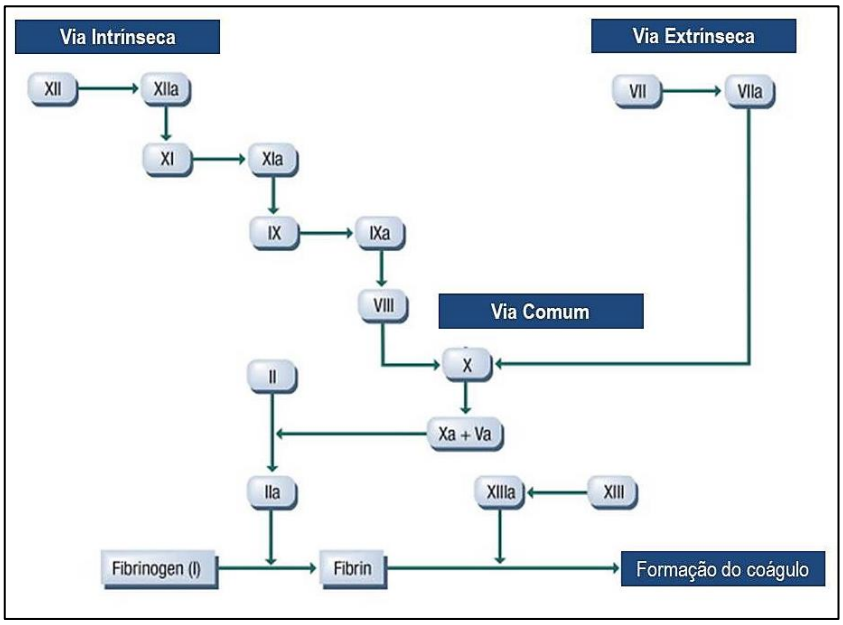
O grupo de proteínas plasmáticas inclui uma variedade de proteínas, tais como albumina (54%), globulinas (38%), fibrinogênio (7%), proteínas reguladoras (<1%) e fatores de coagulação sanguínea (<1%). Embora a concentração de fatores de coagulação no plasma humano seja muito baixa, essas proteínas são de vital importância para manter a hemostasia (SIEKMANN; TURECEK, 2020). Proteínas como imunoglobulina G (IgG), albumina, α-1-antitripsina, transferrina, IgA e haptoglobina representam mais de 85% da massa total de proteínas do plasma humano; apenas albumina e IgG correspondem a mais de 75% (KULLOLLI et al., 2013; URBAS et al., 2009).

Essas proteínas do plasma humano possuem uma ampla gama de funções biológicas e é um dos fluidos biológicos mais amplamente utilizados no diagnóstico clínico de diversas doenças. Como o plasma está em contato direto com todos os órgãos e tecidos, é provável que as alterações na função do corpo sejam refletidas na composição do plasma e, subsequentemente, alterações quantitativas no proteoma do plasma poderiam, assim, servir como biomarcadores de efeito sistêmico e clínicos de doenças (JASIM et al., 2019).

2.6.1 Proteínas da cascata da coagulação sanguínea

De uma forma geral, a cascata da coagulação consiste em uma série de reações proteolíticas através de serinas proteases geradas a partir de zimogênios. O evento final é a formação de um coágulo de fibrina que pode parar uma hemorragia em um vaso danificado. Convencionalmente, estas reações foram divididas em três vias (Figura 14): (i) via extrínseca - fator tecidual e fator VII; (ii) via intrínseca - fatores VIII, IX e XI e (iii) via comum - fatores II, V e X (CUGNO et al., 2014).

Figura 14 - Esquema das três vias da cascata da coagulação sanguínea humana.



Fonte: Adaptado de OVERBEY et al. (2014).

Quando ocorre um dano ao vaso, o fator tecidual (FT) é exposto e liga-se ao fator VII-VIIa, assim a via extrínseca é ativada iniciando o processo de coagulação (FURIE e FURIE, 1988; REZENDE, 2010).

Enquanto que na via intrínseca, a ativação do fator XII ocorre quando o sangue entra em contato com uma superfície contendo cargas elétricas negativas. Tal processo é denominado "ativação por contato" e requer ainda a presença de outros componentes do plasma: pré-calicreína (uma serinoprotease) e cininogênio de alto peso molecular (um cofator não enzimático). O fator XII, assim ativado, ativa o fator XI que, por sua vez, ativa o fator IX. O fator IX ativado, na presença de fator VIII ativado por traços de trombina e em presença de íons cálcio (complexo tenase), ativa o fator X da coagulação desencadeando a geração de trombina e, subsequentemente, formação de fibrina (DAVIE et al., 1991; REZENDE, 2010).

O mecanismo bioquímico do sistema hemostático da coagulação gera enzimas tais como a trombina, que precisam ser inibidas, após exercer sua função e, se houver um distúrbio desta inibição, ocasiona coagulação sistêmica descontrolada (MOORE et al., 2015). Desta forma, o controle fisiológico deste processo é determinado por anticoagulantes naturais, tais como a AT que inibe a trombina e outros fatores de coagulação; a proteína C e S, as quais inibem os fatores V e VIII; e o mecanismo fibrinolítico responsável pela dissolução de fibrina (FERNÁNDEZ; VILLAMEDIANA, 2012).

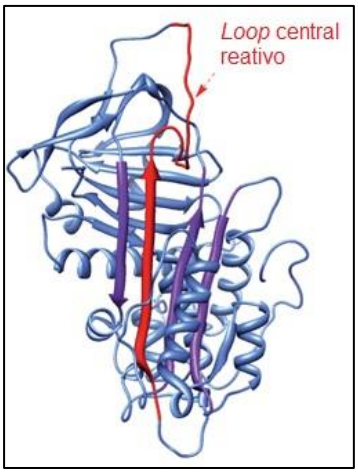
2.6.2 Serpinas

As serpinas consistem em um grupo de proteínas homólogas com diversas funções como por exemplo inibidores de proteases, e estão envolvidas em processos como coagulação sanguínea, fibrinólise, morte celular programada e inflamação. Aproximadamente 500 serpinas com 350 a 400 aminoácidos e peso molecular de 40 a 50 kDa já foram identificadas até hoje e estão presentes em uma variedade de espécies de animais e plantas. No plasma humano, representam aproximadamente 2% da proteína total, desse total 70% corresponde a α-1-antitripsina (α1AT, SERPINA1, também conhecida como inibidor α-1-proteina) (VAN GENT et al., 2003).

Além da α1AT, estão presentes na circulação sanguínea humana a AT (SERPINC1), cofator II da heparina (SERPIND1), inibidor-1 ativador de plasminogênio (SERPINE1) e inibidor da proteína C (SERPINA5), todos, de alguma forma, contribuem para a regulação da coagulação sanguínea (POLDERDIJK; HUNTINGTON, 2018). Certas mutações na constituição das serpinas levam a doenças referidas como serpinopatias e estão associadas ao desenvolvimento de angioedema (inibidor da C1-esterase), trombose (AT), enfisema (αlfa-1-antitripsina) e demência (neuroserpina). Serpinas com certas mutações desestabilizadoras têm uma tendência a se polimerizar, e tais polímeros podem ser encontrados em células e fluidos corporais (MARSZAL; SHRAKE 2006).

A AT é o principal inibidor das proteases da cascata da coagulação tais como fator Xa, trombina e fator IXa (AZHAR et al., 2016). Trata-se de uma glicoproteína do plasma humano que pertence à família dos inibidores de serina protease (serpinas). Além do seu efeito anticoagulante, a AT também possui propriedades anti-inflamatórias, que é observada quando há uma elevada concentração deste inibidor presente no sangue (MARIE et al., 2015). Com um peso molecular de 58 kDa, a AT é sintetizada no fígado e circula no plasma sanguíneo em duas formas principais onde a isoforma predominante (α-isoforma) constitui cerca de 90% do total de AT do plasma. A estrutura terciária da AT (Figura 15) baseia-se numa cadeia simples com cinco folhas α-β e um *loop* central móvel de reação (HEGER et al., 2002).

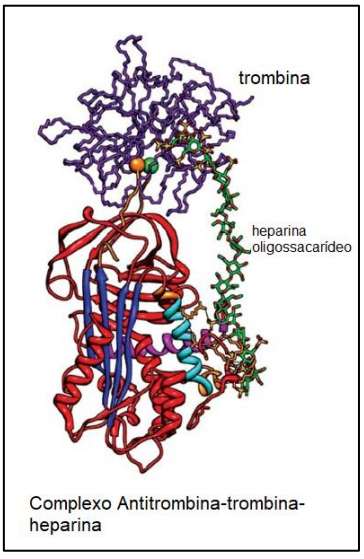
Figura 15 - Estrutura terciária da isoforma α da antitrombina (AT).



Fonte: Adaptado de: http://pubs.rsc.org/services/images/RSCpubs.ePlatform.Service.FreeContent.ImageService.svc/ImageService/Articleimage/2014/SC/c4sc01295j/c4sc01295j-f1.gif. Acesso em: 15/03/2018

Estima-se que a AT proporciona 80% do efeito anticoagulante natural contra a trombina, e o seu amplo espectro de inibição a torna um regulador chave do sistema de coagulação (ORNAGHI et al., 2014). A inibição da trombina realizada pela AT é fortemente aumentada pela sua ligação a sequências específicas de monossacarídeos presentes na heparina (Figura 16), glicosaminoglicanos e outros proteoglicanos que estão na circulação ou ao longo das superfícies endoteliais (KARLSSON; WINGE, 2004).

Figura 16 - Complexo da estrutura formada pela interação entre antitrombina (AT), trombina e heparina.



Fonte: Adaptado de RASHID e seus colaboradores (2014).

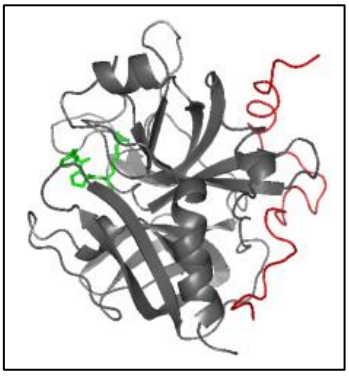
2.6.3 Protrombina

A protrombina ou fator II é o precursor da trombina, uma serina protease, que desempenha um papel fundamental na coagulação sanguínea. A trombina atua convertendo o fibrinogênio circulante em monômeros de fibrina, que depois se polimerizam para formar a fibrina que é o principal constituinte do coágulo sanguíneo. Além disso, a trombina também pode iniciar a inibição do processo de coagulação ativando a proteína C e a proteína S. Os níveis elevados de trombina no organismo podem resultar em doenças tromboembólicas, enquanto sua insuficiência pode induzir sangramento excessivo. Durante o processo de coagulação, a concentração de trombina varia acentuadamente, de nanomolar (nM) para milimolar (mM) (KIM et al., 2018; MELGE et al., 2018).

A protrombina é ativada na superfície de uma membrana fosfolipídica que se liga à extremidade amino da protrombina e aos fatores Va e Xa nas interações dependentes de Ca; o fator Xa remove o peptídeo de ativação e cliva a parte restante em cadeias leves e pesadas. O processo de ativação começa devagar porque o próprio fator V tem que ser ativado pelas pequenas quantidades iniciais de trombina (UNIPROT, 2018).

A estrutura da trombina é formada por duas cadeias polipeptídicas unidas por uma ponte dissulfeto. A cadeia menor é composta por 36 resíduos e é normalmente chamada de cadeia L (*light*) ou A. A cadeia maior possui 259 resíduos e é chamada de cadeia H (*heavy*) ou B (Figura 17). A cadeia L fica localizada no hemisfério oposto de onde está localizado o sítio ativo. Este fica localizado na cadeia H e é caracterizado por uma cavidade no centro da molécula (SOLOMONS, 2011).

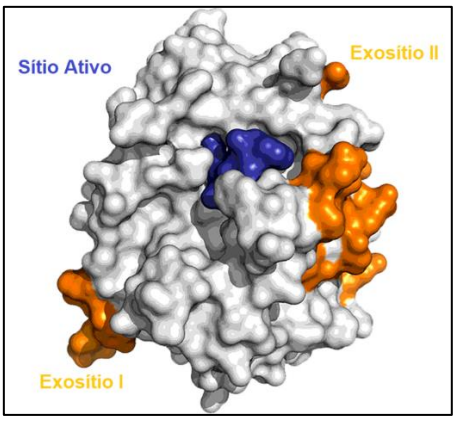
Figura 17 - Representação da estrutura cristalográfica da trombina humana. Vermelho - cadeia L; Grafite - cadeia H.



Fonte: MACHADO (2014)

A trombina possui dois exosítios distintos do sítio ativo de ligação (Figura 18), o primeiro exosítio está relacionado à interação do fibrinogênio com a trombina. O segundo exocítio é o local onde a heparina se liga diretamente à trombina , também chamado de sítio de ligação à heparina, que carrega um número de resíduos com carga positiva, incluindo Arg93, Arg97, Arg101, Arg126, Arg165, Lys169, Arg173, Arg175, Arg233, Lys235, Lys236 e Lys240 (AZIZ; DESAI, 2018).

Figura 18 - Localização dos exosítios I e II da trombina.



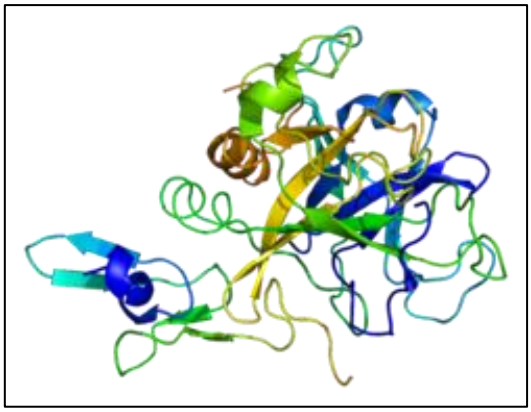
Fonte: Adaptado de PICA et al. (2017).

A heparina atua de forma indireta inibindo a trombina, pois a inibição ocorre através da AT que é auxiliada pela heparina. A heparina se liga à AT de maneira específica e ativa a trombina através de modificação alostérica da estrutura fornecendo um molde para que a AT possa interagir com a trombina (MACHADO, 2014).

2.6.4 Fator X

O fator X participa da fase intermediária da coagulação sanguínea e consiste em uma glicoproteína vitamina K dependente que é sintetizada no fígado e secretada no sangue como um zimogênio de 58,8 kDa (Figura 19). A conversão do fator X em sua forma ativa (fator Xa) é catalisada pelo fator IXa na presença do fator VIIIa, íons cálcio e fosfolipídios. Esta conversão também é catalisada pelo fator VIIa na presença de fator tecidual. O fator Xa cliva a protrombina em trombina na presença do fator Va, íons de cálcio e fosfolipídios (CUGNO et al., 2014).

Figura 19 - Representação da estrutura do fator X da coagulação sanguínea.



Fonte: Wikipedia, Acesso em 10/12/2019.
https://upload.wikimedia.org/wikipedia/commons/thumb/e/eb/Protein_F10_PDB_1c5m.png/250px-

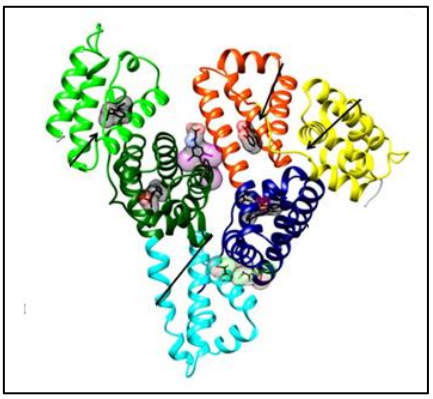
As deficiências adquiridas do fator X têm sido associadas com a não neutralização de anticorpos durante infecções, doenças malignas ou exposição a drogas (FRANCHINI et al., 2012)

2.6.5 Albumina

A albumina humana (HSA) é a proteína mais abundante presente no plasma sanguíneo humano, sua síntese é realizada no fígado. Essa proteína possui várias funções no organismo: manutenção da pressão osmótica coloidal, ligação e transporte de moléculas biologicamente importantes, atividade antioxidante, efeito circulatório, permeabilidade da membrana capilar, efeito neuroprotetor e estabilização do volume de líquido extracelular (RAOFUNIA et al., 2018).

Toda a estrutura da HSA (Figura 20) contém três domínios α-helicoidal homólogos: I (1-195 resíduos), II (196-383) e III (384-585) que são estruturalmente semelhantes. Esses três domínios consistem em dez hélices anti-paralelas e são divididos em dois subdomínios; um subdomínio A com seis hélices (h1-h6) e um subdomínio B com quatro hélices (h7-h10). Além disso, a HSA compreende 35 resíduos de cisteína envolvidos na formação de 17 ponte dissulfeto que estabilizam a conformação desta proteína globular. Assim, a organização globular dos domínios confere propriedades alostéricas à HSA monomérica, tornando-a capaz de interagir com múltiplos ligantes. A HSA contém apenas um resíduo de triptofano (W214), que é posicionado na vizinhança da cavidade hidrofóbica do subdomínio IIA (ASCENZI; FASANO, 2010; BIRKETT et al., 1977; FASANO et al., 2005; RABBANI; AHN, 2018; SUDLOW et al., 1976; ZUNSZAIN et al., 2008)

Figura 20 - Estrutura da albumina sérica humana (HSA). Os diferentes subdomínios da HSA são ilustrados em cores diferentes: subdomínio IA, em amarelo; subdomínio IB, em laranja; subdomínio IIA, em azul; subdomínio IIB, em ciano; subdomínio IIIA, em verde escuro; subdomínio IIIB, em verde.



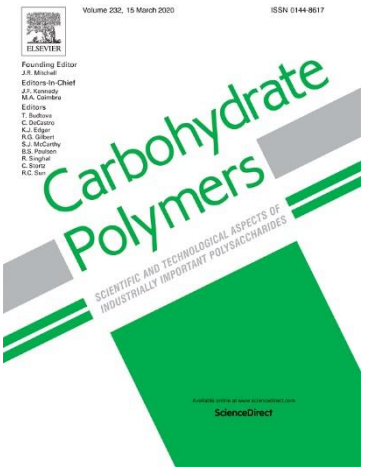
Fonte: RABBANI e AHN (2018).

A síntese da albumina ocorre no fígado a partir de uma proteína precursora chamada pró-albumina que é formada no lúmen do retículo endoplasmático dos hepatócitos na forma de pré-pró-albumina. Para a síntese de pró-albumina, um único gene é responsável por traduzi-lo em pré-albumina e a clivagem ocorre no retículo endoplasmático. O peso molecular da proteína é de 66,5 kDa. A concentração normal de albumina varia entre 35-50 g/L (RABBANI; AHN, 2018). O fracionamento do plasma fornece a possibilidade de obter albumina como hemoderivados, já que essa proteína possui alto valor terapêutico. Cohn et al., em meados do século XX, deram os primeiros passos no campo da tecnologia de fracionamento de plasma e até hoje técnicas tradicionais, como fracionamento, são desenvolvidas e usadas extensivamente (DENIZLI, 2011). Recentemente, além da importância terapêutica, a albumina tem se mostrado como o melhor e mais importante modelo de proteína para a realização de estudos na área de bioquímica e biofísica, incluindo a interação entre nanomateriais e proteínas (LI et al., 2018).

Embora a albumina represente um componente importante do plasma sanguíneo, a sua presença interfere na análise de proteínas de baixa abundância, que funcionam como biomarcadores de doenças. Desta forma para analisar esses componentes, a albumina deve ser removida seletivamente antes da análise e isso pode ser feito por imunoafinidade ou afinidade por ligantes imobilizados (ANDAC et al., 2013).

3 IDENTIFICATION OF BLOOD PLASMA PROTEINS USING HEPARIN-COATED MAGNETIC CHITOSAN PARTICLES

Artigo submetido na revista:



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ABSTRACT

Heparin was immobilized onto magnetic chitosan particles to be used as a tool to human plasma protein identification. Chitosan was magnetized by co-precipitation with Fe^{2+}/Fe^{3+} (MAG-CH). Heparin was activated by carbodiimide and N-hydroxysuccinimide and covalently linked to MAG-CH (MAG-CH-hep). X-ray diffraction confirmed the presence of chitosan and Fe_3O_4 in MAG-CH. This particle exhibited a superparamagnetism behaviour and 100-300 μm. Human plasma diluted with phosphate buffer 10 mM pH 5.5 or Tris-HCl buffer 50 mM pH 8.5 were incubated with MAG-CH-hep and the proteins fixed were eluted with these above buffers containing increasing concentrations of NaCl. The proteins obtained were investigated by SDS/PAGE, LC/MS and biological activity tests (PT, aPTT and enzymatic chromogenic assay). Inhibitors of the serpin family, prothrombin and human albumin were identified in this study. MAG-CH-hep can be used to purify these proteins and presents the following advantages: low-cost synthesis, magnet-based affinity, ionic exchange purification and reusability.

Keywords: bioaffinity; ionic exchange; magnetic; prothrombin; serpin;

1 INTRODUCTION

Immobilization of biomolecules at solid-phase magnetic materials such as magnetic particles is a great tool able to use rapidly and easy in biological separations and recovery from the reaction with an external magnetic field. Modify magnetic particles, for example, magnetite (Fe_3O_4), using some biocompatible polymers with specific functional groups will make them more attractive [1]. Modification of the magnetic particles with thiol, amine or carboxylic groups provide sites for immobilizing specific binders and the magnetic core of such particles are responsible for fast and easy separation of the adsorbed substances in such materials [2].

Chitosan (CS) is a 1,4-linked 2-amino-2-deoxy-β-D-glucan polysaccharide obtained usually by the alkaline deacetylation of chitin and has been widely used in biomedical research because it presents stability, hydrophilic, biocompatible characteristics and non-toxic material [3]. CS coated magnetic particles can be good immobilization support because it has several functional groups such as amino, hydroxyl, and hydroxymethyl groups for binding drugs, proteins, enzymes and other biomolecules [4]. Therefore, CS has both amino and hydroxyl groups that can be used to bind heparin or can be crosslinked with glutaraldehyde [5]. So, these groups are very useful for the covalent attachment onto surface of chitosan and when the CS is magnetized are used to immobilize different biomolecules and could have a high specific activity, easy recovery, and enhanced stability [6].

Heparin (hep) is a highly charged polyanion glycosaminoglycan that widely used as a clinical anticoagulant and structurally constituted of a complex mixture of linear anionic polysaccharides with an average molecular weight of 16 kDa [7]. Their disaccharide repeating units are formed for →4) D-GlcA β(1→4) D-GlcN α(1→ and →4) L-IdoA α(1→4)D-GlcN α(1→, where D-GlcA represents D-glucuronic acid, L-IdoA represents L-iduronic acid, and D-GlcN represents D-glucosamine. Each sugar residue can carry O-sulfo groups and the GlcN can also carry N-acetyl or N-sulfo groups, resulting in a mixture of sulfated molecules [8]. When immobilized heparin acts as an affinity ligand capable of purifying proteins that have an affinity for heparin. Several plasma proteins are known to have strong heparin-binding properties such as antithrombin [9] and thrombin [10]. In these proteins, in the heparin-binding region, there are distributions of positively charged amino acid residues that are involved in electrostatic interaction with the negatively charged heparin. Such electrostatic interactions have been exploited by cation exchange chromatography to purify several positively charged proteins [11]. Mercês et al. [12] described the use of immobilized heparin on Dacron magnetic particles as an affinity matrix for antithrombin purification from human plasma.

Serpins are a group of homologous proteins found in various species of plants and animals with sizes about 400 amino acids and a molecular weight between 40 and 50 kDa. Initially, they were identified for shows protease inhibition activity and are involved in a blood coagulation, fibrinolysis and inflammation process, for example [13]. Several serpins are present in human plasma circulation, including α1-antitrypsin (α1AT, SERPINA1 or α1-proteinase inhibitor), antithrombin (SERPINC1), plasminogen activator inhibitor-1 (SERPINE1) and protein C inhibitor (SERPINA5), all of which contribute in some measure to the regulation of hemostasis process [14]. Serpinopathies are diseases associated with certain conformational mutations in serpins that are associated above all with thrombosis (antithrombin, AT) and emphysema (α1AT; α1-antichymotrypsin, ACT) conditions [15].

Prothrombin is the precursor to thrombin, the main serine protease which plays a key role in blood coagulation. It is involved in the conversion of circulating fibrinogen to fibrin monomers in blood clots at the final step of the coagulation cascade. Moreover, it can also initiate the inhibition of the coagulation process by activating protein C and protein S [16]. The monitoring thrombin is of significant importance for early diagnostics of thromboembolic and hemorrhagic complications because excessive thrombin levels in the body can result in thromboembolic diseases and thrombin insufficiency can induce excessive bleeding [17]. Heparin and unfractionated Heparin (UFH) can bind to thrombin directly by a site called exosite 2, or alternatively, the heparin-binding site, which carries a number of positively charged residues including Arg93, Arg97, Arg101, Arg126, Arg165, Lys169, Arg173, Arg175, Arg233, Lys235, Lys236 and Lys240 [10].

Human albumin (HSA) is the most abundant protein present in human blood plasma and its exhibit several functions, for example, maintenance of colloidal osmotic pressure and binding or transport of biologically important molecules [18]. The fractionation of plasma provides the possibility of obtaining albumin as blood products since this protein has high therapeutic value. In addition, albumin has been shown to be the best and most important protein model for the study of biochemistry and biophysics, including the interaction between nanomaterials and proteins [19]. Although albumin represents an important component of blood plasma, its presence interferes with the analysis of low abundance proteins, which function as disease biomarkers. In order to analyze these components, albumin should be selectively removed prior to analysis and this may be done by immunoaffinity or affinity for immobilized ligands [20].

Therefore, this study aimed to synthesize and to characterize magnetic chitosan particles with immobilized heparin to serve as an alternative tool to human plasma protein bioseparation

or purification. These materials have several advantages including easy synthesis using low-cost reagents and easy removal from the incubation mixture by applying a magnet and the particles are reusable.

2 MATERIAL AND METHODS

2.1 Materials and reagents

Heparin sodium salt (5.000 UI/mL) from Cristália© (São Paulo, Brazil). Carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EDC), N-hydroxysuccinimide (NHS), ferric and ferrous chloride, benzamidine hydrochloride (99%, MW: 156.61), thrombin from bovine plasma, chitosan low molecular weight (50-190 kDa, 75-85% deacetylated) were purchased from Sigma Chemical Compan (Saint Louis, MO, USA). PT and aPTT and PT reagents were obtained from Dade Behring (Marburg, Germany) and stored at 4° C. Chromogenic substrate (Tosyl-Gly-Pro-Arg-AMC) was purchased from Bachem Americas, Inc. (Torrance, CA, USA). Human blood was collected from a volunteer underapproval of the Ethical Committee of the Universidade Federal de Pernambuco.

2.2 Magnetic chitosan particles preparation

Synthesis of magnetic chitosan particles was performed by co-precipitation method similar as was described to levan magnetic particles by Maciel et al. [21]. Suspension of chitosan low molecular weight (2.0% w/v) in distilled water stayed under stirring and then a solution 1:1 of $FeCl_3$ (1.1 M) and $FeCl_2$ (0.6 M) was added and finally the pH was adjusted to 11 with ammonium hydroxide. That mixture remained under manual stirring for 30 minutes at 80 °C. In the end, with a strong magnet, the particles were washed to the neutral pH range and magnetic chitosan particles (MAG-CH) was obtained.

2.3 Morphology, magnetic properties and structure analysis

Distribution and morphology of the particles were analysed by scanning electron microscopy (SEM) TESCAN-Mira3. Structure samples were characterized by X-ray diffraction (XRD) performed at room temperature in the range 10°–90°, in equal 2θ steps of 0,02°, using

Bruker D8 Advance Davinci diffractometer with CuKα radiation (λ = 1,5406 Å). Magnetization measurements (*M*s) was obtained using a vibrating sample magnetometer (VSM), VersaLab, manufactured by Quantum Design, at temperatures of 293 K, 300 K and 313 K, with magnetic fields in the range -30000 Oe to +30000 Oe.

2.4 Immobilization and determination of heparin

The process of immobilizing heparin in the MAG-CH particles was performed according to Mercês et al. [12]. First, a solution of heparin (3 mg/mL) was previously activated with EDC and NHS this reaction is necessary to carboxylic groups activation. An aliquot (1 mL) of this functionalized heparin solution was incubated with 30 mg of MAG-CH for 72 h at 25°C with mild agitation yielding the covalently immobilized heparin on the magnetic chitosan particle (MAG-CH-hep).

These composites were recovered under a magnetic field (0.6 T) and washed three times with distilled water to remove non-immobilized heparin. Under this magnetic field the particles precipitation occurred in about 10 seconds. The supernatant, first washed and second washed were incubated with methylene blue at room temperature. This resulted in a complex of methylene blue with heparin. The solution was measured at 664 nm using a Shimadzu UV Visible Spectrophotometer (UVmini-1240) [22]. The calibration curve was obtained through measurement of the absorbance of a series of standards at heparin concentrations of 10–100 μg/mL. The amount of immobilized heparin was calculated from the calibration curve.

2.5 Interaction study between MAG-CH-hep and plasma proteins

The magnetic composites with immobilized heparin were incubated with: (a) diluted blood plasma (4:1) in 10 mM phosphate buffer pH 5.5 (4:1) and (b) diluted blood plasma (4:1) in 50 mM Tris-HCl buffer pH 8.5. Boths plasmas were treated with benzamidine hydrochloride (2 mM) to prevent proteases degradation. The incubation time was 30 minutes at 4 °C with 30 mg of MAG-CH-hep for each study. After this time, using a magnetic separation plaque (0.6 T), washes and elution were carried out with 10 mM phosphate buffer pH 5.5 or 50 mM Tris-HCl buffer pH 8.5 supplemented 0.15, 1.0, 2.0 M NaCl. The proteins were monitored at 280 nm (Shimadzu UV Visible Spectrophotometer - UVmini-1240). The protein peaks obtained

were pooled, dialyzed and finally dried in a speed vac. Proteins were quantified by the Bradford method [23].

2.6 Proteins identification

After dialysis, the proteins eluted at different concentrations of NaCl have applied (10 µg) on 10.0% SDS-PAGE electrophoresis (non-reduced samples). The gel was stained with a solution of Coomassie brilliant blue, CBB, (R250) Bands of proteins were excised then bleached for further digestion using trypsin (10 ng/µl in 50 mM ammonium bicarbonate). Molecular weight and sequence of major proteins isolated on the electrophoresis gel were analyzed by LC/MS. The analyses were performed on the Synapt G2 HDMS (Waters) mass spectrometer coupled to nanoAcquity UPLC system (Waters). The peptides were analyzed in the BLAST® NCBI online database.

2.7 Coaguation assays in vitro

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were the initial tests to evaluate the inhibitory activity of proteins present in the eluates obtained from different concentrations of NaCl in buffers with two different pH ranges (5.5 and 8.5). The measurements were made using a semi-automated coagulometer (BFT II - Dade Behring) according to Salu et al. [24] and Silva et al. [25]. It was performed dose-response tests to verify the action of the inhibitor according to the amount incubated in the plasma, the controls were performed with 0.15 M and 0.7 M NaCl.

Eluates with a significant presence of inhibitors (1.0 M and 2.0 M in pH 5.5) were subjected to a chromogenic assay with thrombin to assess their inhibition. Inhibition was also evaluated in the presence of heparin. To perform the assay the enzyme used was bovine thrombin (18 nM) in 20 mM Tris-HCl 0.15 M NaCl, the chromogenic substrate was Tosyl-Gly-Pro-Arg-AMC (18 µM), the heparin used was of 0.021 U or 0.0625 U and 40 µL of 1.0 or 2.0 eluate obtained in elution with NaCl in 10 mM phosphate buffer pH 5.5. The reading was made in spectrum fluorimeter: excitation at 380 nm and emission at 460 nm for 90 minutes, with reading, collected every five minutes.

3 RESULTS AND DISCUSSION

3.1 Physical characterization of magnetic particles

After magnetization of chitosan (MAG-CH) by chemical co-precipitation method of Fe(II) and Fe(III) ions, the morphology of the particles analyzed by scanning electron microscopy (SEM) revealed heterogeneous particles with structures ranging between 100 and 300 µm (Figure 1). Furthermore, on the surface of the particles, it is possible to observe lumps corresponding to the magnetite (Fe_3O_4) crystals (Figure 1b - arrows) present in the chitosan structure. Also, in the Figure 1b (MAG-CH), it is possible to observe a very irregular surface. These irregularities increase the contact area of the magnetic chitosan particles, increasing interaction with biomolecules.

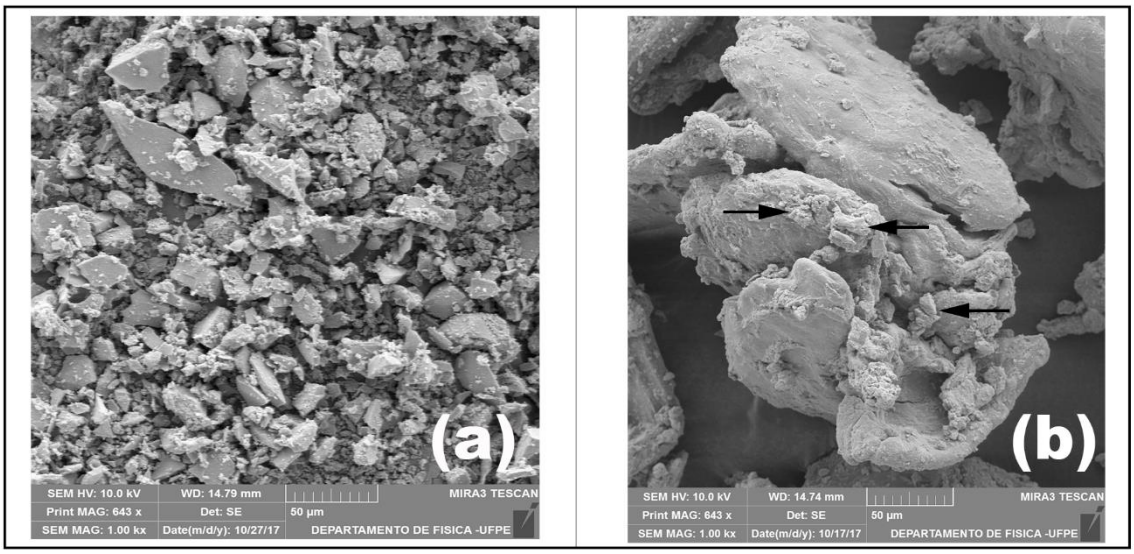

Figure 1. Scanning electron microscopy images of magnetite (a) and magnetic chitosan (b) particles. Black arrows: magnetite (Fe3O4) lumps.

According to the result obtained by X-ray diffraction (XRD) analysis (Figure 2), the magnetic chitosan particles are composed of two phases: an amorphous and a crystalline phase represented by chitosan (organic polymer) and magnetite crystals (Fe_3O_4), respectively. Six peaks for characteristic magnetite were observed in the samples of magnetite (MAG) and magnetite chitosan particles (MAG-CH), corresponding to (220), (311), (400), (422), (511) and (440) planes (Table 1 and Figure 2). Diffractogram of chitosan (CH) and magnetite chitosan particles (MAG-CH) exhibited typical peak of chitosan at $2\theta = 20°$ [26].

Table 1. Peaks obtained from the analysis of the particles by X-ray diffraction.

Particles	*Angles positions (2θ) of peaks and planes*
CH-MAG	10.35°; 19.79°; 30.07° (220); 35.48°(311); 43.23° (400); 53.64°(422); 57.12° (511); 62.81° (440);
CH	10.21°; 19.61°
MAG	18.27° (111); 30.39° (220); 35.62° (311); 43.23° (400); 53.69° (422); 57.3° (511); 62.81° (440);

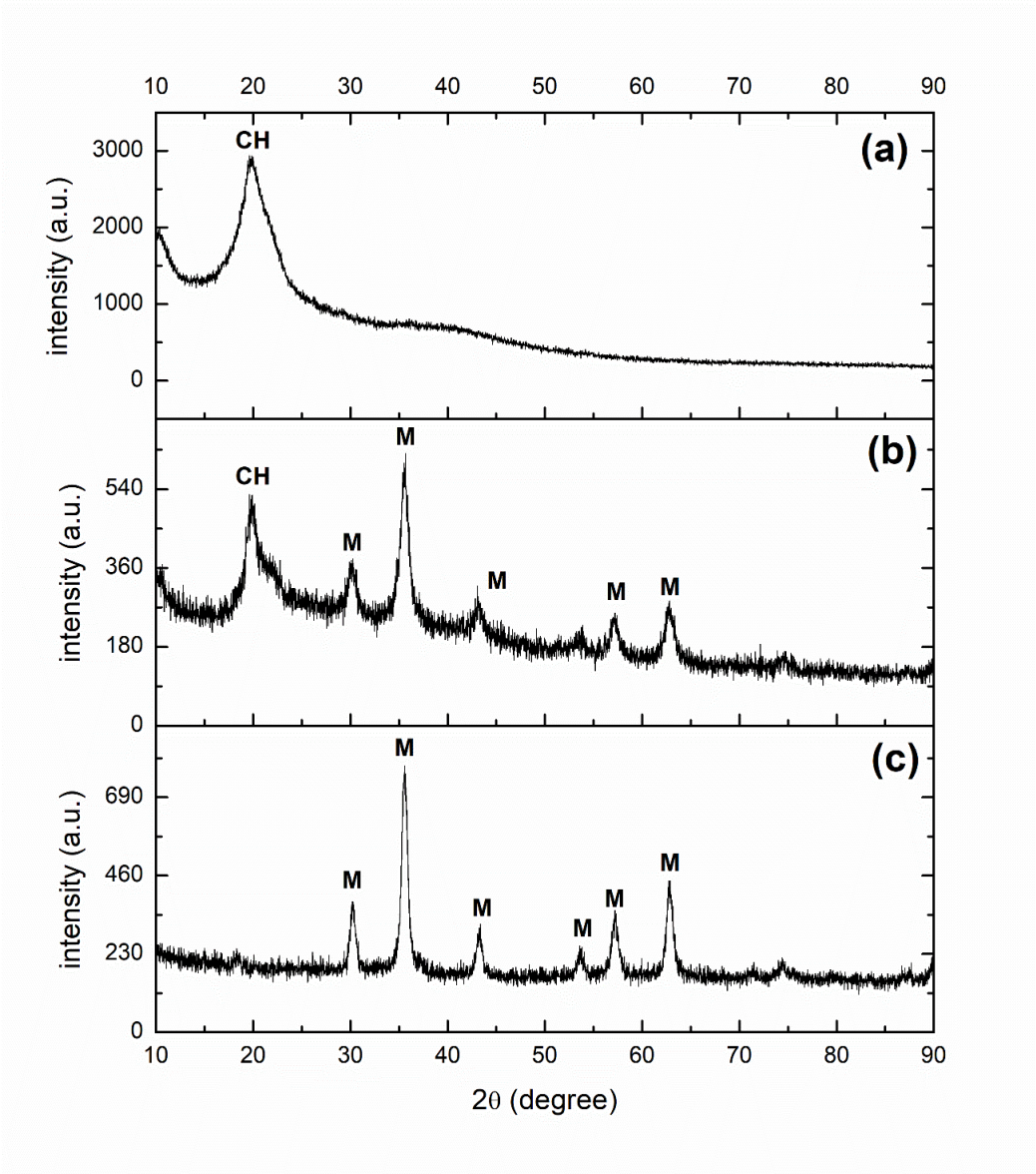

Figure 2. X-ray diffraction patterns of the chitosan (a), magnetic chitosan (b) and magnetite (c) particles. M: magnetite phase. CH: chitosan phase.

Isothermal magnetization curves M(H) measured at 293 K, 300 K and 313 K with magnetic field up to 30 kOe applied at the magnetic particles synthesized are presented in figure 3. The magnetic saturation (*M*s) values obtained for the magnetite (Figure 3a) were 72, 72 and 71 emu/g and for MAG-CH (Figure 3b) were 15, 16 and 15 emu/g at 293 K, 300 K and 313 K, respectively. *M*s determine the value of the magnetization present in a sample that has measured from the application of a constant magnetic field in this magnetized sample. The magnetite particles produced present *M*s similar to the bulk magnetite (*M*s of 92 emu/g) [27]. The magnetic saturation of MAG-CH was 4.8 times lesser to magnetic particles (MAG). The decrease in magnetic saturation of MAG-CH compared to magnetite bare particles is due to the presence of chitosan polymer on the magnetic particles as also observed by other authors [28, 29, 30]. However, separation of the magnetic chitosan particles is done easily with an external magnet [30]. A very similar result described in this work was obtained by Sahin and Ozmen [4] who synthesized particles of magnetic chitosan with a *M*s of 28.7 emu/g.

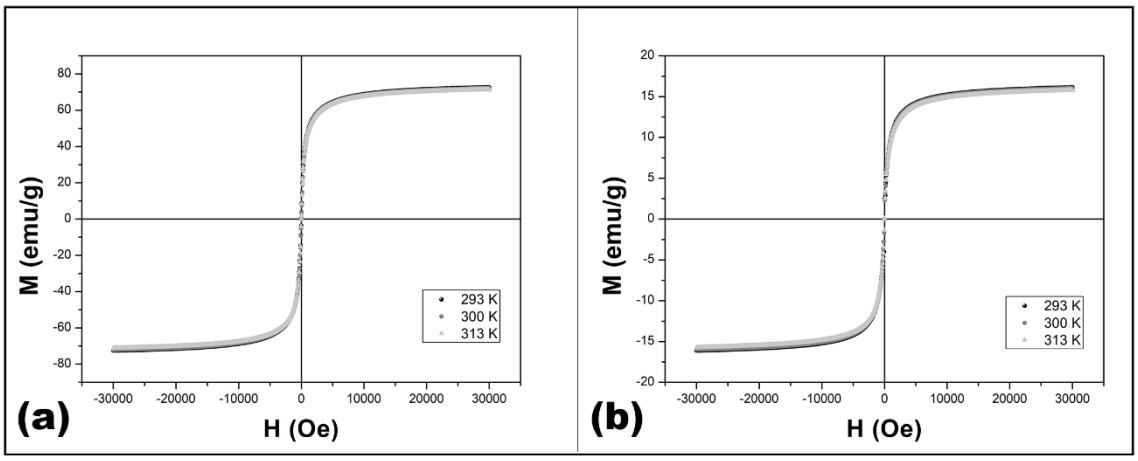

Figure 3. Isothermal magnetization M(H) curves at 293 K, 300 K and 313 K for magnetite (a) and magnetite chitosan (b).

3.2 Immobilization of heparin on magnetic chitosan particles

The amount of immobilized heparin was obtained by the difference between the amount of heparin supplied and the sum of the amount of unfixed heparin present in supernatants and washes, then according to a calibration curve, the amount of heparin immobilized per mg support was obtained. The concentration of heparin supplied (stock solution) was 3.277 mg/mL, the mean amount of heparin immobilized on the particles was 93.8 ± 1.93 μg of heparin per mg

of MAG-CH. Particles without the chitosan coating fixed about 29.4 µg of heparin per mg of magnetite. This result demonstrates the importance of the presence of amine groups at chitosan polymer to allow the covalent immobilization process of heparin. Interaction between the amine groups of the particles and the functionalized carboxyl groups of heparin is in agreement with the literature where we find different approaches to immobilize covalently the heparin in biomaterials through covalent attachment to support using EDC and NHS [31]. Modifications of the Fe_3O_4 particles using synthetic, biocompatible or biodegradable polymers with specific functional groups make them more attractive because the superparamagnetic magnetite particles coated with polymers are usually formed by magnetic cores responsible for a strong magnetic response and a polymeric layer to provide functionalized groups and them can be used in the biotechnological applications [32]. Different applications are described in the literature for heparin immobilized, such as heparin immobilized on microspheres to improve blood compatibility in hemoperfusion [33]; iron oxide nanoparticles were modified with a poly (ethylene oxide) based coating and then further functionalized with heparin and used in the treatment of neointimal hyperplasia [34]. Mercês et al. [12] synthesized Dacron-heparin magnetic composites to use as a tool to human antithrombin purification.

3.3 Interaction between MAG-CH-hep and human plasma proteins

Proteins are present in human plasma at a pH range between 7.35 - 7.45. Because of this, many of these proteins are negatively charged. According to Paull and Michalski [35], ion chromatography is used to analyze inorganic and organic analytes in samples origin from many industries, such as chemical and pharmaceutical. The study about the role of some molecules organic in the body fluids is of great importance. Ion chromatography is a very practical analytical tool for the analysis of various biological fluids such as blood serum. Recently, the application of this method for routine biological analysis became more and more popular. Due to the specific interactions between heparin and various proteins, it can be used for protein purification using the heparin affinity chromatography method. In this method, heparin is covalently immobilized on support or particle and its acts as a specific affinity linker [36].

Immobilized heparin in magnetic composites has a high negative charge that can function as a protein purification tool by ion exchange and/or affinity. Heparin interacts with positively charged basic amino acid residues present on target proteins [37]. In addition, the use of heparin affinity chromatography can be applied as a strategy to selectively remove some

proteins of great abundance, facilitating the analysis of proteins of low concentration in the plasma. It has already been demonstrated that albumin can be removed, for example, by immunoaffinity column techniques, isoelectric entrapment and affinity chromatography [38].

Therefore, plasma proteins were diluted in buffers pH 5.5 or 8.5 and subsequently incubated in MAG-CH-hep and eluted with different concentrations of NaCl in the same buffers at pH 5.5 or 8.5 to observe the standards of protein binding with heparin immobilized on the magnetic particles.

The chromatogram of the human plasma protein elution with 10 mM phosphate buffer supplemented 0.15, 1.0 and 2.0 M NaCl, pH 5.5 or 50 mM Tris-HCl pH 8.5 are indicated in the Figures 4 and 5, respectively. The magnetic particles and the same plasma were three times reused in both experiments. Washings between the reuses were performed with 10 mM phosphate buffer pH 5.5 or 50 mM Tris-HCl pH 8.5 to keep the equilibrium.

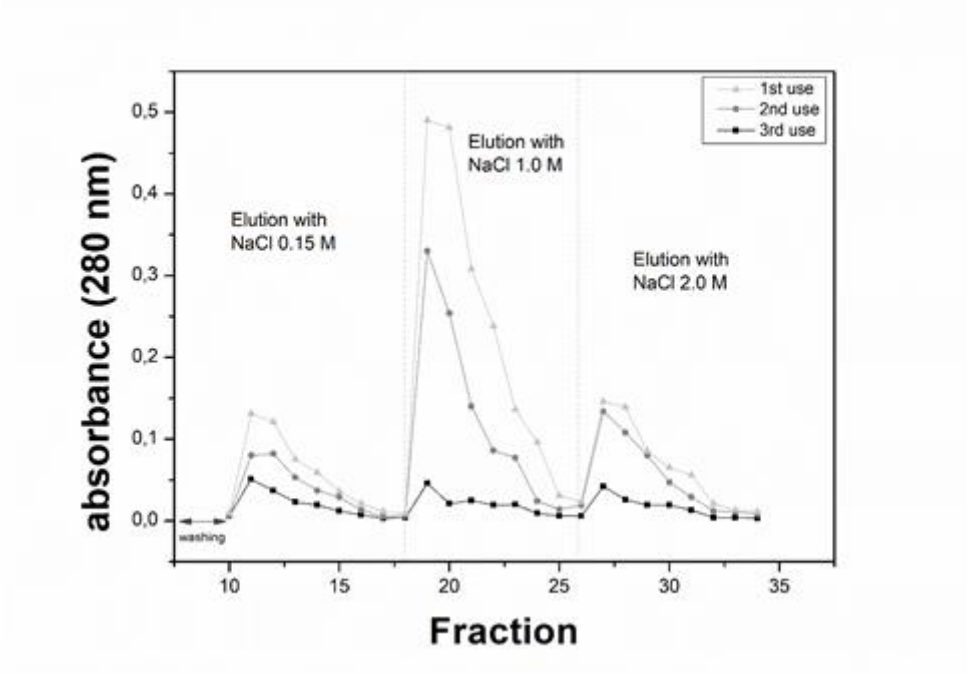

Figure 4. Chromatogram of proteins eluted with NaCl 0.15; 1.0 and 2.0 M in 10 mM phosphate buffer pH 5.5. The same plasma and the same MAG-CH-hep composites has been used 3 times.

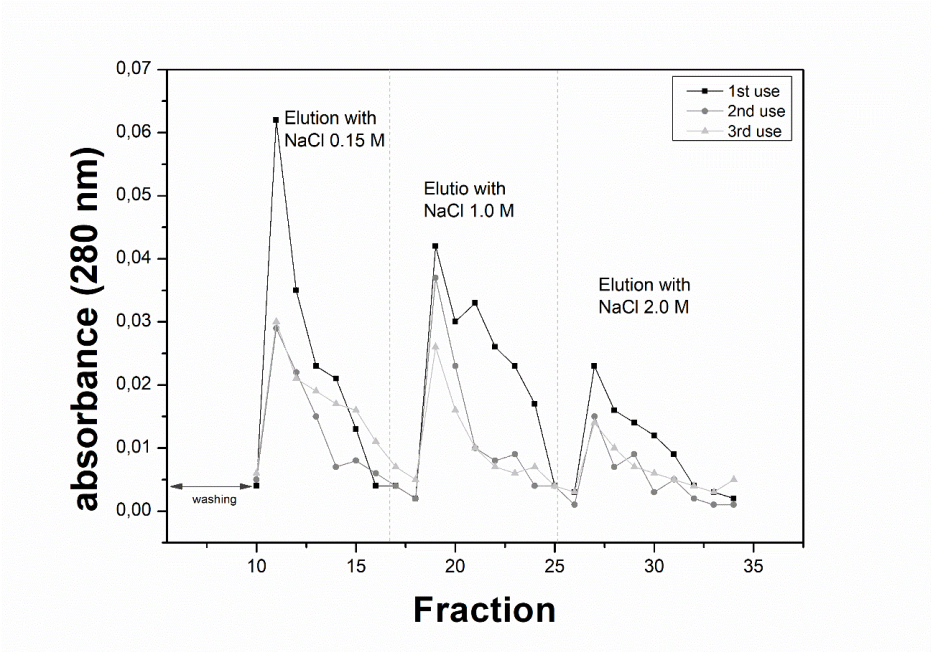

Figure 5. Chromatogram of proteins eluted with NaCl 0.15; 1.0 and 2.0 M in in 50 mM Tris-HCl pH 8.5. The same plasma and the same MAG-CH-hep composites has been used 3 times.

The amount of protein present in the volume of incubated plasma corresponds to 133.4 mg. Table 2 shows the amount of protein after 3 uses that have fixed and then eluted with 10 mM phosphate buffer 0.15; 1.0 and 2.0 M NaCl pH 5.5 or 50 mM Tris-HCl pH 8.5. A higher amount of fixed protein or a higher yield was obtained by incubating diluted plasma proteins in 10 mM phosphate buffer pH 5.5. In addition, elution with 1.0 M NaCl in 10 mM phosphate buffer pH 5.5 corresponds to most of the purified proteins: 2.024 mg. Plasma proteins diluted in pH 5.5 buffer showed a higher interaction with MAG-CH-hep composites because in this pH range these proteins had a positive charge. In contrast, the proteins diluted in pH 8.5 buffer were fixed in low quantity because of its negative charge. In general, some charged solutes can be eluted from ion-exchange columns by the addition of salts [39].

Table 2. Amount of purified plasma protein with MAG-CH-hep composites after three reuses.

Samples	*Elution buffer*	*Amount of purified protein (yield)*
A	0.15 M NaCl in 10 mM phosphate buffer pH 5.5	797 µg
B	1.0 M NaCl in 10 mM phosphate buffer pH 5.5	2024 µg
C	2.0 M NaCl in 10 mM phosphate buffer pH 5.5	438 µg
D	0.15 M NaCl in 50 mM Tris-HCl pH 8.5	187 µg
E	1.0 M NaCl in 50 mM Tris-HCl pH 8.5	116 µg
F	2.0 M NaCl in 50 mM Tris-HCl pH 8.5	53 µg

3.4 Identification by SDS-PAGE and LC-MS of the proteins isolated

Interactions between heparin and heparin-binding proteins occur due to protein shows basic clusters with a density of high positive charge. The acidic groups of heparin were electrostatically interacting with these basic clusters [37, 40]. The results of SDS-PAGE electrophoresis of proteins eluted at in 10 mM phosphate buffer supplemented 0.15, 1.0 and 2.0 M NaCl, pH 5.5 and 50 mM Tris-HCl pH 8.5 are demonstrated at Figures 6 and 7, respectively. It is possible to observe that there is a significant difference in the plasma protein profile that was fixed to the heparin immobilized in MAG-CH-hep after incubation and elution of proteins with the same ionic strength, but in different pH ranges. The majority proteins separated in the electrophoresis of proteins eluted with NaCl in 10 mM phosphate buffer pH 5.5 (Figure 6), were sequenced by LC/MS and the results are shown in table 3. The proteins were identified by the UniProt database and correspond to inhibitors of the serpin family (P36955 and P05155), prothrombin (P00734) and albumin (P02768). Some proteins like antithrombin that belongs to the serpine family, is already a well-known example of heparin-protein interactions model [37, 41, 42]. Besides that, thrombin, a serine protease, is described as a protein with a strong affinity to heparin [37, 42, 43].

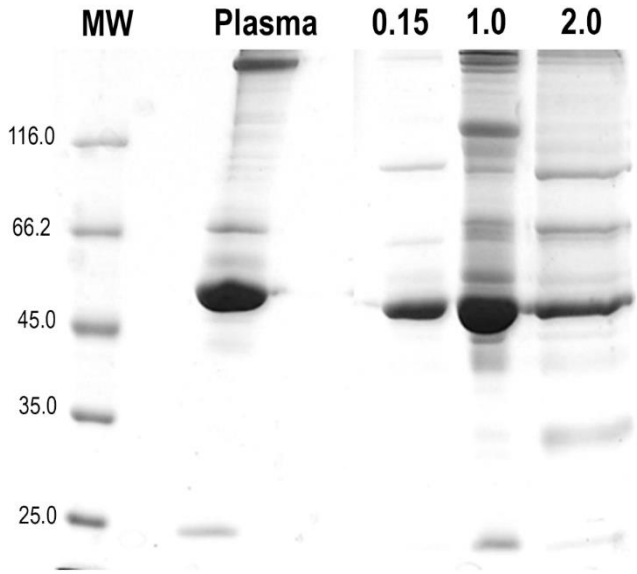

Figure 6. SDS/PAGE electrophoresis of the purified plasmatic proteins has been eluted with NaCl 0.15; 1.0 e 2.0 M in 10 mM phosphate buffer pH 5.5, using MAG-CH-hep composites. MW: molecular weight. Samples not reduced and stained with Brilliant Blue Comassie (R250).

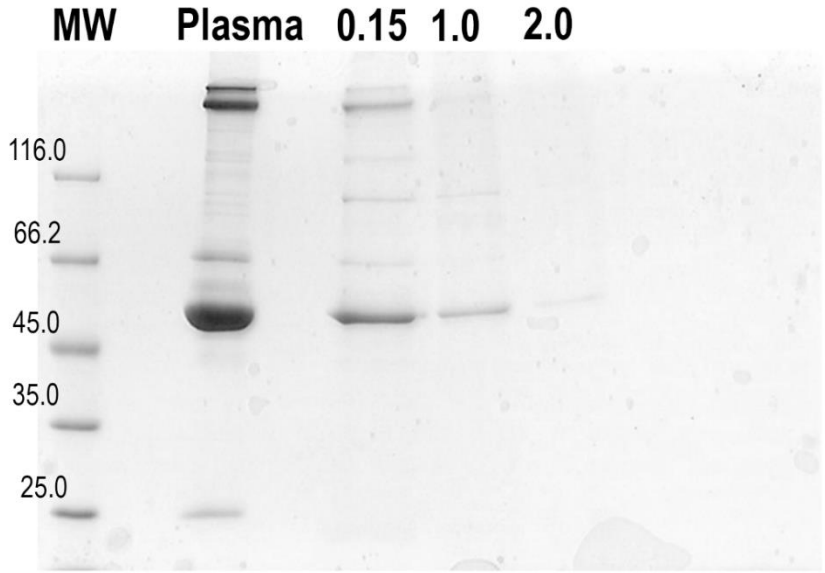

Figure 7 SDS/PAGE electrophoresis of the purified plasmatic proteins has been eluted with NaCl 0.15; 1.0 e 2.0 M in 50 mM Tris-HCl pH 8.5, using MAG-CH-hep composites. MW: molecular weight. Samples not reduced and stained with Brilliant Blue Comassie (R250).

Table 3. Identification of proteins by LC/MS.

Protein	*Protein Entry*	*SCORE*	*Molecular Weight (kDa)*	*Peptide sequence*
Serpin F1	P36955	172.429	46.5	LQSLFDSPDFSK DTDTGALLFIGK ALYYDLISSPDIHGTYK LAAAVSNFGYDLYR
Plasma protease C1 inhibitor	P05155	139.9614	55.4	FQPTLLTLPR GVTSVSQIFHSPDLAIR
Prothrombin	P00734	2270.451	71.5	GQPSVLQVVNLPIVERPVCK LAVTTHGLPCLAWASAQAK TATSEYQTFFNPR TFGSGEADCGLRPLFEK HQDFNSAVQLVENFCR ELLESYIDGR SPQELLCGASLISDR SEGSSVNLSPPLEQCVPDR NPDSSTTGPWCYTTDPTVR SGIECQLWR ETAASLLQAGYK KPVAFSDYIHPVCLPDRETAASLLQAGYK

				LKKPVAFSDYIHPVCLPDRETAASLLQAGYK
				KSPQELLCGASLISDR
				SEGSSVNLSPPLEQCVPDRGQQYQGR
				IVEGSDAEIGMSPWQVMLFR
				GQPSVLQVVNLPIVERPVCK
Serum albumin	P02768	2533.552	71.3	VFDEFKPLVEEPQNLIK
				AVMDDFAAFVEK
				SHCIAEVENDEMPADLPSLAADFVESK
				QNCELFEQLGEYK
				SHCIAEVENDEMPADLPSLAADFVESK
				SHCIAEVENDEMPADLPSLAADFVESKDVCK

3.5 Inhibitory activity of purified proteins

An assessment was made of possible inhibitory activity of the eluted proteins from the analysis of prothrombin time (PT) and activated partial thromboplastin time (aPTT) of the human plasma after incubation with these purified protein eluates. The results of PT and aPTT are shown in Figure 8 and 9, respectively.

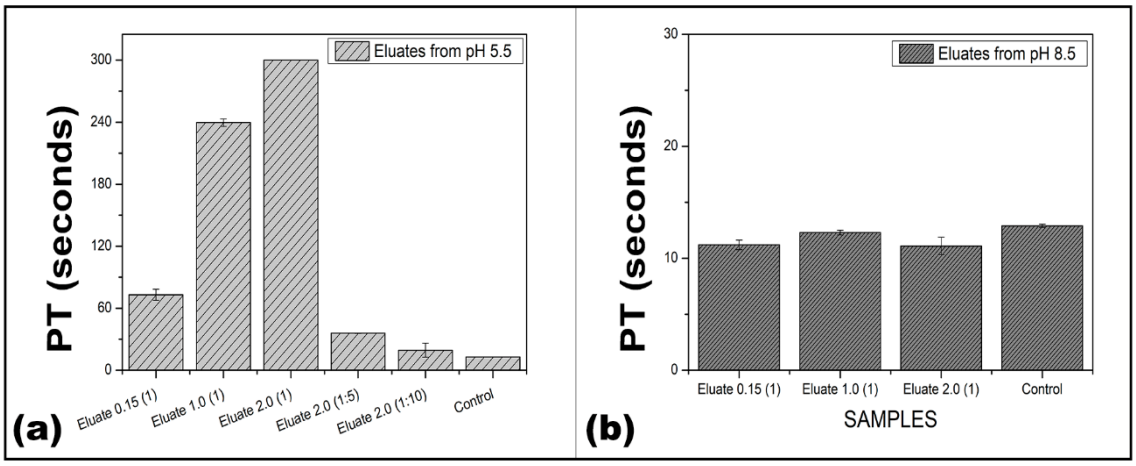

Figura 8. Plasma TP values after incubation of the purified eluates with NaCl 0.15; 1.0; 2.0 M in phosphate buffer pH 5.5 (a) and its in 50 mM Tris-HCl pH 8.5 (b).

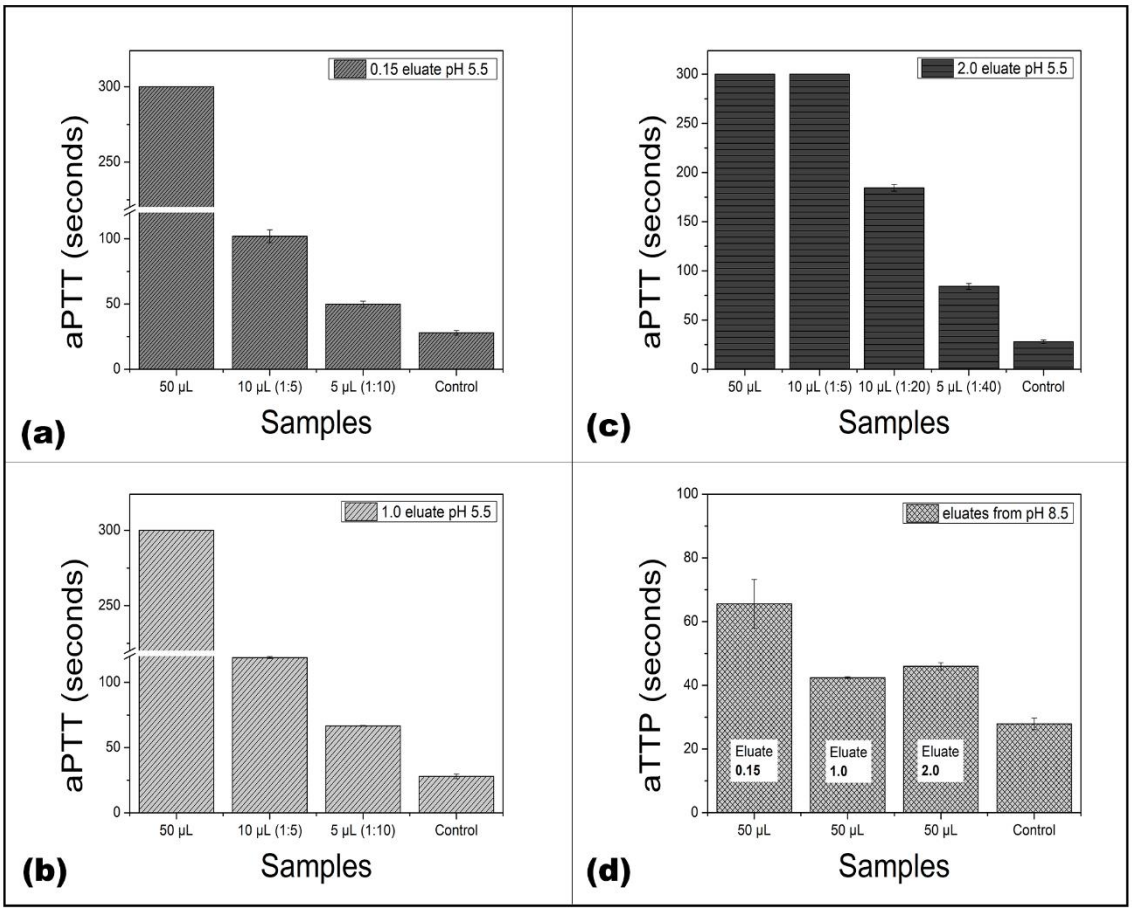

Figura 9. Plasma aPTT values after incubation of the purified eluates with NaCl 0.15 M (a), 1.0 M (b), 2.0 M (c) in phosphate buffer pH 5.5 and the purified eluates obtained from NaCl 0.15; 1.0; 2.0 M in 50 mM Tris-HCl pH 8.5 (d).

Eluates obtained with 0.15, 1.0 and 2.0 M NaCl in 10 mM phosphate buffer pH 5.5 after incubation with normal plasma obtained high values in the PT and aPTT tests. These results indicate the presence of inhibitors capable of prolonging the time of human blood coagulation. The eluates obtained in ionic strength in 50 mM Tris-HCl buffer pH 8.5 did not present a significant inhibitor capable of prolonging the coagulation time. These results demonstrate that there was a greater interaction of the diluted proteins in 10 mM phosphate buffer pH 5.5 (positive charge) with the MAG-CH-hep particles (negative charge).

3.6 Thrombin inhibition assay by using chromogenic method

The elution of plasma proteins in MAG-CH-hep using 1.0 and 2.0 M NaCl in 10 mM phosphate buffer pH 5.5 eluates were obtained with a highest amount of inhibitors, as was demonstrated in the previous step in the coagulation inhibition assays blood.

The results of the thrombin inhibition assay with the proteins eluted with 1.0 M and 2.0 M NaCl are shown in figure 10a and 10b, respectively. The presence of the inhibitor eluted with 1.0 M NaCl was able to decrease the activity of thrombin and this decrease was more pronounced when using 0.0625 U of heparin (figure 10a). Probably the inhibitor present in this eluate has similarity to antithrombin, since it is known that heparin has the property of increasing the antithrombin inhibitory activity hundreds of times. The inhibitor present in eluate 2.0 (Figure 10b) was able to decrease thrombin activity but its inhibitory activity was not altered in the presence of heparin.

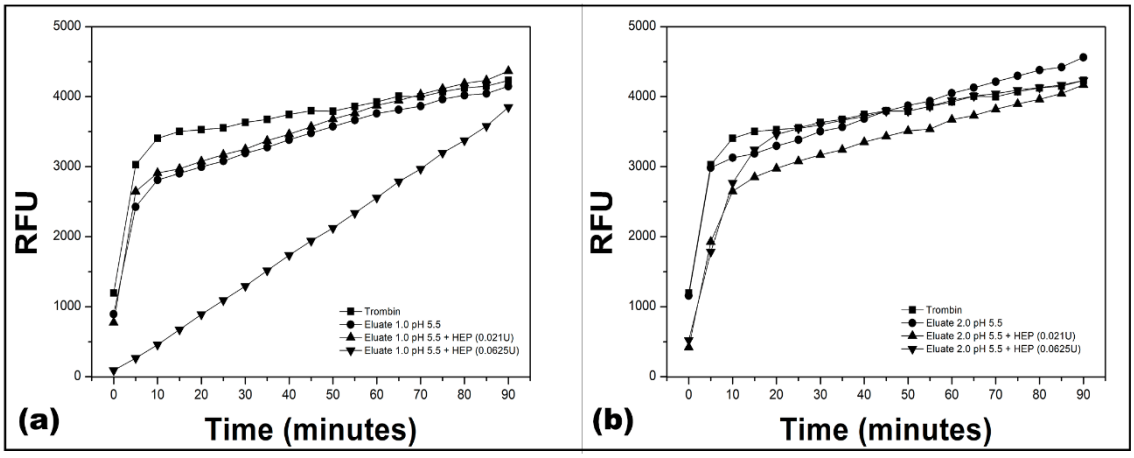

Figura 10. Inhibitory activity of the protein present in the eluate obtained with 1.0 M NaCl (a) and 2.0 M NaCl (b) in 10 mM phosphate buffer pH 5.5. HEP: heparin.

4 CONCLUSION

In this study, magnetic chitosan particles were synthesized and characterized by SEM, XRD and VSM methods. These particles were used to covalently heparin immobilization yielding the MAG-CH-hep composite that was used to interaction/purification study of human plasma proteins. Human blood plasma was diluted in two different buffers: 10 mM phosphate buffer pH 5.5 or 50 mM Tris-HCl pH 8.5, for the purpose of becoming, respectively, proteins charged positively or negatively. After the incubation of MAG-CH-hep composites with these diluted plasmas using a magnetic separation plaque, washes and elution had made with high NaCl concentrations and these experimental was reused three times. The separated proteins in each eluate had dosed and investigated by SDS/PAGE, LC/MS and biological activity tests. Plasma proteins diluted with 10 mM phosphate buffer pH 5.5 had a greater binding capacity to MAG-CH-hep particles as compared to proteins diluted with 50 mM Tris-HCl pH 8.5. This happens because of the composite MAG-CH-hep acts as an ion exchange column and the heparin as an affinity ligand. So, using this method it was possible to identify/purify some important proteins of the blood plasma as inhibitors (serpin family), thrombin and albumin. Therefore, the heparin-coated magnetic composite synthesized in this study may serve as a simple, specific and inexpensive tool to investigate these proteins or others of biomedical interest.

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4 HUMAN PROTEINS BIOSEPARATION AND DEPLETION BY AFFINITY TO HEPARIN IMMOBILIZED ONTO MAGNETIC CHITOSAN PARTICLES.

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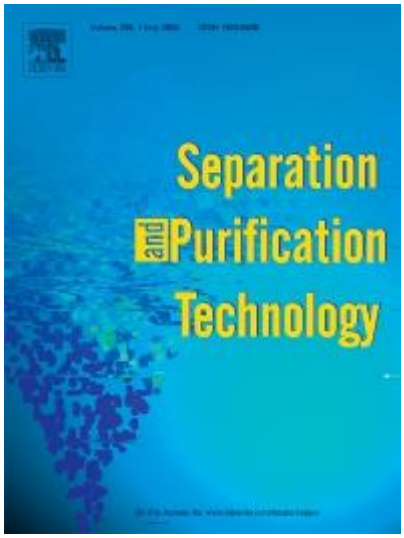

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ABSTRACT

Magnetic chitosan particles (MAG-CH) were prepared by co-precipitation method with Fe^{+3}/Fe^{+2}. Heparin functionalized with carbodiimide and N-hydroxysuccinimide was covalently immobilized onto MAG-CH yielding MAG-CH-hep composites. Differents volumes of a plasma pool (1 or 7 or 20 mL) were incubed for 40 minutes at 4 °C with each 30 mg of MAG-CH-hep. After, using an external magnet (0.6 T), washes and elution were carried out with 10 mM phosphate buffer pH 7.4 supplemented with 0.15, 0.25, 0.5, and 1.0 M NaCl. Eluted proteins were separated by gel filtration chromatography and investigated by SDS-PAGE. Inhibitory activity of the eluted proteins was analyzed by coagulation assays: aPTT and PT. Around 1.3 mg of proteins were depleted after incubation of 20 mL pool plasma, using MAG-CH-hep. These depleted proteins showed a molecular weight between 50 and 115 kDa, among them was human serum albumin (69 kDa). Proteins eluted with 0.25 M NaCl demonstrated inhibitory biological activity on the extrinsic pathway of blood coagulation, whereas proteins eluted with 1.0 M NaCl inhibited the coagulation factors of intrinsic pathway. Finally, the proteins eluted with 0.5 M NaCl showed inhibitory activity on coagulation factors of both intrinsic and extrinsic coagulation pathways. Therefore, using an easy and low cost methodology, MAG-CH-hep has proved to be an alternative tool in obtaining and depleting of inhibitory or high abundance proteins of human plasma.

Key words: bioaffinity; chitosan; heparin; magnetite;

1 INTRODUCTION

Human plasma contains proteins with a wide range of biological functions and is one of the most widely used biological fluids in clinical diagnostics. The ability to identify and quantify low abundance proteins in plasma facilitates plasma proteomic analysis and the search for disease biomarkers [1]. Proteins such as immunoglobulin G (IgG), albumin, α-1-antitrypsin, transferrin, IgA and haptoglobin represent more than 85% of the total protein mass of human plasma; only albumin and IgG correspond to more than 75%. These high abundance proteins make it difficult to detect low abundance proteins that are potential markers for various diseases. To improve detection of low abundance proteins, proteins such as IgG and albumin need to be removed [2]. Proteomics is a fast growing field of study, very relevant in discovering possible biomarkers. However, the use of this technology is somewhat limited due to the presence of some high molecular weight proteins in blood plasma [3], as mentioned earlier. Affinity chromatography is a powerful tool in proteomics. This separation method is based on the interaction between specific immobilized ligands and their target proteins. As this method is versatile, it can be adapted to different needs [4].

Over the past decades, it has been shown that heparin is involved in many biological processes through its interaction with a high number of proteins [5]. Heparin is a highly sulfated polymeric straight chain polysaccharide formed by repeated disaccharide units consisting of an uronic acid (glucuronic or iduronic) and a glucosamine [6]. Due to this structure heparin has the highest negative charge density when compared to any other biologically active macromolecule [7]. Because it has a unique structure, heparin can interact with different proteins. This interaction occurs because protein binding sites always contain basic amino acids (Lys and Arg) whose positive charges probably interact with the negatively charged sulfate and carboxylate groups present in GAG chains [8]. Furthermore, these functional groups facilitate immobilization of heparin in a matrix, so immobilized heparin on solid supports is widely used in affinity chromatography for the purification and identification of proteins capable of binding to its structure [9].

Magnetic particles have wide applications in many areas, such as materials science, medicine, chemistry, and the environment. Thus, the use of these particles has great advantages over conventional techniques, such as their noninvasive character and the non-use of radiation during the analysis. In industrial biotechnology, magnetic particles have been used for biomolecules immobilization, biocatalysis and biosensors [10]. Chitosan is an organic polymer obtained from the N-deacetylation of chitin. The combination of chitosan and magnetic

particles could produce an ideal support for the immobilization of several molecules, with easy functionalization with reactive groups for immobilization of molecules and, at the same time, providing magnetic separability, allowing easy reuse [11].

Because heparin interacts with several proteins, the present study aims to immobilize heparin in magnetic chitosan particles, for use as a tool for depletion and fractionation of plasma proteins by magnetic bioseparation.

2 MATERIAL AND METHODS

2.1 Materials and reagents

Heparin sodium salt (5.000 UI/mL) from Cristália© (São Paulo, Brazil). Carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EDC), N-hydroxysuccinimide (NHS), ferric and ferrous chloride, benzamidine hydrochloride (99%, MW: 156.61), chitosan low molecular weight (50-190 kDa, 75-85% deacetylated) were purchased from Sigma Chemical Compan (Saint Louis, MO, USA). PT and aPTT reagents were obtained from Dade Behring (Marburg, Germany) and stored at 4° C. Human blood was collected from a volunteer underapproval of the Ethical Committee of the Universidade Federal de Pernambuco.

2.2 Magnetic chitosan particles preparation

Synthesis of magnetic chitosan particles was performed by chemistry co-precipitation of iron salts according Maciel et al. 2012 [12]. Suspension of chitosan low molecular weight (2.0% w/v) in distilled water stayed under stirring and then a solution 1:1 of $FeCl_3$ (1.1 M) and $FeCl_2$ (0.6 M) was added and finally the pH was adjusted to 11 with ammonium hydroxide. That mixture remained under manual stirring for 30 minutes at 80 °C. In the end, with a strong magnet, the particles were washed to the neutral pH range and magnetic chitosan particles (MAG-CH) was obtained.

2.3 Immobilization and determination of heparin

Heparin was immobilized in the MAG-CH particles according to Mercês et al. [13]. Initially, a solution of heparin (3 mg/mL) was previously activated with EDC and NHS this

reaction is necessary to carboxylic groups activation. An aliquot (1 mL) of this functionalized heparin solution was incubated with 30 mg of MAG-CH for 72 h with mild agitation yielding the covalently immobilized heparin on the magnetic chitosan particle (MAG-CH-hep).

These composites were recovered under a magnetic field (0.6 T) and washed three times with distilled water to remove non-immobilized heparin. Under this magnetic field the particles precipitation occurred in about 10 seconds. The supernatant, first washed and second washed were incubated with methylene blue at room temperature. This resulted in a complex of methylene blue with heparin. The solution was measured at 664 nm using a Shimadzu UV Visible Spectrophotometer (UVmini-1240) [14]. The calibration curve was obtained through measurement of the absorbance of a series of standards at heparin concentrations of 10–100 μg/mL. The amount of immobilized heparin was calculated from the calibration curve.

2.4 Interaction between human plasma protein and MAG-CH-hep

The interaction between plasma proteins with MAG-CH-hep was performed from different experimental conditions related to different volumes of a plasma pool. Thus, experimental assays were performed with 1.0 or 7.0 or 20.0 ml plasma incubated each in 30 mg MAG-CH-hep. All plasmas were treated with benzamidine hydrochloride (2 mM) to prevent proteases degradation. The incubation time was 40 minutes at 4 °C with 30 mg of MAG-CH-hep for each study. After this time, using a magnetic separation plaque (0.6 T), washes and elution were carried out with 10 mM phosphate buffer pH 7.4 supplemented with 0.15, 0.25, 0.5 and 1.0 M NaCl. The same magnetic composites and the same blood plasmas were reused 3 times. The proteins were monitored at 280 nm (Shimadzu UV Visible Spectrophotometer - UVmini-1240). The protein peaks obtained were pooled, dialyzed (cut off 10-14 kDa) and finally dried in a speed vac. Proteins were quantified by the Bradford method [15].

2.5 Proteins identification and separation

After dialysis, the proteins eluted at different concentrations of NaCl (0.15; 0.25; 0.5 and 1.0 M) have applied (10 μg) on 12.0% SDS-PAGE electrophoresis (non-reduced samples). The gel was stained with a solution of Coomassie brilliant blue, CBB, (R250) and next silver nitrate.

The dialyzed protein eluates obtained with 0.15 and 0.25 M NaCl in 10 mM phosphate buffer pH 7.4 were pooled and studied by fast protein liquid chromatography (FPLC) using the

high resolution Superdex-G75 column (gel filtration) - ÄKTA Purifier (GE) - equipped with a fraction collector and a 280 nm UV absorption detector, UPC-900. The applied volume was 500 µl using 10 mM phosphate buffer pH 7.4 as elution buffer, flow of 0.5 mL/min and fractions were collected every 0.5 mL. After separation by gel filtration, the obtained peaks were investigated in SDS/PAGE and stained with a solution of Coomassie brilliant blue, CBB, (R250).

2.6 Proteins activity assays in vitro

Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were the initial tests to evaluate the inhibitory activity of proteins present in the eluates obtained from 0.15, 0.25, 0.5 and 1.0 M NaCl 10 mM phosphate buffer pH 7.4. The measurements were made using a semi-automated coagulometer (BFT II - Dade Behring) according to Salu et al. [16] and Silva et al. [17]. It was performed dose-response tests to verify the action of the inhibitor according to the amount incubated in the plasma, the control were performed with normal plasma.

3 RESULTS AND DISCUSSION

3.1 Magnetic chitosan composites and heparin immobilization

Magnetic particle modifications using some polymers such as chitosan make them attractive as biomolecule immobilization matrices [18]. Thus, modifications of the Fe_3O_4 particles using synthetic, biocompatible or biodegradable polymers with specific functional groups make them more attractive because the superparamagnetic magnetite particles coated with polymers are usually formed by magnetic cores responsible for a strong magnetic response and a polymeric layer to provide functionalized groups and them can be used in the biotechnological applications [19].

The amount of immobilized heparin was obtained by the difference between the amount of heparin supplied and the sum of the amount of unfixed heparin present in supernatants and washes, then according to a calibration curve, the amount of heparin immobilized per mg support was obtained. Thus, the amount of covalently immobilized heparin was 78.13 ± 1.4 µg heparin per mg MAG-CH. Particles without the chitosan coating fixed about 29.4 mg heparin per mg magnetite. This result demonstrates the importance of the presence of amine groups at

chitosan polymer to allow the covalent immobilization process of heparin. Interaction between the amine groups of the particles and the functionalized carboxyl groups of heparin is in agreement with the literature where we find different approaches to immobilize covalently the heparin in biomaterials through covalent attachment to support using EDC and NHS [20].

3.2 Affinity-fixed proteins in MAG-CH-hep composite

In recent years, magnetic particles have attracted much attention due to their biological applications such in depleting high-abundant proteins in human serum [21], in bovine serum albumin separation processes [22] and protein separation in multiple myeloma serum samples [23]. Currently, magnetic particles have attracting more attention as an important tool or strategy for protein separation of biological samples, because they provide simple and cheap applications in the discovery of human disease biomarkers [24].

Table 1 indicates the amount of protein fixed in the MAG-CH-hep composites after 3 reuses. Elutions were performed with sodium chloride (NaCl) at concentrations of 0.15; 0.25; 0.5 and 1.0 M in 10 mM sodium phosphate buffer pH 7.4. A larger amount of human plasma protein was obtained from the larger plasma volume incubation. A larger amount of human plasma protein was obtained from the larger plasma volume incubation. About 1.3 mg of proteins affinity bound to 30 mg of MAG-CH immobilized heparin following incubation of 20 mL of a plasma pool. A smaller volume of incubated plasma (1 mL) also corresponds to a lower value of fixed proteins around 120 µg.

Table 1. Amount of protein fixed in MAG-CH-hep composites.

	Incubated plasma pool	
Plasma volume	***Plasma protein amount***	***Amount of protein fixed in MAG-CH-hep***
1.0 mL	66.0 mg	120 µg
7.0 mL	462.0 mg	642 µg
20.0 mL	1320.0 mg	1340 µg

Elutions performed after incubation with the different plasma volumes showed similar absorption at 280 nm, and the chromatogram obtained is shown in Figure 1. Therefore, the proteins eluted after 3 reuses and in all volumes were separated and pooled according to the NaCl concentration (0.15, 0.25, 0.5, 1.0 M) used in their elution. Finally, they were dialyzed (cut off 12 kDa) in 0.07 M NaCl to prevent protein precipitation. They were then dried in *speed vac* and resuspended in 1 mL of deionized water for subsequent gel filtration separation, electrophoresis identification and biological activity.

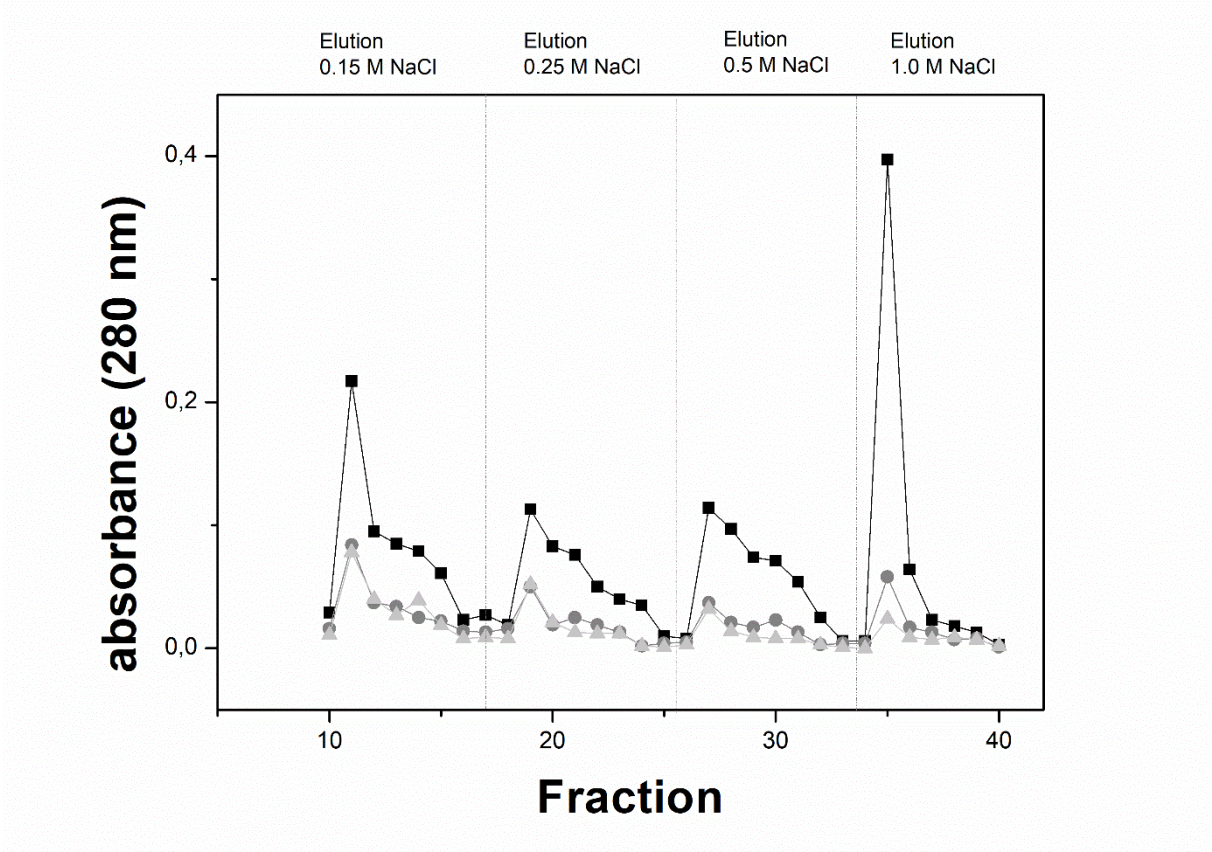

Figure 1. Chromatography of plasma proteins eluted with different NaCl concentrations after incubation with MAG-CH-hep. first reuse (■), second reuse (●) and third reuse (▲).

3.3 SDS-PAGE electrophoresis

According to Figure 2, it can been observe the fractionation of proteins present in human plasma that were fixed in the immobilized heparin (MAG-CH-hep) composites. The proteins showed different molecular weights ranging from 50 to 115 kDa. The results demonstrate that by using magnetic bioseparation and immobilized heparin affinity it is possible to deplete high

molecular weight proteins. Among the proteins we can visualize bands that correspond to human serum albumin (HSA) with 69 kDa. HSA accounts for 55–60% of the measured serum proteins in healthy humans [25]. Besides that, HSA is a main carrier for a variety of substances in the body and also plays a key role in maintaining normal osmolarity in plasma and interstitial fluid [26].

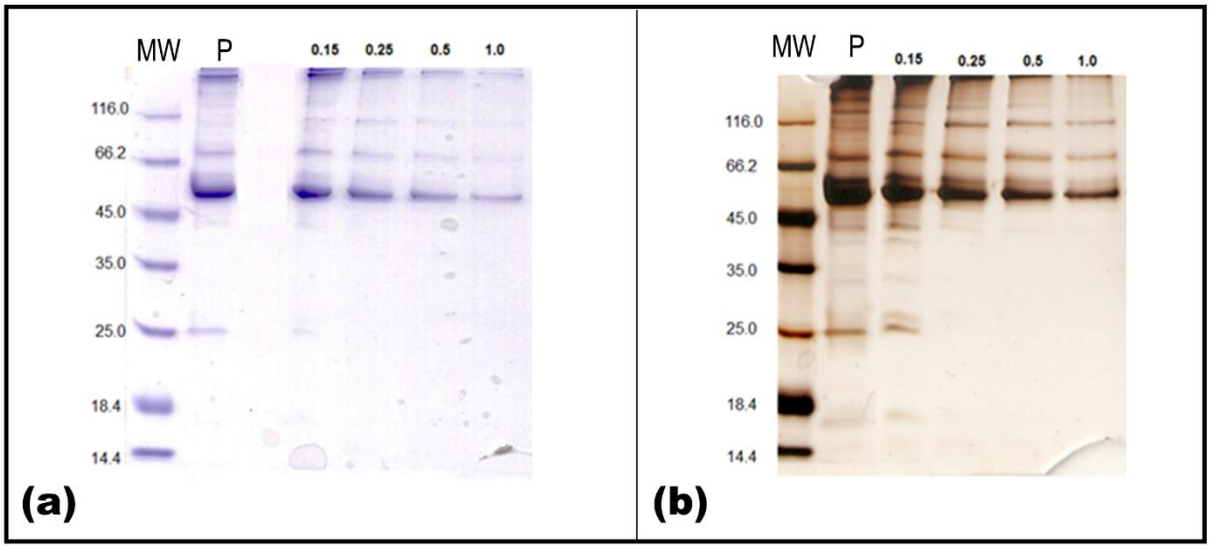

Figure 2. SDS-PAGE 12% of plasma eluted proteins with 0.15, 0.25, 0.5 and 1.0 M NaCl in 10 mM sodium phosphate buffer pH 7.4; stained with a coomassie bright blue dye solution (R250) (a) and stained with silver nitrate (b). Unreduced samples. MW: molecular weight. P: human blood plasma.

3.4 Protein separation by gel filtration – FPLC, ÄKTA Purifier

In gel filtration chromatography, proteins and peptides are separated by their size. However, the separation result can be affected by several kinds of non-size related effects such as ion exchange and hydrophobic interactions [27]. The 0.15 and 0.25 M protein eluates obtained were pooled in a final volume pool of 500 µL and applied to Superdex-G75 (FPLC - ÄKTA Purifier) to separate the proteins into solution. The profile of these proteins separated by gel filtration is shown in Figure 3. According to the chromatogram shown in Figure 3, after the separation process 4 protein peaks (280 nm) are displayed, the larger proteins correspond to the peaks: “a, b and c”; the smaller proteins correspond to the peak d.

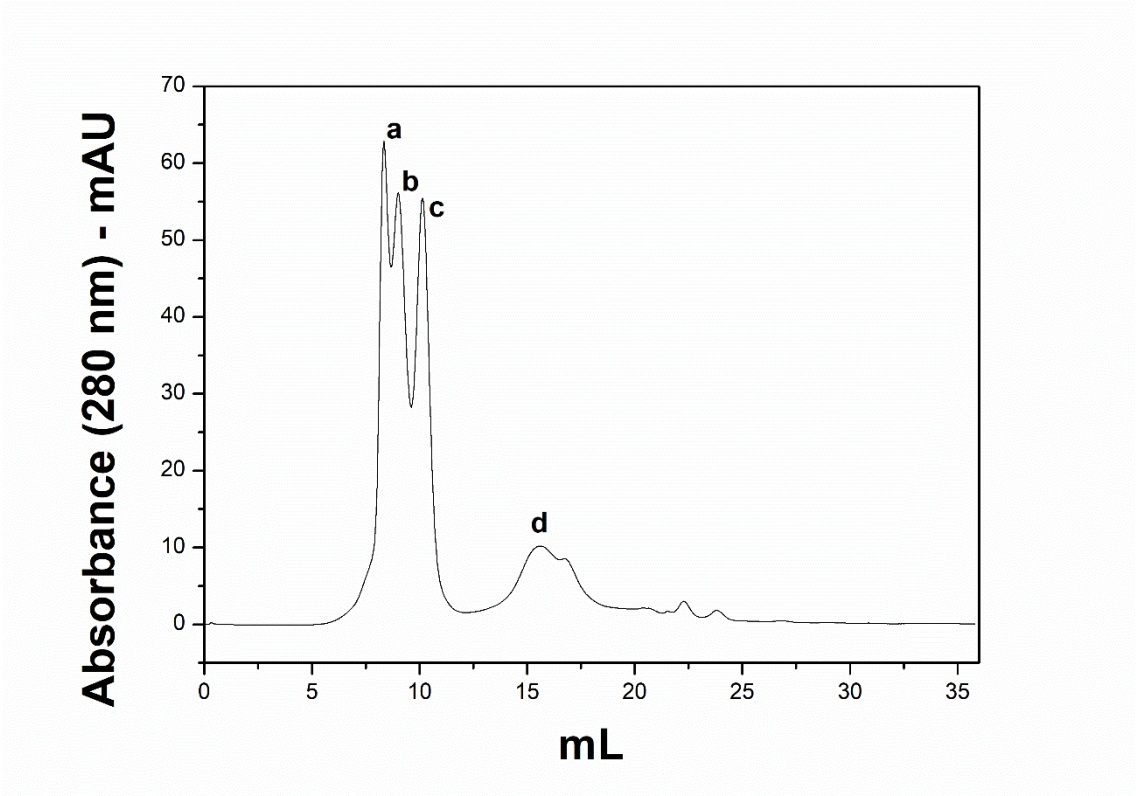

Figure 3. Protein separation by gel filtration. Superdex G-75; Flow 0.500 mL / min - 10 mM sodium phosphate buffer 137 mM NaCl pH 7.4; 500 µL applied and collected. Elution: 10 mM sodium phosphate buffer 137 mM NaCl pH 7.4.

Protein peaks (a, b, c, d) obtained after gel filtration chromatography (Fig. 3) were investigated by electrophoresis to obtain approximate values of their molecular weight (Figure 4). According with the obtained results, a protein weighing 50-60 kDa corresponding to peak "d" was purified using magnetic separation and filtration gel separation chromatography. The proteins present in the peaks “a, b and c” presented lower molecular weights and some with a weight greater than 100 kDa, thus showing the efficiency of the methodology in the depletion of high molecular weight plasma proteins.

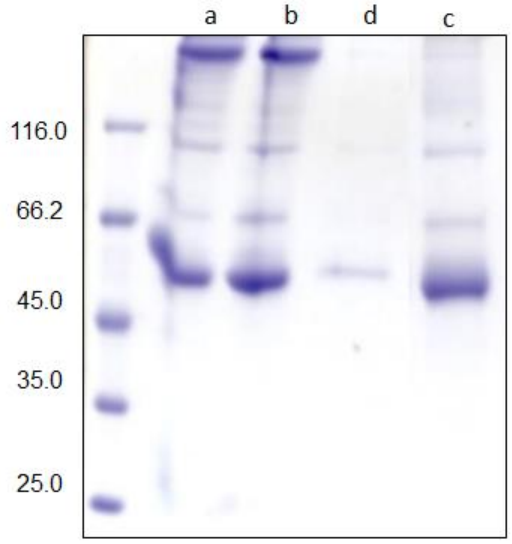

Figure 4. SDS-PAGE 12% of proteins after gel separation (Superdex-G75, FPLC - ÄKTA Purifier); stained with a coomassie bright blue dye solution (R250).

3.5 Proteins biological activity

In order to evaluate a possible inhibitory biological activity of the possible proteins obtained on the blood coagulation cascade, coagulation assays were performed from the analysis of prothrombin time (PT) and activated partial thromboplastin time (aPTT). Thus, understanding the clotting inhibition made by the proteins present in the eluates has impact to investigate the biological activity of these serum proteins obtained. aPTT is a screening test for factors II, V, VIII, IX, X, XI and XII of the intrinsic and common pathways [28, 29], whilst PT is a screening test for factors II, V, VII and X of the extrinsic and common pathways [29].

Figure 5 shows the different aPTT values obtained after incubation of human blood plasma with proteins eluted in 10 mM phosphate buffer pH 7.4 with 0.15; 0.5; and 1.0 M NaCl. aPTT measurements are the most commonly used sensitive index endogenous coagulation function monitoring [30].

According to Figure 5d, there is a strong inhibitor present which was fixed in MAG-CH-hep and eluted at 1.0 M NaCl in 10 mM phosphate buffer pH 7.4. In the graph we can analyze the dose response profile of the inhibitor in its total concentration, and also diluted 5, 10, 20 and 40 times that showed the ability to prolong the normal plasma aPTT. The inhibitor in question acted by inhibiting the activity of factors of the intrinsic and common coagulation pathway. No significant inhibition was observed after plasma incubation with the eluates from

0.15 and 0.25 M NaCl in 10 mM phosphate buffer pH 7.4. Probably the same inhibitor present in the eluate obtained in 1.0 M NaCl was also present in the eluate obtained in 0.5 M NaCl, but in smaller quantity.

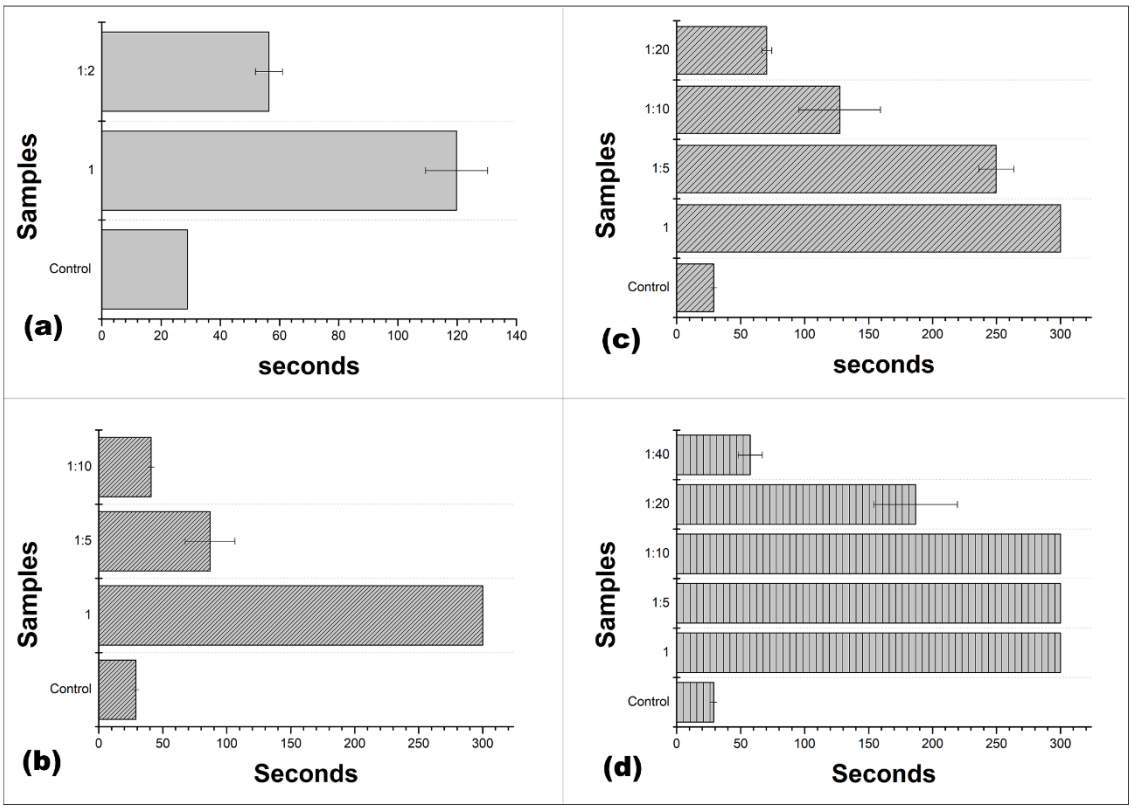

Figure 5. aPTT value of proteins present in the eluates obtained with 0.15 M (a), 0.25 M (b), 0.5 M (c) and 1.0 M (d) NaCl in 10 mM phosphate buffer pH 7.4. Control: normal human plasma.

Figure 6 shows the different PT values obtained after incubation of human blood plasma with proteins eluted in 10 mM phosphate buffer pH 7.4 with 0.15; 0.5 and 1.0 NaCl. According to Figure 6b there was a significant coagulation prolongation after incubation of the eluate obtained with 0.25 M NaCl. After incubation with eluate obtained with 0.5 M NaCl (Fig. 6c), there was a prolongation, but lower when compared to the first. The inhibitor acted by inhibiting the activity of factors of the extrinsic and common coagulation pathway. No significant inhibition was observed after plasma incubation with eluates obtained by 1.0 M NaCl in 10 mM phosphate buffer pH 7.4 (Fig. 6d).

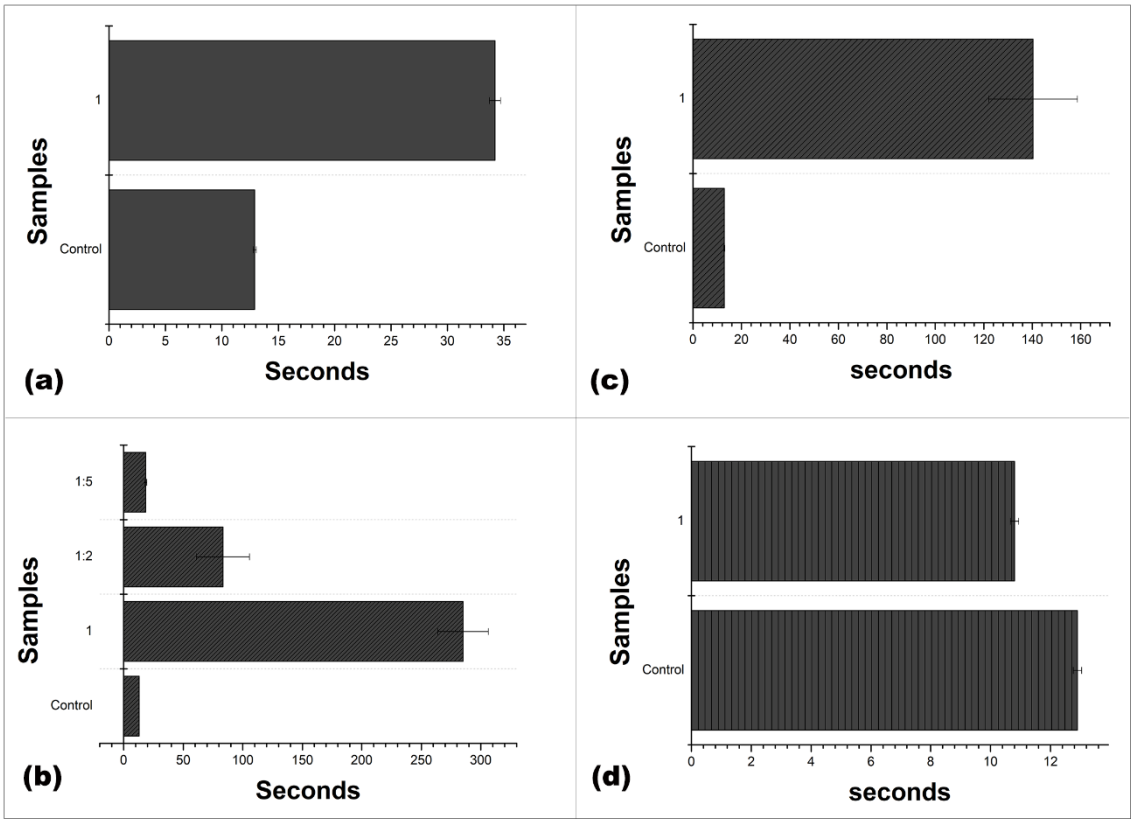

Figure 6. PT value of proteins present in the eluates obtained with 0.15 M (a), 0.25 M (b), 0.5 M (c) and 1.0 M (d) NaCl in 10 mM phosphate buffer pH 7.4. Control: normal human plasma.

From the above results (Fig. 5 and Fig. 6), there are presence of different inhibitors that were fixed and eluted after incubation with MAG-CH-hep. Thus analyzing the prolongation and or inhibition of coagulation from aPTT and PT it is possible to state that in the eluate obtained with 0.25 M NaCl in PBS pH 7.4 buffer there are proteins with inhibitory activity under the extrinsic pathway. The eluate obtained with 0.5 M NaCl in PBS pH 7.4 buffer contains proteins with inhibitory activity under both extrinsic and intrinsic pathways. Finally, the eluate obtained with 1.0 M NaCl in 10 mM phosphate buffer pH 7.4 contains proteins with inhibitory activity under ~~both~~ intrinsic pathways. These results may assist in studies of the proteins involved in the clot formation process and inhibition of the coagulation cascade.

CONCLUSION

The magnetic chitosan particles were correctly synthesized by the chemical co-precipitation method and demonstrated excellent magnetic ordering. Heparin was covalently immobilized on magnetic chitosan particles and the composite formed was used as a fractionation and depletion tool of plasma proteins. Incubating different plasma volumes with heparin immobilized onto magnetic chitosan, it was observed that around 1.3 mg of proteins were fixed after incubation of 20 mL of plasma. Therefore the larger the volume of incubated plasma the greater the amount of protein depleted. Proteins eluted with 0.15; 0.25; 0.5 and 1.0 M NaCl in 10 mM phosphate buffer pH 7.4 were investigated in SDS/PAGE and the different eluted proteins showed molecular weights ranging from 50 to 115 kDa. After gel filtration chromatography, some proteins depleted showed a molecular weight of over 100 kDa. In addition, proteins eluted with 0.25 M NaCl demonstrated inhibitory biological activity on the extrinsic pathway of blood coagulation, whereas proteins eluted with 1.0 M NaCl inhibited the coagulation factors of intrinsic pathway. Finally, the proteins eluted with 0.5 M NaCl showed inhibitory activity on coagulation factors of both intrinsic and extrinsic coagulation pathways. Therefore, in view of the above, MAG-CH-hep composites have proved useful as an alternative tool for depletion, fractionation and purification of blood plasma inhibitors. This tool has many advantages such as low cost, easy synthesis, possibility of reuse and can be used in proteomics studies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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5 INTERACTION BETWEEN HUMAN α-THROMBIN AND FACTOR Xa WITH HEPARIN IMMOBILIZED IN POLYANILINE-COATED MAGNETITE NANOPARTICLES.

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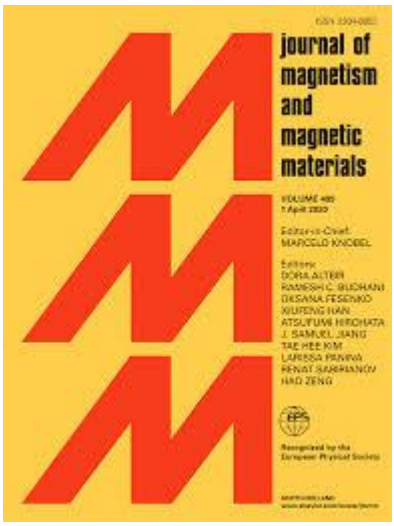

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Qualis CAPES - Ciências Biológicas 1: B1

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ABSTRACT

This works aimed to immobilize heparin onto magnetite coated with polyaniline nanoparticles (MAG-PANI) and to use for interaction study or bioseparation of α-thrombin and Factor Xa. Magnetite (Fe_3O_4) was obtained by chemistry co-precipitation method (Fe^{2+}/Fe^{3+}). Magnetite coated with polyaniline was obtained by chemistry aniline oxidation. Heparin was activated by carbodiimide and N-hydroxysuccinimide and covalently linked to MAG-PANI (MAG-PANI-hep). SEM (scanning electron microscopy) and TEM (transmission electron microscopy) analysis showed that MAG-PANI has heterogeneus morphology and size of 11.7 ± 2.2 nm and XRD (X-ray diffraction) analysis suggests the presence of polyaniline and Fe3O4 in MAG-PANI that it exhibited superparamagnetism behavior (magnetic analysis). Human α-thrombin (10 nM) or Factor Xa (10 nM) was incubed with MAG-PANI-hep in 20 mM Tris-HCl pH 7.4 containing increasing concentrations of NaCl. After incubation, each supernatant was collected for the thrombin or Factor Xa enzyme activity test. Under conditions with 800 mM NaCl was observed a thrombin and Factor Xa supernatant enzymatic activity of around 85.0 ± 4 % and 55.4 ± 12 %, respectively. Under salt-free conditions the residual supernatant activity was 5.3 ± 0.4 and 10.9 ± 0.4% for thrombin and Factor Xa, respectively. These results suggest that the enzymes showed high affinity for immobilized heparin in MAG-PANI. This method can be applied to the blood products industry since it proved useful as a tool for the bioseparation of coagulation factors.

Key words: factor Xa; Fe_3O_4; heparin; polyaniline; thrombin;

1 INTRODUCTION

Iron oxide nanoparticles have been investigated extensively studied and applicate in biochemistry, biomedical and materials sciences. Magnetite (Fe_3O_4) and hematite (α-Fe_2O_3) are examples of iron oxide class nanoparticles offering various application such as medical diagnosis, therapy, drug delivery, magnetic separation, magnetic resonance imaging and catalysis [1, 2]. Magnetite is very popular due to easy synthesis, low toxicity and excellent superparamagnetic properties. Various methods have been developed for the synthesis of magnetite nanoparticles according of the desired shape, size and structure. The main methods used are reduction of ferric oxide under hydrogen environment, reduction of ferric ions using reducing agents, sonochemical, hydrothermal and coprecipitation method [3]. Coprecipitation is the easer and is quasi-immediate at room temperature and need less equipmente to handle. A number of parameters are required to control during coprecipitation method such as pH control, stirring rate and temperature for example [4, 5].

The modified magnetic nanoparticles have a polymer-coated iron oxide core. This coating has active groups that can be conjugated to biomolecules such as carbohydrates, proteins and enzymes [6, 7]. Polymer coated superparamagnetic magnetite particles are usually formed by magnetic core responsible for a strong magnetic response and a polymeric shell to provide functionalizable and characteristic groups [8].

Polyethylene-terephthalate [9], polyaniline [10], chitosan [11] and poly(acrylic acid) [12] are examples of polymers used to modify magnetic nanoparticles. Magnetite coated with polyaniline (PANI) are efficient for the removal of dyes or heavy metals from waste water. Also, these composites can serve as support for the immobilization of enzymes or antibodies, as well as for the development of biosensorsor as magnetic resonance imaging contrast agents [13]. Due to the simplicity of the synthesis and redox activity polyaniline (PANI) is one of the most important and interesting electroconductive polymers. It is applicable, for example, as a material for sensors, indicators and others. The most frequently used method for synthesis of PANI is the chemical oxidative polymerization [14, 15].

Heparin is a linear polysaccharide of the glycosaminoglycan family, has a highly sulfated chain formed by repeating disaccharide units consisting of an uronic acid (iduronic or glucuronic) and glucosamine [16]. Heparin when immobilized may interact with various proteins including the coagulation factors, acting as an affinity ligand capable of interacting with these proteins [17]. Cardim and Weintraub [18] showed that heparin-binding proteins contain known sequences that are responsible for interaction and specificity with heparin. Some

of the heparin-binding segments were XBBXBX, XBBBXXBX and XBBXXBBBXXXXBBX, where B is one of three basic amino acids (arginine, lysine or histidine) and X is any of the other 17 natural amino acids [19].

Prothrombin is the precursor to thrombin, a serine protease, which plays a key role in blood clotting. Thrombin is very effective and acts by converting circulating fibrinogen into fibrin monomers, which then polymerize to form the fibrin that is the major constituent of the blood clot [20, 21]. Thrombin structure is formed by two polypeptide chains joined by a disulfide bridge. The smaller chain is composed of 36 residues and is usually called the L (light) or A chain. The larger chain has 259 residues and is called the H (heavy) or B chain. The L chain is located in the opposite hemisphere from which it is located [22]. Thrombin has two distinct exocites from the active binding site, the first exocite being related to the interaction of fibrinogen with thrombin. The second exocyte is the site where heparin binds directly to thrombin, also called the heparin binding site, which carries a number of positively charged residues, including Arg93, Arg97, Arg101, Arg126, Arg165, Lys169, Arg173, Arg175, Arg233, Lys235, Lys236 e Lys240 [23].

Factor X (FX) participates in the intermediate phase of blood coagulation and consists of a vitamin K dependent glycoprotein that is synthesized in the liver and secreted into the blood as a 58.8 kDa zymogen. The conversion of factor X into its active form (factor Xa - FXa) is catalyzed by factor IXa in the presence of factor VIIIa, calcium ions and phospholipids. This conversion is also catalyzed by factor VIIa in the presence of tissue factor. Factor Xa (FXa) cleaves prothrombin to thrombin in the presence of factor Va, calcium ions and phospholipids [24].

Therefore, this study aimed to synthesize and to characterize magnetite coated with polyaniline particles to be used as a matrix to heparin imobilization and to serve as an alternative tool to trombin and Factor Xa bioseparation or purification. These materials have several advantages including easy synthesis using low-cost reagents and easy removal from the incubation mixture by applying a magnet.

2 MATERIAL AND METHODS

2.1 Materials and reagents

Heparin sodium salt (5.000 UI/mL) from Cristália© (São Paulo, Brazil). Aniline, ammonium hydroxide, carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EDC), N-

hydroxysuccinimide (NHS), ferric and ferrous chloride were purchased from Sigma Chemical Compan (Saint Louis, MO, USA). Chromogenic substrates: S2238 (H-D-Phe-Pip-Arg-pNA) 625.6 g/mol and S2222 (Bz-Ile-Glu-(γ-OR)-Gly-Arg-pNA) 741.3 g/mol were purchased from Chrromogenix©. Human α-Trombin (36 kDa) and Factor Xa were purchased from Molecular Innovations (USA).

2.2 Synthesis of magnetite nanoparticles.

Magnetite nanoparticles (Fe_3O_4) were synthesized by chemistry co-precipitataion method according to Neri et al. [10]. Briefly: a solution (1:1, v/v) with 0.6 M $FeCl_2$ and 1.1 M $FeCl_3$ was prepared, next it was added in 50 mL of distilled water under stirring. The pH values was adjust to 11 with ammonium hydroxide to precipitate the magnetite nanoparticles. Finally, this suspension remained at 90 °C for 30 min under constant manual agitation. After this step the magnetic nanoparticles were externally recovered with a magnet (0.6 T) and washed with distilled water until pH neutral range. At the end was obtained a black solid residue that was filtered and dried at 50 °C for 24 h. This material was macerated and sieved (pore < 250µm) to obtain a black powder yielding the magnetite nanoparticles (MAG).

2.3 Coating of magnetite nanoparticles (Fe_3O_4) with polyaniline.

Magnetite nanoparticles coated with polyaniline was obtained by aniline chemical oxidation according to Maciel et al. [25]. Briefly: 500 mg of MAG was incubated for 1 hour with 50 ml of a 0.1 M $KMnO_4$ at 25 °C under slight agitation. After, this functionalized magnetic nanoparticles were recupered with a magnetic separation plate (0.6 T) and them were washed with distilled water to remove $KMnO_4$ excess. Next step was to incubate the functionalized magnetic nanoparticles in 50 mL of the 0.5 M aniline (in 1.0M HNO_3) solution for 1 hour at 4 °C under slight agitation. Then with an external magnet the particles were recovered and washed with 3 steps: distilled water, 0.1M citric acid and distilled water again. Finally, the polyaniline coated magnetite nanoparticles (MAG-PANI) was obtained.

2.4 Physical characterization.

Distribution and morphology of MAG and MAG-PANI nanoparticles synthesized were analyzed by scanning electron microscopy (SEM) TESCAN-Mira3. In addition, the structure of these nanoparticles synthesized was established by transmission electron microscopy (TEM) TECNAI (20-200kv). Particle size was obtained TEM images using ImageJ 1.48v software (Wayne Rasband, National Institutes of Health, USA). Magnetization measurements (*M*s) of MAG and MAG-PANI were obtained using a vibrating sample magnetometer (VSM), VersaLab, manufactured by Quantum Design, at temperatures of 293 K, 300 K and 313 K, with magnetic fields in the range -30000 Oe to +30000 Oe. Structure samples of MAG and MAG-PANI were characterized by X-ray diffraction (XRD) performed at room temperature in the range 10°–90°, in equal 2θ steps of 0,02°, using Bruker D8 Advance Davinci diffractometer with CuKα radiation (λ = 1,5406 Å).

2.5 Heparin immobilization onto MAG-PANI nanoparticles.

Heparin was covalently immobilized onto MAG-PANI nanoparticles according to Mercês et al. [26]. Initially, the carboxylic groups of heparin was previously functionalized with EDC and NHS. Aliquots of this functionalized heparin solution (3 mg/mL) were incubated on each 30 mg of MAG-PANI by 72 h, under slow agitation at 25 °C. At the end, the heparin covalently immobilized onto polyaniline coated magnetite nanoparticles (MAG-PANI-hep) was finally obtained. These magnetic composites were recovered under a magnetic field (0.6 T) and washed three times with PBS (phosphate buffered saline) to remove non-immobilized heparin. Under this magnetic field the particles precipitation occurred in about 10 seconds.

The supernatant, first washed and second washed were incubated with methylene blue at room temperature. This resulted in a complex of methylene blue with heparin. The solution was measured at 664 nm using a Shimadzu UV Visible Spectrophotometer (UVmini-1240) [27]. The calibration curve was obtained through measurement of the absorbance of a series of standards at heparin concentrations of 10–100 μg/mL. The amount of immobilized heparin was calculated from the calibration curve.

2.6 Interaction of thrombin and factor Xa with the magnetic nanoparticles.

The interaction/affinity assay of immobilized heparin in MAG-PANI with human thrombin and coagulation FXa had been based on a study conducted by Lira et al. [28]. Initially, 10 nM thrombin and 10 nM FXa solutions were prepared in enzymatic assay buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 0.5% PEG 8000) containing increasing concentrations of 20, 50, 100, 200, 400, 600, 800 and 1000 mM NaCl. Then 125 µl of a suspension of MAG-PANI-hep (in 20 mM Tris-HCL pH 7.5) were incubated for 3 minutes at 25 °C with 1 mL of thrombin (10 nM) or FXa (10 nM) solutions that had been prepare. Besides that, MAG-PANI nanoparticles and MAG-PANI-hep composites were incubated with 1 mL of a thrombin (10 nM) or factor Xa (10 nM) solution in Tris-HCl buffer without salt (0 mM) also for 3 minutes at 25 °C. After the incubation step, the magnetic composites were recovered with a magnetic rack (MagRack ™ 6, GE) and then 200 µL of the each supernatant was collected for the thrombin or factor Xa enzyme activity test.

2.7 Fluorescence enzymatic assay by chromogenic method.

The enzymatic assays of thrombin and FXa in the supernatant collected after incubation with MAG-PANI-hep and MAG-PANI were performed in a Flexstation III from Molecular Devices (Sunnyvale, CA) at 25 C. 200 µL of thrombin or FXa supernatant were added in a 96-well plate. Next, 5 µL of the thrombin chromogenic substrate S2238 (H-D-Phe-Pip-Arg-pNA) or 5 µL FXa chromogenic substrate S2222 (Bz-Ile-Glu- (γ-OR)-Gly-Arg-pNA) were added to the respective well.

Progress curve of the enzyme reaction (absorbance readings over time) were obtained at 10 seconds intervals for a maximum of 30 minute. The assay were performed in duplicate. Data were corrected by subtracting the substrate-only absorbance and finally analyzed by Origin85© software.

3 RESULTS AND DISCUSSION

3.1 Electron microscopy characterization

MAG-PANI analyzed by SEM (Figure 1b) shows irregular morphology and distribution with clumps containing nanoparticles with heterogeneous distribution between 10-100 µm. Moreover, according TEM analyses (Figure 2) the MAG-PANI particle size had an average size of 11.7 ± 2.2 nm. Magnetite bare particles (Figure 2A) had a particle size about 9.94 ± 1.5 nm, a size smaller due to the absence of polyaniline in MAG. According to Maciel et al. [29], the coating is responsible for changes in particle size. In addition, similar results are shows by Chelminiak et al. [12] who synthesized magnetite nanoparticles coated with a poly (acrylic acid) polymer and these particles after TEM analysis showed a size of about 13 nm, larger than compared to pure magnetite.

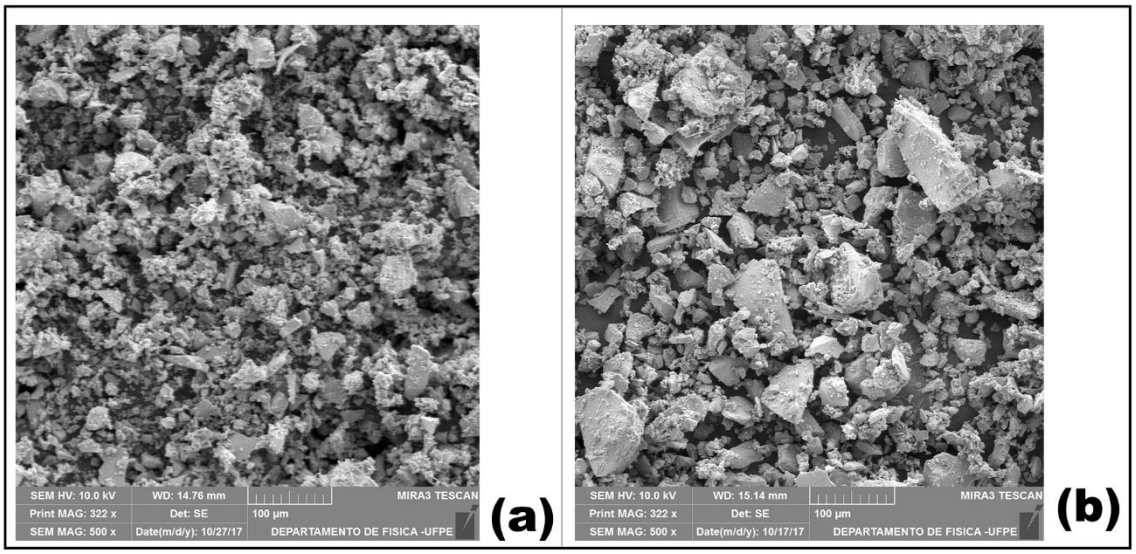

Figure 1. Distribution and morphology of magnetite (a) and MAG-PANI (b) obtained by scanning electron microscopy. 500x magnification.

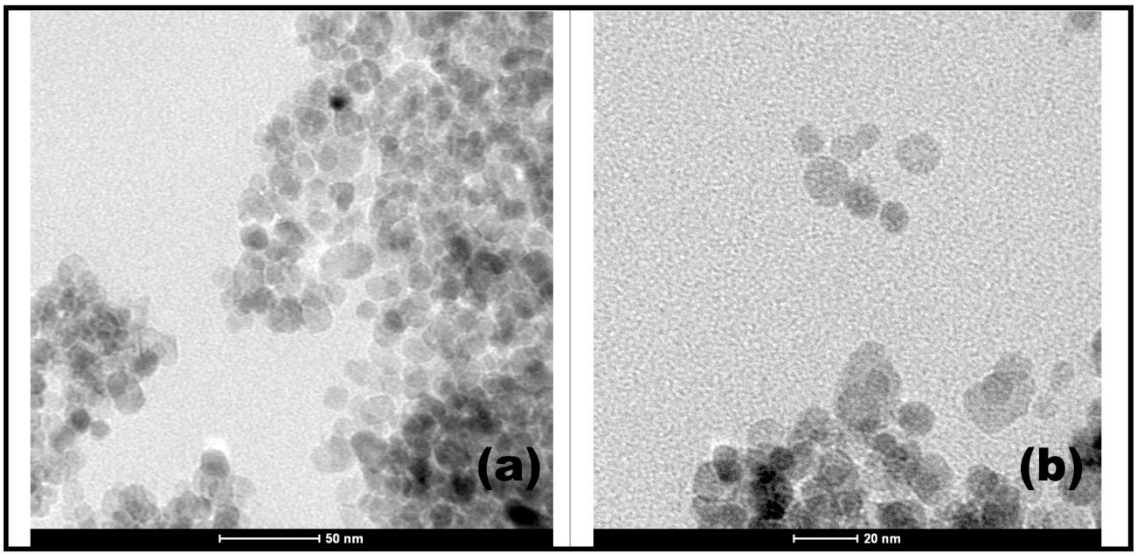

Figure 2. Particle size and structure of magnetite (a) and MAG-PANI (b) by transmission electron microscopy.

3.2 Structural analysis and magnetic saturation of nanoparticles

XRD confirmed the formation of pure magnetite crystals (Fe_3O_4) and polyaniline coating (Figure 3). The peaks corresponding to the pure magnetite and MAG-PANI nanoparticles are shows in table 1. The MAG-PANI diffractogram (Fig 3b) contains all reflections of pure magnetite at the 2θ angles according with Mišurović et al. [15]. The magnetite phase it was dominant and polyaniline phase correspond to 10.53° and 14.4° peaks, absent in the uncoated Fe_3O_4 crystals. Neri et al. [10] reports a difficult differentiation between magnetite and polyaniline coated magnetite nanoparticles by XRD pattern because their lines are close.

Table 1. Peaks obtained from X-ray analysis of magnetite and MAG-PANI particles.

Nanoparticles	***Peaks 2Θ (degrees)***
MAG-PANI	10.53°; 14.4°; 18.13°; 30.39°; 35.48°; 43.23°; 53.69°; 57.12°; 62.9°; 71.33°; 74.02°.
Magnetite crystals	18.27°; 30.39°; 35.62°; 43.23°; 53.69°; 57.3°; 62.81°; 74.02°.

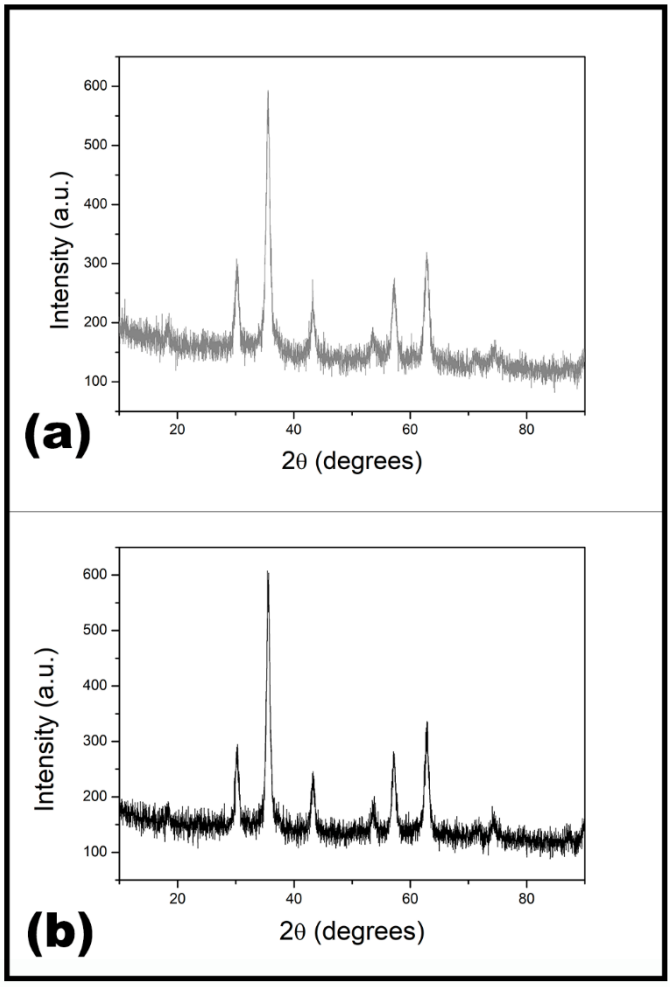

Figure 3. Structural analysis by XRD of magnetite (a) and MAG-PANI (b) nanoparticles.

The magnetic measurements analyzed by isothermal magnetization curves M(H) in Figure 4 determine the value of the magnetization (emu/g) present in MAG-PANI e magnetite nanoparticles as a function of applied magnetic field (Oe) at 293 K, 300 K and 313 K. The magnetic saturation (M_S) to MAG-PANI was 68 emu/g and 72 emu/g to magnetite (Table 2). In addition MAG-PANI shows a superparamagnetic behavior because at the isothermal magnetization curves M(H) no remnant magnetization or coercivity was observed [30].

The bare magnetite (uncoated) obtained a higher M_S value compared to MAG-PANI nanoparticles, the presence of a coating or polymer in the magnetite crystal (Fe_3O_4) causes a reduction in its magnetic saturation.

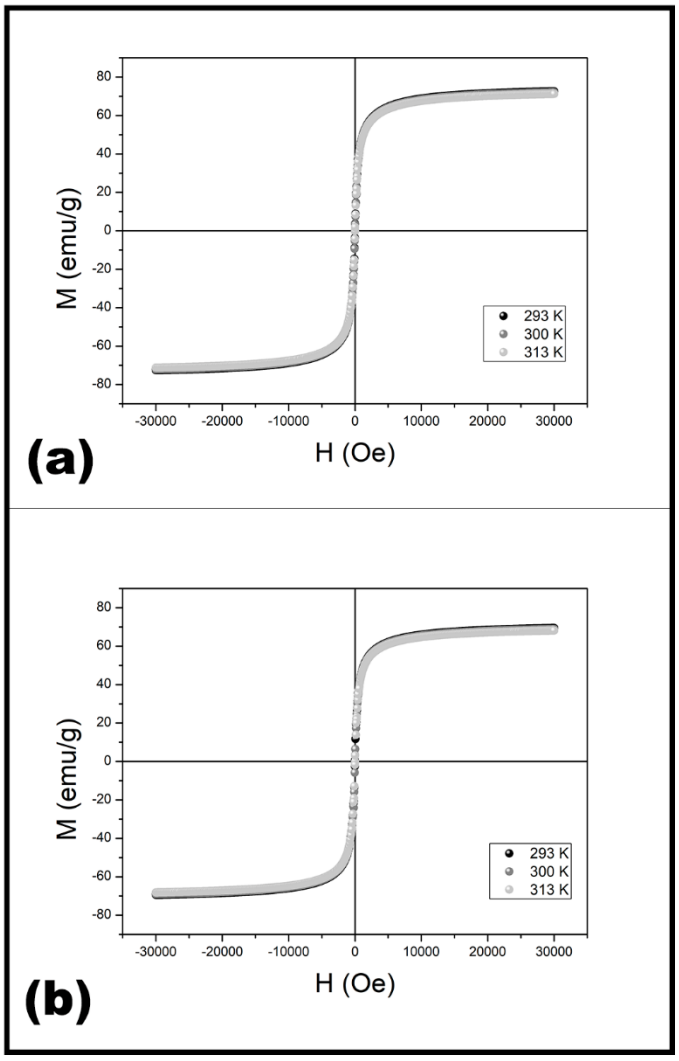

Figure 4. Isothermal magnetization M(H) curves at 293 K, 300 K and 313 K for magnetite (a) and MAG-PANI (b).

Table 2. Magnetic saturation measurements of the polyaniline coated magnetite and magnetite particles at temperatures of 293, 300 and 313K.

Nanoparticles	***M_s (293K)***	***M_s (300K)***	***M_s (313K)***
Magnetite crystals	72 emu/g	72 emu/g	71 emu/g
MAG-PANI	69 emu/g	68 emu/g	68 emu/g

3.3 Immobilization and determination of heparin

Magnetite crystal (Fe_3O_4) coated with polyaniline become this material very attractive as a matrix to biomolecules immobilization [31]. Neri et al. [10] synthesized and used polyaniline-coated magnetite particles for β-galactosidase immobilization and Maciel et al. [25] used to tripsin immobilization. Recently, Cabrera et al. [32] demonstrated that polyaniline-coated magnetic diatomite nanoparticles can be used as a matrix for immobilization of invertase, β-galactosidase, and trypsin. Lima et al. [33] immobilized tannase onto magnetic diatomaceous earth nanoparticles coated with polyaniline.

In the present study, heparin was immobilized onto MAG-PANI nanoparticles due interaction between functionalized carboxyl groups of heparin and the amine groups of the nanoparticles; this proposal is in agreement with the literature where we find different approaches to immobilize covalently the heparin in biomaterials through covalent attachment to support using EDC and NHS [34]. Heparin solution initially offered was 3.411 mg/mL, the average amount of immobilized heparin in the particles was 43 ± 6.2 μg heparin per mg MAG-PANI nanoparticles. Magnetite (uncoated) fixed approximately 29.4 μg of heparin per mg of magnetite crystals (Fe_3O_4).

3.4 Enzyme activity and interaction

Heparin exerts its anticoagulant activity via antithrombin-mediated inhibition of thrombin and factors Xa, IXa, and XIa [35]. Figure 5 shows the thrombin residual activity present in the supernatant collected after its incubation with heparin immobilized onto MAG-PANI nanoparticles. About 9.0 ± 3 % of thrombin enzymatic activity was detected in 20 mM NaCl. Under conditions with 800 mM NaCl, thrombin enzymatic activity of around 85.0 ± 4 % was observed.

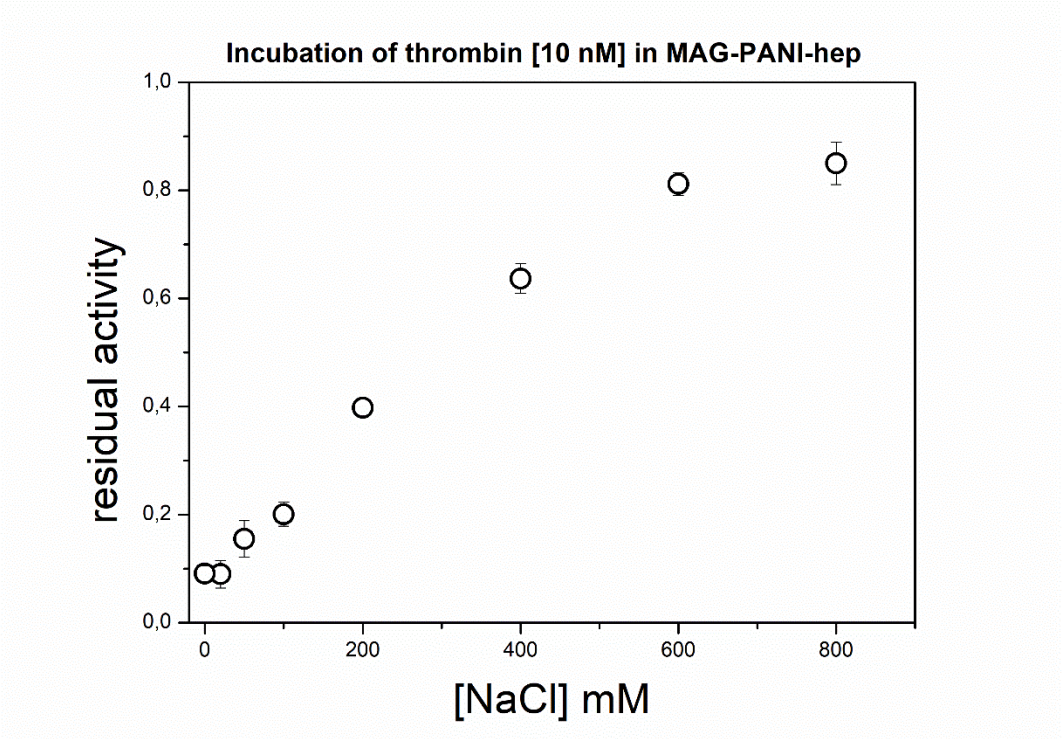

Figure 5. Thrombin activity residual in 10 mM Tris-HCl buffer pH 7.4 with 0, 20, 50, 100, 200, 400, 600, 800 mM NaCl after incubation with MAG-PANI-hep.

Results of the factor Xa residual activity in the supernatant collected after its incubation with heparin immobilized onto MAG-PANI nanoparticles is present in the Figure 6. The observed values indicate that about 25.2 ± 1 % of factor Xa enzymatic activity was detected in 20 mM NaCl. Under conditions with 800 mM NaCl, factor Xa enzymatic activity of around 55.4 ± 12 % was observed.

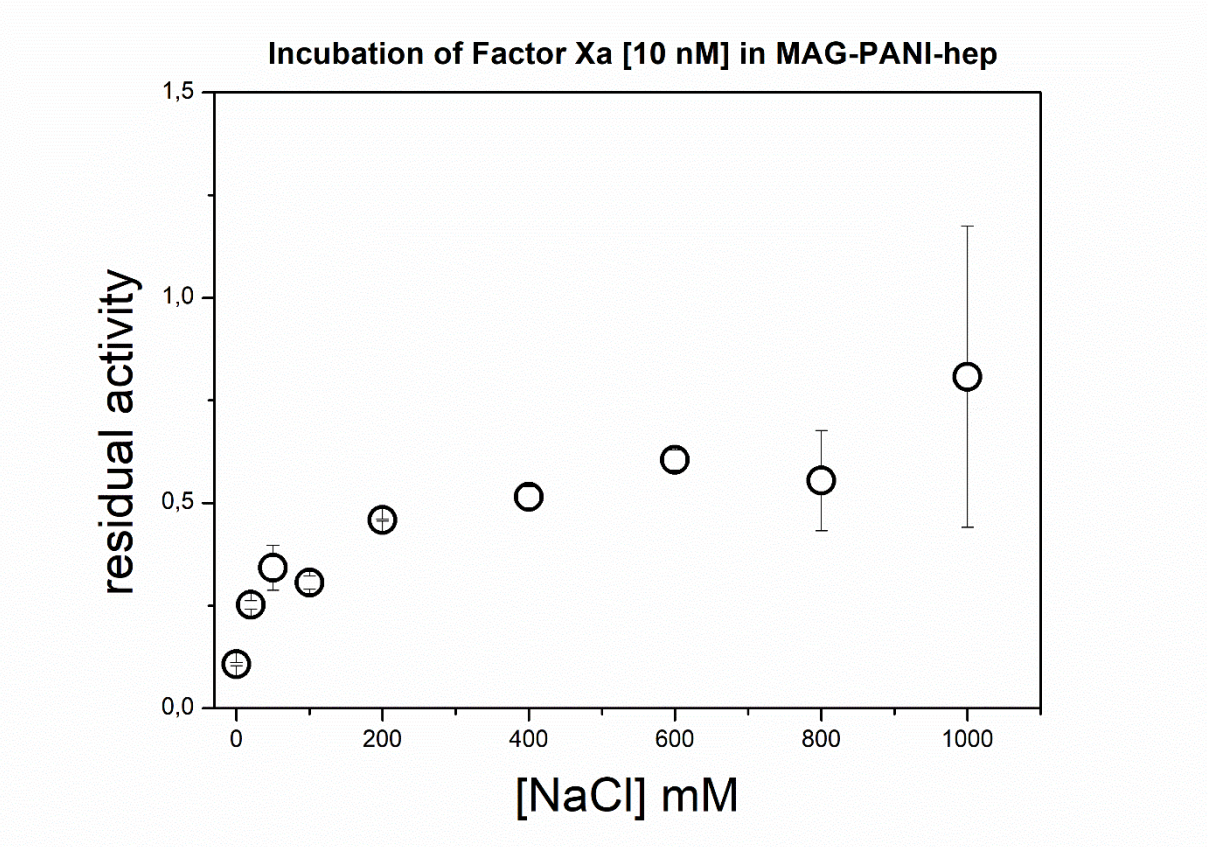

Figure 6. Factor Xa activity residual in 10 mM Tris-HCl buffer pH 7.4 with 0, 20, 50, 100, 200, 400, 600, 800 mM NaCl after incubation with MAG-PANI-hep.

Also according to Figure 7, thrombin and factor Xa demonstrated a low interaction with MAG-PANI in the absence of heparin, the enzymatic activity in the supernatant after incubation with MAG-PANI at 0 mM NaCl was about 85.0 ± 3.9 % and 62.1 ± 2% respectively. Therefore, the above results suggest that thrombin and factor Xa demonstrated affinity to heparin immobilized onto MAG-PANI.

Under salt-free conditions the residual supernatant activity after incubation in MAG-PANI-hep was 5.3 ± 0.4 and 10.9 ± 0.4% for thrombin and factor Xa, respectively. This demonstrates that in the absence of ionic strength the enzyme is fixed to immobilized heparin thus decreasing its activity and when in high NaCl concentrations an amount of enzyme is still fixed to heparin.

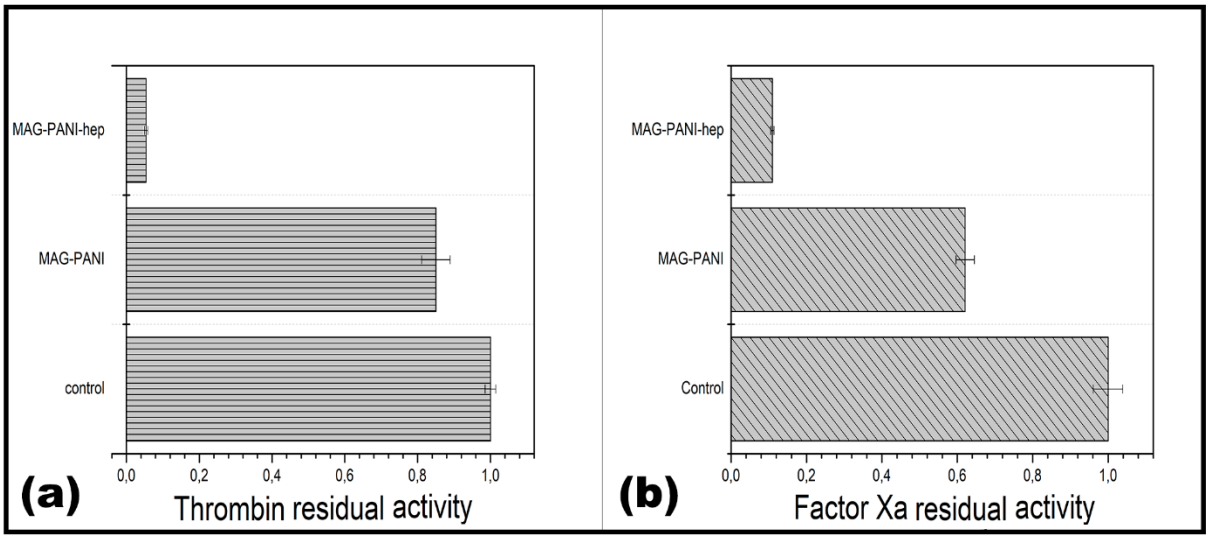

Figure 7. Thrombin (a) and Factor Xa (b) activity residual after incubation with 0 mM NaCl (Tris-HCL buffer pH 7,4) in MAG-PANI-hep e MAG-PANI. Control: free enzyme activity.

Thrombin and factor Xa showed a stronger interaction with heparin immobilized onto MAG-PANI at differents NaCl solutions (Figure 8). Therefore, at higher sodium chloride concentrations (600 - 800 mM) there is less residual activity in the supernatat after MAG-PANI-hep incubation, which suggests an affinity to heparin. Factor Xa and thrombin are important enzymes of the human blood coagulation cascade and are known to have binding sites that recognize heparin. Thrombin is capable of interacting with heparin due to the presence of an exocyte, also called the heparin binding site, which carries a number of positively charged residues, including Arg93, Arg97, Arg101, Arg126, Arg165, Lys169, Arg173, Arg175, Arg233, Lys235, Lys236 e Lys240 [23].

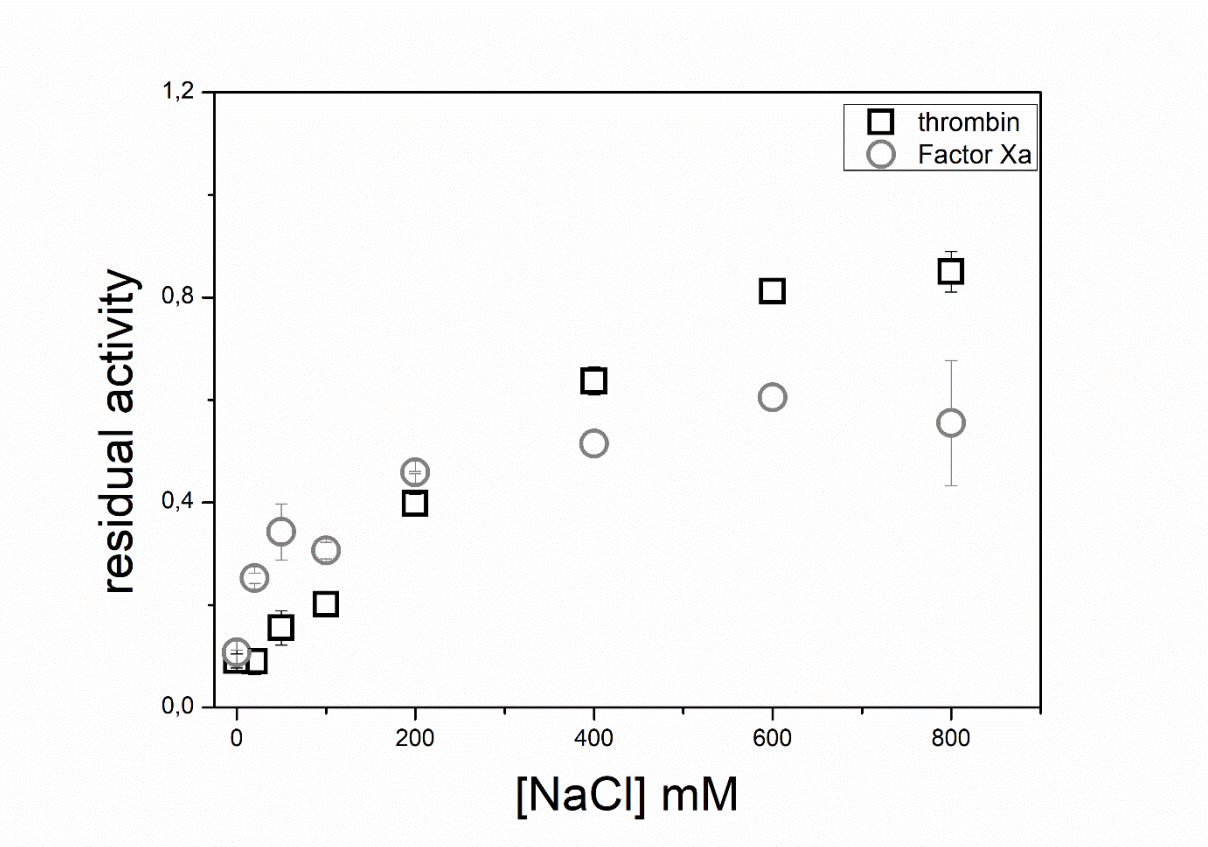

Figure 8. Comparison of thrombin and factor Xa residual activity in 10 mM Tris-HCl buffer pH 7.4 with 0, 20, 50, 100, 200, 400, 600 and 800 mM NaCl after interaction with heparin immobilized (MAG-PANI-hep). □: thrombin residual activity; ○: factor Xa residual activity.

4 CONCLUSION

According to the above results, the magnetite nanoparticles were obtained satisfactorily by the co-precipitation method and coated with polyaniline. TEM and SEM analysis showed that MAG-PANI particles have a heterogeneous distribution and a size of 11.7 ± 2.2 nm. Moreover, the presence the phase magnetite and polyaniline have been proven through XRD analysis. In addition, MAG-PANI nanoparticles exhibited a superparamagnetic behavior when subjected to a magnetic field of +30000 Oe to -30000 Oe at of 293 K, 300 K and 313 K. thrombin and factor Xa demonstrated affinity for immobilized heparin in MAG-PANI. These results were confirmed by measuring enzyme activity in the supernatant after incubation with magnetic composites. Under conditions with 800 mM NaCl the residual activity was 85.0 ± 4% and 55.4 ± 12% for thrombin and factor Xa, respectively. Under salt-free conditions the residual supernatant activity was 5.3 ± 0.4 and 10.9 ± 0.4% for thrombin and factor Xa, respectively.

This demonstrates that in the absence of ionic strength the enzyme is fixed to immobilized heparin thus decreasing its activity and when in high NaCl concentrations an amount of enzyme is still fixed to heparin. Thus, MAG-PANI-hep composite can be used as a tool for obtaining blood products and assisting in the therapy of some diseases. In addition this tool can be used for further studies of ligand-protein interaction /modification. The proposed method based on the affinity magnetic separation is effective, easy and specific

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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6 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

- As partículas magnéticas sintetizadas: mDAC, QS-MAG e MAG-PANI demonstraram ser úteis como suporte para imobilização covalente da heparina. O tamanho da partícula e o polímero utilizado na modificação das partículas magnéticas influenciaram na quantidade de heparina imobilizada. As partículas de QS-MAG apresentaram uma maior quantidade de heparina imobilizada (93,8 ± 1,93 μg de heparina por mg de suporte) e também um maior tamanho de partícula (100-300 μm);

- Além disso a heparina imobilizada funcionou como um ligante de afinidade e quando capaz de interagir por afinidade e troca iônica com diferentes proteínas do plasma humano. A heparina presente em mDAC e MAG-PANI foi capaz, respectivamente, de purificar e interagir por afinidade com proteínas da cascata da coagulação. Uma maior quantidade de proteína plasmática foi depletada e purificada a partir da interação por troca iônica com a heparina imobilizada em QS-MAG.

- Importantes proteínas do plasma humano como antitrombina, protrombina, trombina, fator Xa, albumina humana e serpinas, foram capazes de interagir com a heparina imobilizada nos compósitos magnéticos produzidos;

- O método proposto possui vantagens como por exemplo: fácil síntese, baixo custo e reutilização;

- Diante dos resultados obtidos, mais estudos podem ser realizados a fim de se avaliar os diferentes sítios de ligação responsáveis pela interação entre as proteínas e a heparina imobilizada em suportes magnéticos;

- Além disso, estudos de afinidade entre proteínas com outros glicosaminoglicanos podem ser investigados;

- Os resultados obtidos neste trabalho fortalecem as pesquisas com partículas magnéticas, bem como sua utilização em diferentes aplicações biotecnológicas, todas justificadas pelas diversas vantagens já citadas anteriormente.

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APÊNDICE A – ARTIGO CIENTÍFICO PUBLICADO: SYNTHESIS AND CHARACTERISATION OF MAGNETISED DACRON-HEPARIN COMPOSITE EMPLOYED FOR ANTITHROMBIN AFFINITY PURIFICATION.

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Synthesis and characterisation of magnetised Dacron-heparin composite employed for antithrombin affinity purification

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ABSTRACT

Human antithrombin is a blood derivative widely used in the treatment of coagulation dysfunction. Affinity chromatography using heparin (HEP) derivatives is usually used for antithrombin purification. In this study, an affinity procedure based on a magnetic Dacron-HEP composite is proposed. Dacron was firstly converted to Dacron-hydrazide and magnetised by co-precipitation with of Fe^{2+}/Fe^{3+} (mDAC). HEP was activated by carbodiimide and *N*-hydroxysuccinimide and covalently linked to mDAC (mDAC-HEP). EDX and infrared spectra analyses confirmed each synthesis step of mDAC-HEP. This composite exhibited superparamagnetism behaviour. Human plasma was incubated with mDAC-HEP (fresh and stored over a long period) and washed with phosphate buffer containing increasing concentrations of NaCl. Human plasma antithrombin activity was reduced by approximately 20% in the presence of the 1.0 M NaCl fraction, and this eluate was able to prolong coagulation time (aPTT) using both preparations. Electrophoresis of the eluates revealed bands corresponding to the expected size of antithrombin (58 kDa). The mDAC-HEP particles are reusable. This method presents the following advantages: easy, low-cost synthesis of the composite, magnet-based affinity purification steps, and reusability.

1. Introduction

Antithrombin (AT), as a serine proteinase inhibitor, can interfere with blood coagulation and, consequently, has been used in coagulation dysfunction therapies, such as acute thrombotic episodes, for prophylaxis during surgery and in patients with AT shortage [1]. AT is usually purified by heparin (Hep) affinity chromatography [2] from human plasma fractions [3]. In the last four decades, Hep covalently linked to both Sepharose and Sephadex has been used for AT purification [4–7]. Currently, there are few reports of the use of alternatives to these commercial resins. Here, an alternative procedure is proposed for human AT purification based on immobilised Hep on magnetic Dacron. Hep is a highly sulphated linear polysaccharide, belonging to the glycosaminoglycan family, which has been used in clinics because of its anticoagulant properties [8]. Hep's structure is composed of a linear chain of repeating disaccharide units comprised of an uronic acid (iduronic or glucuronic) [9] and glucosamine that can be either *N*-acetylated or *N*-sulfonated [10]. As a result of this structure, Hep has the highest negative charge density compared to any other biologically active macromolecule [11]. The negative charge of Hep has been associated to its strong binding activity to various proteins [12]. In addition, Hep has reactive functional groups (carboxylates and sulphates) that permit its covalent immobilisation onto water-insoluble matrices [13]. Immobilised Hep can interact, for example, with coagulation factors and act as an affinity ligand capable of purifying such proteins [14]. This method is known as heparin affinity chromatography, which is used to purify or fractionate proteins and other biological molecules that may interact with heparin. Burnouf and Radosevich [15] reviewed the importance of affinity chromatography for purification of plasma proteins, such as AT, for therapeutic use and reported that the best way of obtaining AT purified is through the use of immobilised Hep.

Magnetic particles are among the most common materials used for separation techniques in biomedical analysis and

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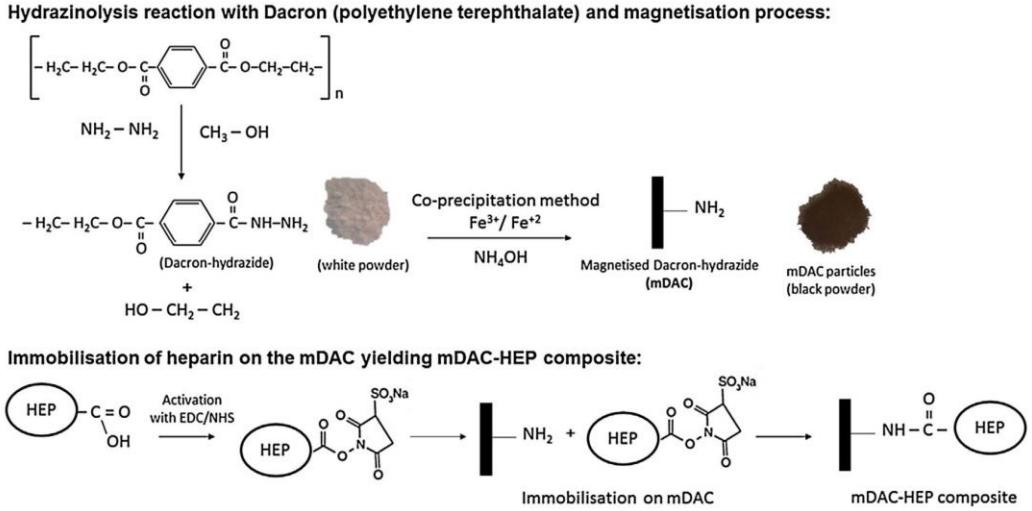

Fig. 1. mDAC-HEP composite synthesis. mDAC: Dacron-hydrazide magnetic; HEP: heparin; EDC: 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide and NHS: N-hidroxissuccinimida.

biotechnology. According to Laurent et al. [16] a co-precipitation technique is the most efficient way to obtain magnetic particles. For example, iron oxides such as magnetite (Fe_3O_4), are generally prepared by a stoichiometric mixture of ferric and ferrous salts in an alkaline aqueous environment. The chemical reaction of Fe_3O_4 formation can be written according to the equation: $Fe^{+2} + 2Fe^{+3} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O$. The magnetic properties of these particles allow the easy isolation of products in solution by attracting them with the aid of an external magnetic field. Thus, suspended magnetic particles tagged with analytes can be removed using a magnet, and isolation and purification are easier and faster than with other methods [17]. In our laboratory, Dacron magnetic particles have been proposed for enzyme immobilisation [18–20]. Dacron, or Polyethylene terephthalate, is a widely used polymer derived from a condensation reaction between terephthalic acid and ethyleneglycol [21]. Dacron is an inert hydrophobic polymer without functional groups. Therefore, chemical modifications must be performed to introduce functional groups in order to alter the surface properties. Such modifications can create groups that can form covalent linkages with various biomolecules such as carbohydrates, peptides and proteins [22]. For instance, the synthesis of magnetic Dacron particle composites described by Carneiro Leão et al. [23] was initially performed by converting the polymer into Dacron-hydrazide. Therefore, this study aimed to characterise and use Dacron-hydrazide-Hep magnetic particles to serve as an alternative tool for affinity purification of AT. Dacron-hydrazide-Hep magnetic particles have several advantages compared to the resin derivatives, including easy synthesis using low cost reagents and, easy removal from the incubation mixture by applying a magnet, and the particles are reusable.

2. Material and methods

2.1. Materials and reagents

Terphane Ltda (Cabo de Santo Agostinho, PE, Brazil) kindly donated Dacron. Heparin was purchased from Cristália Chemicals & Pharmaceuticals Ltda (São Paulo, Brazil). Carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EDC), *N*-hydroxysuccinimide (NHS), hydrazine hydrate, antithrombin III from human plasma (lyophilized powder, ≥95% – SDS-PAGE),

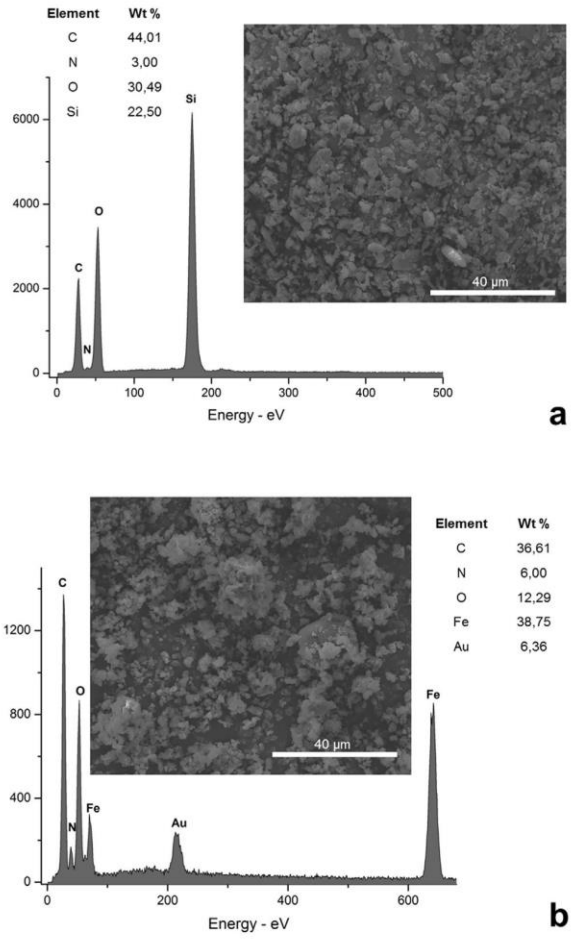

Fig. 2. SEM images and EDX of Dacron-hydrazide (a) and mDAC (b).

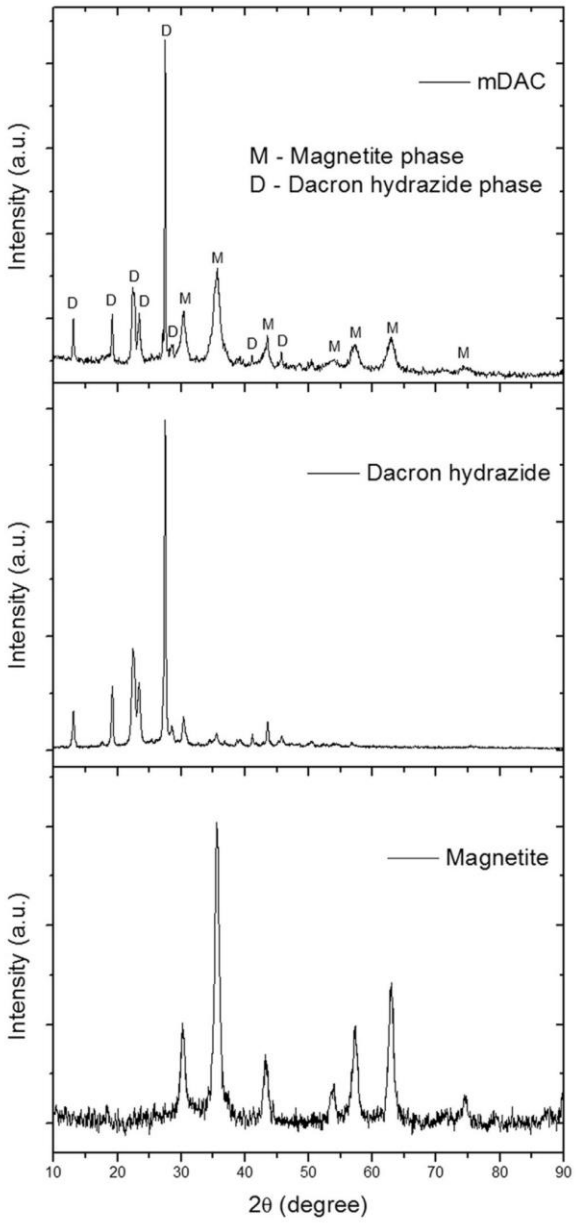

Fig. 3. X-ray diffraction patterns of magnetite, Dacron hydrazide and mDAC particles.

ferric and ferrous chloride were purchased from Sigma-Aldrich Ltda (São Paulo, Brazil). The antithrombin amidolytic activity kit (TriniCHROM™ Antithrombin IIa) was acquired from Trinity Biotech SA (New Jersey, USA), and the activated partial thromboplastin time (aPTT) kit was from Labtest Diagnóstica (Minas Gerais, Brazil). Human blood was collected from a volunteer under approval of the Ethical Committee of the Universidade Federal de Pernambuco.

2.2. Synthesis and characterisation of magnetic particles

Dacron (polyethylene terephthalate) was converted to Dacron-hydrazide by hydrazinolysis and then magnetised by co-precipitation of Fe^{2+}/Fe^{3+} (mDAC) according to Amaral et al. [18]. The particle size, morphology and chemical composition of Dacron-hydrazide and mDAC were established by scanning electronic microscopy (SEM) using a QUANTA 200 FEG electron microscope equipped with an Energy Dispersive X-ray (EDX). The structural properties were characterised by X-ray diffraction (XRD) performed at room temperature in the range 10–90°, in equal 2θ steps of 0.02°, using a Bruker D8 Advance diffractometer with CuKα radiation (λ = 1. 5406 Å). Magnetisation measurements were made as a function of applied magnetic field (M × H) using a vibrating sample magnetometer (VSM), Versalab, manufactured by Quantum Design. The measurements were obtained at 293 K, 300 K and 313 K, with magnetic fields in the −30 KOe to +30 KOe range.

2.3. Immobilisation of heparin on the magnetic particles

First, a solution of heparin (3 mg/mL) was treated with EDC/NHS according to Oliveira et al. [24] to activate heparin carboxylic groups. An aliquot of this functionalized heparin (1 mL) was incubated with 30 mg of mDAC for 72 h with mild agitation, yielding covalently immobilised heparin on mDAC (mDAC-HEP). The composite was recovered under a magnetic field (6000 Oe) and washed ten times with deionized water to remove non-immobilised heparin. Under this magnetic field, particle precipitation occurred in approximately 30 s, but one minute was used to assure that all particles were collected. The supernatant (SB), first wash (L1) and second wash (L2) were reserved for heparin determination according to Oliveira et al. [24] based on the property of heparin to form complexes with basic dyes such as methylene blue. This magnetic composite was suspended in water at 4 °C and used immediately (fresh preparation) or after 2 years of storage (stored over a long period preparation) in order to investigate the stability of the composite for antithrombin purification.

2.4. Physical-chemical analysis

Infrared spectra of magnetite (MAG), mDAC and mDAC-HEP were analysed using a BRUKER instrument model IFS 66.

2.5. Plasma antithrombin purification

Citrated human plasma (1.0 mL) was incubated with mDAC-HEP (30 mg) and incubated with stirring for 1 h at 4 °C. Both fresh and stored over a long period preparations of mDAC and mDAC-HEP were used for AT purification from human plasma. The magnetic composites were collected by a magnetic field (6000 Oe), and the supernatant (human plasma) was stored for further analysis. The composites were washed with 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl (PBS), and the composites were subsequently eluted with the same buffer containing an increasing gradient of NaCl (0.25 M; 0.5 M; 1.0 M). Both mDAC-HEP preparations (fresh and stored over a long period) were reused ten times, washing fifty times with PBS between each successive use. The eluates obtained from the increasing concentration gradient of NaCl (0.25 M; 0.5 M; 1.0 M) were analysed by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE; 12.5%) according to Laemmli [25]. Two mDAC-HEP preparations were used: fresh and stored over a long period.

2.6. Measurement of antithrombin activity

Plasma antithrombin activity was evaluated by amidolytic inhibition using a chromogenic substrate assay (Tos-Gly-Pro-Arg-ANBA-IPA) and aPTT. These procedures were performed according to the manufacturer's instructions. The aPTT test for the plasma was

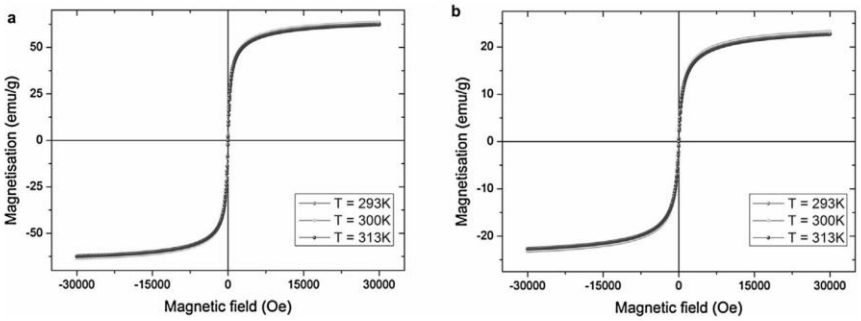

Fig. 4. Hysteresis curves at 293 K, 300 K and 313 K for MAG (a) and mDAC (b).

performed by adding 20 μL of eluate obtained from the 1.0 M NaCl gradient to the reaction system kit.

3. Results and discussion

The synthesis method for obtaining mDAC-HEP composite is illustrated in Fig. 1. According to this scheme, the initial process starts with Dacron being converted to Dacron-hydrazide (hydrazinolysis), which contains amine groups. Afterward, this modified polymer (white powder) is magnetised with iron salts (black powder). In parallel, heparin was functionalized with EDC/NHS. Finally the modified heparin was covalently immobilised on the mDAC.

3.1. Morphology and size of particles

Images obtained by SEM (Fig. 2) showed heterogeneous morphology and similar size for both Dacron-hydrazide (Fig. 2a; 0.7–2.0 μm) and mDAC (Fig. 2b; 1.0–2.0 μm) particles, whereas EDX analysis demonstrated the presence of the elements C, N and O in Dacron-hydrazide and the additional presence of iron (Fe) in mDAC particles. These findings indicate the presence of Dacron in the magnetic composite.

3.2. X-ray analysis

The presence of magnetite phase in the Dacron-hydrazide particles was indicated by X-ray analysis. The X-ray diffraction patterns of MAG, Dacron-hydrazide and mDAC particles can be observed in Fig. 3. In the magnetite XRD pattern, the 2θ peaks corresponding to this phase appear at 18.39°, 30.26°, 35.65°, 43.33°, 53.74°, 57.76°, 62.94°, 74.49° and 79.50°. These peaks of magnetite also appear in mDAC's XRD pattern in addition to the peaks relative to Dacron hydrazide material. Peaks corresponding to the Dacron hydrazide: 11.24°, 13.10°, 17.57°, 19.25°, 22.35°, 22.58°, 23.44°, 25.33°, 26.54°, 27.49°, 28.51°, 30.33°, 32.66°, 34.43°, 35.49°, 36.76°, 38.71°, 39.23°, 41.17°, 45.54°, 45.75°, 48.42°, 50.64°, 52.18°, 54.24°, 56.75°. The magnetite diffractogram revealed a cell parameter of 8.37 Å for the magnetite phase. For the magnetite phase formed in mDAC, the cell parameter value is 8.36 Å, notably close to the value obtained by Jamarillo-Tabares et al. [26]. The peaks corresponding to magnetite are according to Jamarillo-Tabares et al. [26] and Cabrera et al. [27]. The XRD peaks of the mDAC particles are slightly broader than those of the Dacron-hydrazide powder, which is characteristic for amorphous materials and also of ultrafine crystalline particles. This finding suggests the presence of polyethylene terephthalate in mDAC particles.

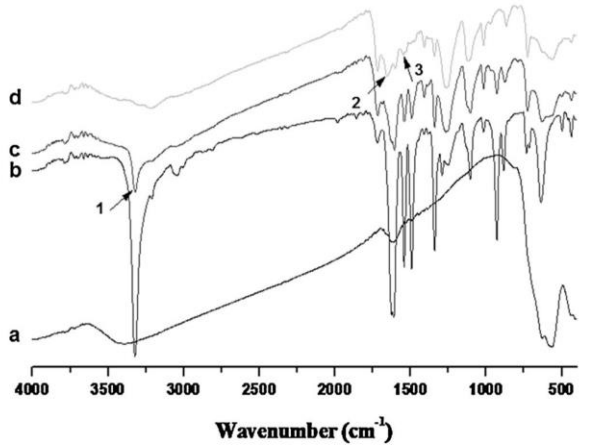

Fig. 5. Infrared spectra of MAG (a), Dacron-hydrazide (b), mDAC (c) and mDAC-HEP (d). 1: Band of NH_2 group of the hydrazide; 2: Band of amide I; 3: Band of amide II.

3.3. Magnetisation measurements

The magnetisation curves dependent on the magnetic field for pure magnetite and mDAC particles are shown in Fig. 4a and b, respectively. The hysteresis curves revealed a magnetic saturation (M_S) to mDAC of 23.13 emu/g (293 K), 23.02 emu/g (300 K) and 22.67 emu/g (313 K). As expected, the mDAC particles had magnetic ordering but the degree of M_S was lower (2.74 times) at 293 K, 300 K and 313 K compared to MAG. For all of these temperatures, the decrease in saturation could be related to magnetite modification in the mDAC particles due to the presence of polyethylene terephthalate. Cabrera et al. [27] and Maciel et al. [28] synthesized mineral composites and magnetic levan, respectively, and reported similar results. Furthermore, according to the analysis of MAG and mDAC magnetisation, neither remnant magnetization nor coercivity was observed, suggesting that both magnetic materials exhibited superparamagnetism. Nicolás et al. [29] also reported that the magnetic particles produced by the co-precipitation method and modified by the addition of stabilizers presented superparamagnetic behaviour.

3.4. Heparin immobilisation

Approaches to covalently immobilise Hep on biomaterials by linking it to EDC and NHS supports have been described in the literature [30]. Thus, covalent immobilisation of Hep was established between the functionalized Hep carboxyl groups and mDAC amino groups, yielding an amide bond. The mDAC-HEP presented 51 ± 0.1 μg of heparin per mg of mDAC, whereas 13 ± 0.4 μg of hep-

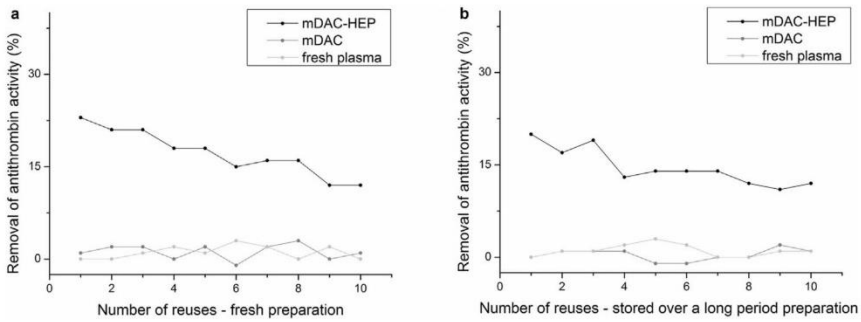

Fig. 6. Removal of antithrombin activity present in fresh plasma and after direct contact with the mDAC derivatives. Fresh preparation (a) stored over a long period preparation (b).

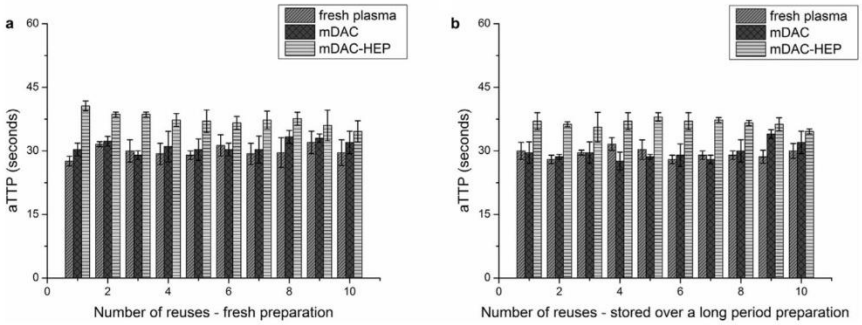

Fig. 7. Activated partial thromboplastin time (aPTT) of fresh plasma evaluated by the addition of a small aliquot of the eluate (1.0 M) of mDAC derivatives. Fresh preparation (a) stored over a long period preparation (b).

arin was adsorbed to each mg of MAG. About half of the added heparin was covalently immobilised on the mDAC particles. This immobilisation rate is larger than previously reported values, for example, Funahashi et al. [31] reported a preparation containing 16 μg of Hep per mg of wet gel (Sepharose-4B modified to contain amine groups) using EDC as a coupling agent.

3.5. Assignment of infrared absorption bands

The infrared spectrum of mDAC-HEP showed a pattern of bands in the region between 1700 and 1500 cm^{-1} that indicate Hep immobilisation (Fig. 5). In this region, amide I bands (1650 cm^{-1}, arrow 2) and amide II bands (1544 cm^{-1}, arrow 3) suggest that the amide group was formed by linkage of the carboxyl group of heparin with the hydrazide group of Dacron. Dacron-hydrazide spectrum presents a band approximately 3300 cm^{-1} (NH_2 group, arrow 1) that is absent in mDAC-HEP, suggesting that the hydrazide groups were consumed during the immobilisation reaction. Infrared spectra of the three constituent particles (MAG, Dacron-hydrazide and heparin) have been described in the literature. Yamaura et al. [32] observed strong bands approximately 632 cm^{-1} and 585 cm^{-1} in their magnetic nanoparticles and attributed these to the presence of magnetite. Yamaye et al. [33] reported bands at 3330, 1630 and 1540 cm^{-1} and characteristics of dihydrazide from Dacron. In this study, the infrared spectra of the composites did not denote any band characteristic of heparin, probably because Dacron-hydrazide bands overlapped them. Infrared spectra of heparin should present bands approximately 3400, 1624, 1425 and 1236 cm^{-1} [24].

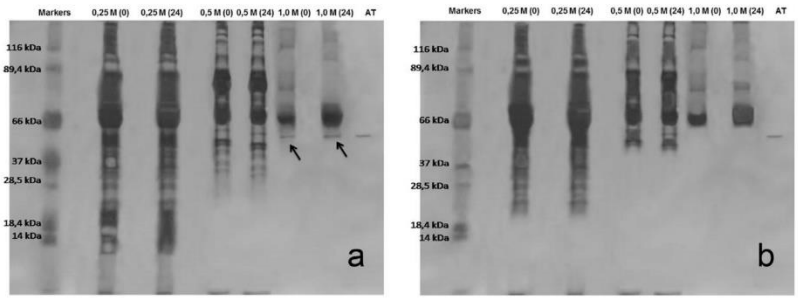

Fig. 8. Electrophoresis of protein fractions from plasma obtained with the use of magnetic particles. Elutions were carried out using increasing NaCl concentrations (0.25; 0.5 and 1.0 M) in the magnetic derivatives mDAC-HEP (a) and mDAC (b). AT: Standard antithrombin. (0): Elutions were performed in supports with immediately synthesized (fresh preparation). (24): Elutions performed in supports that were stored for 2 years (stored over a long period preparation). Arrows: Antithrombin (58 kDa).

3.6. Plasma antithrombin purification

Heparin affinity chromatography, eluting with increasing concentrations of NaCl is the most common method for separating human plasma AT [2,34]. Fig. 6 shows plasma AT activity before and after incubation with mDAC-HEP and mDAC using the fresh and stored over a long period preparations. AT was removed after incubation with fresh (Fig. 6a) and stored over a long period (Fig. 6b) mDAC-HEP preparations at the 1st use (approximately 20%, namely, a fifth of total AT). This property was preserved during all reuses with a small decrease, but approximately 10% was still removed on the 10th reuse. Negligible removal of AT was observed for the mDAC (Fig. 6) compared to antithrombin activity in fresh plasma. Purification yields have been reported in the literature higher than these values [2,7], but these studies used pre-prepared human plasma. In this study, whole plasma was used, and the procedure can be reused.

3.7. In vitro anticoagulant assay and electrophoresis

Fig. 7 displays the aPTT for eluates obtained from the mDAC-HEP-plasma antithrombin complex washes with PBS containing 1.0 M NaCl. It is well-known that AT strongly binds to heparin and is eluted at very high salt concentrations [35]. Higher aPTT values were seen for the eluates from mDAC-HEP compared with fresh plasma and mDAC, using either fresh (Fig. 7a) or stored over a long period (Fig. 7b) preparations at the 1st use and all subsequent reuses. The mDAC eluates did not show an increase in coagulation time and values were similar to fresh plasma. Fig. 8 presents plasma proteins eluted from fresh or stored over a long period mDAC-HEP-AT complex preparations (Fig. 8a) and mDAC (Fig. 8b) using 0.25, 0.5 and 1.0 M NaCl solutions stepwise. No protein was eluted with higher NaCl concentrations (data not shown). The number of protein bands decreased as the NaCl concentrations increased for the eluates from fresh and stored over a long period preparations. As displayed in Fig. 6, antithrombin activity was only demonstrated in the 1.0 M NaCl elution. Miller-Anderson et al. [4] obtained similar results using a heparin-sepharose column and 0.02 M Tris-HCl 1.0 M NaCl. Electrophoresis of this elution showed a band at the same position of the antithrombin standard (58 kDa) in both preparations. It is important to note that a strong band is present above 58 kDa, and one might predict that this band corresponds to albumin (66.5 kDa). Furthermore, the mDAC preparation did not produce a protein band at the expected size for antithrombin (Fig. 8b). The purification profile observed in the control can be attributed to the presence of absorbed heparin. Nevertheless, this heparin was not capable to purify antithrombin in detectable amounts. Similar results were reported by Zhao et al. [36] using SDS-PAGE to demonstrate AT purification by viscose-Hep fibres composite.

4. Conclusions

According to the results presented in this contribution, one can conclude that mDAC microparticles (1–2 μm) exhibited good magnetic ordering and a suggestive superparamagnetic behaviour; magnetite and Dacron-hydrazide were demonstrated in mDAC by EDX and XRD analyses; heparin was covalently fixed onto the mDAC as demonstrated by Infrared spectra; this derivative (mDAC-HEP) formed a complex with human plasma proteins, including antithrombin, and these proteins could be released into solutions with increasing ionic strength; mDAC-HEP preparations can be reused at least ten times and stored up to 2 years while preserving these affinity properties.

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APÊNDICE B – ARTIGO DE REVISÃO PUBLICADO: BIOQUÍMICA PARA ESTUDANTES DA ÁREA DA SAÚDE: IMPORTÂNCIA E ALTERNATIVAS DE ENSINO.

Review

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Bioquímica para estudantes da área da saúde: importância e alternativas de ensino

Biochemical for health students: importance and teaching alternatives

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RESUMO

Introdução: O estudo de bioquímica envolve assuntos complexos e abstratos, o que, muitas vezes, dificulta a apreciação dos alunos por seus conteúdos. Dessa forma, os alunos sentem-se desinteressados e não conseguem visualizar a importância dessa disciplina, que é imprescindível para o entendimento dos distúrbios metabólicos e interpretação de exames laboratoriais e clínicos. **Objetivo:** Buscar informações na literatura científica sobre as dificuldades e alternativas do ensino de bioquímica para estudantes da saúde. **Métodos:** Trata-se de uma pesquisa exploratório-descritiva baseada na análise de artigos científicos publicados em língua portuguesa e inglesa no período de 2008 a 2018, orientada a partir da questão "Como o ensino das ciências básicas (bioquímica) para alunos das áreas de ciências da saúde é realizado atualmente e quais os seus objetivos, métodos alternativos e resultados?", utilizando os descritores: educação em bioquímica, educação em ciências básicas, área da saúde e prática docente. **Desenvolvimento:** A disciplina de bioquímica é considerada como de difícil aprendizado pelos alunos devido à complexidade e grande quantidade de assuntos. A falta de conhecimento prévio de alguns conceitos sobre bioquímica atrapalham o desempenho na faculdade e também a omissão e passividade de alguns docentes influencia neste cenário. Materiais multimidiáticos e o ensino baseado em problemas tornam os alunos mais ativos em sala de aula, portanto são alternativas promissoras para aumentar o interesse e importância do ensino e aprendizagem da bioquímica. **Conclusão:** O maior desafio do ensino da bioquímica é abordar os conhecimentos básicos para os diferentes cursos de saúde unindo a construção do conhecimento com a prática profissional.

Palavras-chave: Bioquímica, ensino-aprendizagem, prática docente.

ABSTRACT

Introduction: The study of biochemistry involves complex and abstract subjects, which often makes it difficult for students to appreciate their contents. In this way, the students feel disinterested and fail to visualize the importance of this discipline that is essential for the understanding of metabolic disorders and interpretation of laboratory and clinical exams. **Objective:** Search for information in the scientific literature about difficulties and alternatives in biochemistry teaching to health students. **Methods:** This is an exploratory-descriptive research based on the analysis of scientific articles published in Portuguese and English language in the period from 2008 to 2018, guided by the question "How do basic science teaching (biochemistry) for students in science health is currently carried out and what are its objectives, alternative methods and results? ", using the descriptors: education in biochemistry, education in basic sciences, health area and teaching practice. **Development:** The discipline of biochemistry is considered to be difficult for students to learn due to its complexity and large amount of subjects. The lack of prior knowledge of some concepts of biochemistry hinder performance in college and also the omission and passivity of some teachers influence in this scenario. Multi-mediatic materials and the project based learning make the students more active in the classroom, so they are promising alternatives to increase the interest and importance of teaching and learning biochemistry. **Conclusion:** The greatest challenge of teaching biochemistry is to approach the basic knowledge for the different health courses linking the construction of knowledge with professional practice.

Keywords: Biochemistry, teaching-learning, teaching practice.

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1. INTRODUÇÃO

A disciplina de bioquímica faz parte dos componentes do ciclo básico oferecida em alguns cursos da área de saúde, tais como medicina, fisioterapia, biomedicina e enfermagem. Essa disciplina atende a grupos muito heterogêneos de alunos e sua característica multidisciplinar

é um indicativo da sua imperiosa aplicação nos mais diversos campos de atuação profissional (GARRIDO *et al.*, 2010). Trata-se de uma disciplina que requer conhecimentos interdisciplinares, o que permite a troca de conteúdos entre as mais diversas áreas, a fim de entender processos patológicos, farmacológicos e fisiológicos do organismo humano (ALMEIDA *et al.*, 2014).

O estudo de bioquímica envolve assuntos complexos e abstratos, o que, muitas vezes, dificulta a apreciação dos alunos por seus conteúdos. Dessa forma, os alunos sentem-se desinteressados, porque não conseguem perceber a relação entre os conteúdos que estão estudando com seu perfil profissional (SABINO *et al.*, 2009). O reconhecimento da importância das disciplinas básicas, como a bioquímica, ocorre tardiamente, quando os conceitos que exploram são necessários para o prosseguimento do curso ou para o exercício profissional. Por isso, os benefícios que essas disciplinas poderiam trazer para a formação integrada dos estudantes ficam muito comprometidos (SCATIGNO e TORRES, 2016). O número de alunos reprovados na disciplina de bioquímica é expressivo e uma parte desses alunos repete a disciplina já cursada por mais de uma vez (SABINO *et al.*, 2009). Os alunos também apresentam dificuldades sobre os temas abordados na disciplina desde os primeiros contatos com temas relacionados ainda no ensino médio (SCHIMIDT *et al.*, 2014). Portanto, como ocorre em outras disciplinas da graduação, parte das dificuldades encontradas no ensino da bioquímica, deve-se também ao grande volume de informações associado a uma reduzida carga horária e às ferramentas utilizadas pelos docentes para abordar o conteúdo (FARKUH e PEREIRA-LEITE, 2014).

Mesmo diante de tantas dificuldades no ensino-aprendizagem, é imprescindível o conhecimento prévio em bioquímica para o entendimento dos distúrbios metabólicos ou para interpretação de exames laboratoriais e clínicos. Isto se torna especialmente importante na prática dos profissionais da saúde como médicos e enfermeiros, onde, atualmente, além de se exigir na prática assistencial, tornaram-se essenciais à pesquisa e ao diagnóstico. Apesar disso, os estudantes da área da saúde mostram, em geral, bastante dificuldade e desinteresse pelas disciplinas de biociências (GARRIDO *et al.*, 2010).

Diante dessas considerações, o presente trabalho teve como objetivo buscar informações na literatura científica sobre as dificuldades no ensino de bioquímica para estudantes de cursos de graduação das áreas de ciências da saúde. Além disso, através dessa pesquisa, propor alternativas para aumentar o interesse e a importância do ensino e aprendizagem da bioquímica na formação do conhecimento que é tão útil para a futura prática profissional.

2. MÉTODOS

Trata-se de uma pesquisa exploratório-descritiva, de natureza qualitativa, baseada nos pressupostos da análise de conteúdo e nos preceitos da revisão de literatura (FIGUEREDO *et al.*, 2014). A pesquisa foi orientada a partir da questão: "Como o ensino das ciências básicas (bioquímica) para alunos das áreas de ciências da saúde é realizado atualmente e quais os seus objetivos, métodos alternativos e resultados?", por meio de artigos científicos publicados em revistas brasileiras e internacionais indexados no período de 2008 a 2018. Foram utilizados os descritores: "educação em bioquímica", "educação em ciências básicas", "área da saúde", "prática docente" e "*biochemistry teaching*". Como critérios de inclusão, optou-se por selecionar maior quantidade de artigos publicados em revistas brasileiras, escritos em português, disponíveis online e em texto completo.

3. DESENVOLVIMENTO

3.1. Importância da bioquímica na formação dos profissionais da saúde

Os conhecimentos científicos fazem parte das diretrizes curriculares dos cursos de nível superior no Brasil, estejam eles presentes em conteúdos do ciclo básico ou profissional, sendo todos necessários para a formação acadêmica, inclusive daqueles da área da saúde. Entre esses conteúdos encontra-se a bioquímica, considerada o ramo das ciências naturais que estuda a química da vida, definição apresentada por Amabis e Martho (2010).

A bioquímica contempla conteúdos nas áreas de biologia e química, sendo muito promissora para abordagens interdisciplinares. Essencialmente, essa disciplina faz parte do ciclo de conteúdos básicos, sem os quais muitas informações relevantes sobre importantes processos fisiológicos e patológicos deixam de ser compreendidas em sua plenitude. Ao mesmo tempo em que os avanços na área de pesquisa em ciências básicas vão aumentando, fica cada vez mais difícil abordar os temas clássicos e atuais em disciplinas com cargas horárias limitadas, como bioquímica, que está presente nos cursos de graduação como medicina, biomedicina, fisioterapia, enfermagem e odontologia, por exemplo. Além disso, devido à complexidade dos assuntos associada à imaturidade dos estudantes nos primeiros anos, muitos discentes podem não assimilar e perceber a importância desses conteúdos em sua formação profissional ou correlacionar com outras disciplinas (PINHEIRO *et al.*, 2009).

3.2. Dificuldades no ensino da bioquímica para os cursos da área da saúde

A disciplina de bioquímica é considerada, pela maioria dos alunos, como de difícil aprendizado devido ao alto nível de complexidade, o que dificulta a assimilação do conteúdo e, muitas vezes, desmotivando o interesse em parte dos alunos. Os assuntos são considerados difíceis porque os alunos precisam lidar com conceitos abstratos e ainda necessitam de conhecimentos prévios, muitas vezes ausentes, sobre características essenciais das moléculas e substâncias orgânicas. Ministrar uma disciplina de bioquímica também é considerado difícil por parte dos professores devido à complexidade e grande quantidade de conteúdos a serem abordados (COSTA *et al.*, 2013).

Infelizmente, a disciplina de bioquímica apresenta historicamente uma considerada rejeição entre os alunos de graduação e o reconhecimento da importância das disciplinas básicas para sua formação ocorre depois de cursar tais disciplinas, e em boa parte, os benefícios dos conhecimentos já foram comprometidos. As necessidades impostas pelo mercado de trabalho combinam uma formação profissional atrelada apenas com habilidades técnicas e, muitas vezes, percebe a ausência de uma formação crítica e autossuficiente. As atuais condições de ensino de disciplinas básicas nas instituições públicas e privadas de nível superior são pouco estudadas. Para tornar a disciplina de bioquímica

mais efetiva na formação dos profissionais da saúde, faz-se necessário estudar as principais dificuldades apresentadas pelos alunos durante as aulas e propor alternativas que possam estimular aluno e professor a consolidar o ensino e aprendizagem.

Em um estudo realizado por Zeni (2010), observou-se que 50,6% dos alunos do curso de medicina e odontologia obtiveram um conhecimento prévio de alguns conceitos de bioquímica durante o ensino médio e outros 16,1% a partir de cursinhos preparatórios de vestibular. No mesmo estudo, porém, entre os alunos de fisioterapia e farmácia um total de 61,2% não obtiveram esses conhecimentos, enquanto que 19,2% do total adquiram contato com a bioquímica durante o ensino médio. Esses dados sugerem que a grande maioria dos alunos no nível superior não tem base de conhecimentos em bioquímica o que pode atrapalhar seu desempenho durante o curso.

Pinheiro e seus colaboradores (2009) realizaram um estudo sobre o ensino de bioquímica para alunos do curso de fisioterapia, os resultados da pesquisa demonstraram que 67% dos alunos estudam periodicamente os conteúdos de bioquímica, mas ainda assim 54% não gostam de estudar bioquímica e outros 29% não conseguem aprender satisfatoriamente os assuntos ministrados em sala de aula pelo professor.

De acordo com Scatigno e Torres (2016), um dos maiores desafios enfrentados pelo docente de nível superior é obter uma participação ativa dos alunos que facilite o aprofundamento da aprendizagem. Além disso, as atitudes de omissão, passividade e desinteresse, as quais podem ser apresentadas por ambos, aluno e professor, não fazem parte de um processo de ensino construtivista. Embora muitos professores possam ter o domínio sobre os assuntos, faltam ainda novas ferramentas alternativas e estratégias para solidificar o aprendizado.

As autoras possuem experiência na prática docente tradicional, que também contribui para a formação do aluno; porém, atualmente, apresenta dificuldades para manter-se em um ambiente cheio de fontes de informação e com alunos de uma geração altamente tecnológica. Devido a essas dificuldades e ao baixo aproveitamento na disciplina, o curso de enfermagem da Universidade Federal de Roraima (UFRR) tem tentado inserir novos métodos de ensino, como a aprendizagem baseada em equipes, do inglês *Team Based Learning* - TBL (MICHAELSEN e SWEET, 208), para alunos do segundo ano do curso. Essa metodologia prioriza o estudo individual e o trabalho em equipe, mas também permite ao professor, no momento em que este realiza o *feedback*, apresentar suas contribuições por meio de explicações fundamentadas sobre as respostas dos testes propostos. Os resultados da aplicação desse novo método mostraram-se promissores com a primeira turma, apresentando uma aprovação de 96,4% (resultados não publicados). Espera-se dar continuidade ao método e adequá-lo à realidade dos alunos do curso de enfermagem da UFRR, visando atender às necessidades de sua formação profissional.

3.3. Ferramentas e alternativas para associar o ensino/ aprendizagem com a prática profissional.

Algumas tentativas para diminuir a resistência em aprender ou ensinar bioquímica estão relacionadas em despertar o interesse dos discentes em odontologia, medicina, fisioterapia e enfermagem, por exemplo, em desenvolver projetos de iniciação científica e monitorias na área de disciplinas básicas. Albuquerque *et al.* (2012) relatam que experiências vivenciadas por monitores da disciplina de bioquímica metabólica, em um curso de medicina de uma universidade pública em Pernambuco, contribuem para uma boa formação profissional. Isso porque as atividades desenvolvidas estavam relacionadas à pesquisa, ensino e extensão o que possibilita uma conexão entre o ensino teórico e prático adquirido nas salas de aula. Logo, eles concluem a importância da participação dos discentes junto à motivação dado pelo professor para a construção do conhecimento nessa área.

Em um estudo feito por Iano e seus colaboradores (2009), foi investigado o perfil dos alunos de graduação que estavam envolvidos em projetos de iniciação científica em um laboratório de bioquímica de uma faculdade pública de odontologia, 77,78% dos entrevistados afirmaram que a partir da iniciação científica foi possível integrar os conhecimentos entre a teoria e a prática. Além disso, todos os alunos afirmaram que os conceitos de bioquímica têm relevância em sua formação profissional, pois se trata de uma matéria básica que serve como alicerce para a compreensão das outras e que são importantes nas atividades do cotidiano clínico.

As aulas expositivas e experimentações realizadas em laboratório são os recursos didáticos mais utilizados pelos docentes para o ensino de bioquímica; porém, a aprendizagem a partir de metodologias criativas e alternativas, com ambientes virtuais estimula a compreensão dos conceitos básicos da disciplina. Uma forma lúdica encontrada por Marinho, Castro e Marinho (2014) foi a inserção de aplicativos, através de dispositivos móveis, para proporcionar ao aluno visualizar de forma dinâmica e tridimensional estruturas químicas e moleculares de uma lectina (proteína).

Uma alternativa para os docentes é utilizar a tecnologia da informação para proporcionar o uso de diversas ferramentas, tais como sites e softwares educacionais para serem utilizadas no contexto educacional. Isso permite a criação de novos métodos e estratégias para resolução dos problemas enfrentados no ensino/aprendizagem (ALCÂNTARA e MORARES-FILHO, 2015). Uma proposta também muito interessante, para driblar as dificuldades enfrentadas pelos professores no ensino da bioquímica, foi apresentada por Dias e seus colaboradores (2013), os quais vêm desenvolvendo materiais multimidiáticos que combinam conteúdo audiovisual como animações, filmes que ilustram a dinâmica molecular de proteínas e enzimas, para melhorar a aprendizagem na disciplina de bioquímica. Os resultados foram satisfatórios, demonstrando interesse e maior participação dos alunos no início da graduação na área da saúde.

Outra grande dificuldade enfrentada pelos discentes de nível superior é conseguir agregar à futura prática profissional os conteúdos que são vistos em bioquímica. Sabendo disso, Matta e Sodré Neto (2015) descrevem um trabalho com uma proposta de aliar os conteúdos abordados em bioquímica metabólica com a possível prática docente dos alunos, associando a formação em bioquímica com uma carreira profissional. Neste caso, grupos de alunos elaboraram projetos com problemáticas e conteúdos relacionados à bioquímica e ministravam aulas hipotéticas

de ensino básico. Sendo assim foi possível construir uma estratégia de autonomia para os alunos através de uma instrumentação de ensino paralelo à aprendizagem dos conteúdos específicos de bioquímica.

Selecionar tópicos dos conteúdos a serem abordados em sala de aula através do interesse dos alunos, parece uma forma atrativa e alternativa de construir o conhecimento em bioquímica. Apostar em competências para o desenvolvimento de conhecimento, habilidades e atitudes através da metodologia de aprendizagem baseada em projetos (do inglês *Project Based Learning*) foi uma forma que Garcês, Santos e Oliveira (2018) conseguiram trabalhar com alunos que cursavam a disciplina de bioquímica. Os estudantes foram questionados sobre quais assuntos gostariam de aprender em bioquímica e eles optaram por temas que contemplassem conteúdos para compreender melhor doenças, resultados de exames laboratoriais e alimentação. Assim, o pedido foi atendido mediante explicação e exemplos de doenças metabólicas. Isso demonstra o quanto é importante uma participação ativa e direta dos alunos no processo de ensino e aprendizagem.

A aprendizagem baseada em problemas (ABP, ou do inglês *Problem Based Learning* - PBL) parece ser uma das alternativas mais utilizadas para o ensino e aprendizado de bioquímica em outros países. Por exemplo, Hartfield (2010), da Faculdade de Ciência e Tecnologia da Universidade de Tecnologia de Queensland (Austrália), reforça que nas universidades é importante introduzir um ensino construtivista em bioquímica através da aprendizagem baseada em casos e que os alunos aprendem de forma mais eficaz se forem mais ativos. Ao utilizar informações para resolverem um problema específico, os alunos têm maior chance de lembrar o que aprenderam, de processar as informações que estão recebendo e de refletir sobre como aprenderam. Dessa forma, o ambiente de aprendizado ativo permite a oportunidade de desenvolver pensamentos críticos e habilidades orais e escritas associadas à comunicação de análise de problemas.

Estudantes de medicina de uma universidade na Índia participaram de um estudo no departamento de bioquímica realizado por Nair *et al.* (2013), os alunos foram divididos em dois grupos: um deles com o ensino baseado em problemas e o outro apenas com ensino a partir de palestras didáticas. Os alunos relaram que o ensino baseado em problemas foi o método mais interessante para adquirir conhecimentos, pois conseguiram vivenciar uma experiência de aplicação do conhecimento adquirido em situações voltadas à prática profissional. Com esse método os alunos foram expostos aos problemas médicos reais, que os ajudaram a desenvolver habilidades de análise. Resultados positivos com a proposta de ensino baseado em problemas também foi relatada por Fardilha *et al.* (2010), na Universidade de Aveiro em Portugal, ao propor esse método aos alunos de biomedicina para entender o metabolismo dos ácidos graxos na disciplina de bioquímica médica.

O processo de avaliação utilizado pela grande maioria dos professores de nível superior nem sempre leva à construção do conhecimento, muitas vezes implica na obrigação do aluno em memorizar os conceitos, sem que haja uma aprendizagem significativa dos mesmos. Quando há, por exemplo, assuntos sobre as vias metabólicas, exige uma preocupação dos alunos em memorizar os nomes dos compostos intermediários, porém não reconhecem suas fórmulas estruturais ou importância quando lhes são apresentadas. Atitudes lúdicas a partir da construção de um jogo para o entendimento das vias glicolíticas e da gliconeogênese foram demostradas por Oliveira e seus colaboradores (2015) como forma de aprendizado colaborativo entre alunos do curso de odontologia de uma universidade pública.

O papel da universidade em desenvolver políticas pedagógicas junto ao corpo docente também é importante para auxiliar no processo de aprendizagem. Recentemente Tomelin e seus colaboradores (2018) defenderam a abordagem no ensino superior da chamada "prática inclusiva". É notável a existência de uma deficiência na educação básica dos alunos que atualmente estão ingressando nas universidades, o fato do estudante estar presente em sala de aula não significa, necessariamente, a sua participação plena no meio universitário e a absorção dos conteúdos propostos. Por isso, é necessário que a universidade realize adaptações para que a inclusão seja efetiva, reconhecendo e atendendo às necessidades individuais dos alunos, adaptando-se aos vários estilos e ritmos de avaliação e aprendizagem, de modo a garantir uma qualidade de educação para todos.

4. CONCLUSÃO

O estudo realizado demonstra que são muitas as dificuldades encontradas no processo de ensino-aprendizagem de bioquímica para alunos dos cursos de graduação da área da saúde. Esses problemas podem estar relacionados, por exemplo, à falta de percepção dos alunos de que conteúdos básicos estão conectados com a sua futura prática profissional, bem como à dificuldade de compreender conceitos abstratos. Apesar desses problemas , foi verificado que há formas de mudar esse cenário. O professor precisa abordar os conteúdos de forma a estimular seus alunos na compreensão dos tópicos de forma crítica e construtivista, e não focar apenas em decorar nomes e fórmulas. Atualmente, existem muitas metodologias de ensino, em que o aluno participa ativamente do seu processo de aprendizagem, construindo o seu conhecimento, como é o caso do ensino baseado em problemas e de abordagens a partir de recursos multimidiáticos. O grande desafio é encontrar a metodologia mais adequada em cada turma/curso e mostrar que a bioquímica pode ser bem compreendida e contribuir para a formação profissional de todos os estudantes da área da saúde.

CONFLITO DE INTERESSE

Os autores declaram que não existe qualquer conflito de interesse.

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APÊNDICE C – ARTIGO SUBMETIDO: MAGNETIC HEPARIN COATED PARTICLES FOR LECTIN PURIFICATION.

MAGNETIC HEPARIN COATED PARTICLES FOR LECTIN PURIFICATION.

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ABSTRACT

Lectins can easily be purified by using magnetic particles-chitosan composite coated with heparin. Here, CrataBL, lectin obtained from bark of *Crataeva tapia* is purified according to this approach as a model. CrataBL has been showed potential biomedical application as anti-inflammatory and antitumor activities. Magnetic chitosan particles were synthesized by co-precipitation method of ferric and ferrous ions with chitosan (MAG-CH). Heparin activated with carbodiimide and N-hydroxysuccinimide was covalently coated the magnetic particle (MAG-CH-hep). *Crataeva tapia* bark extract was incubated with MAG-CH-hep for 30 minutes at 4° and then, using a magnetic separation rack, the complex MAG-CH-hep-CrataBL was recovered and washed with 10 mM citrate phosphate buffer pH 5.5. Afterwards, CrataBL was eluted with increasing ionic strength. SDS/PAGE showed 20 and 40 kDa bands suggesting the presence of CrataBL eluted with 0.25 M and 0.5 M NaCl, which were also confirmed by size-exclusion chromatography (Superdex G75). CrataBL presents high affinity for heparin immobilized on magnetic chitosan particles and it can be purified using ionic strength and magnetic separation by ease, simple and low-cost methods. MAG-CH-hep composites were reused three times extracting CrataBL from bark extract similarly.

Keywords: affinity chromatography, CrataBL, chitosan, magnetic separation.

1. INTRODUCTION

Lectins are proteins that bind reversibly and specifically to carbohydrates and compounds that contain sugars in their structure. In addition, they differ in amino acid sequence and molecular structure, as well as according to their specificity for carbohydrates. Because of this binding ability, affinity chromatography purification techniques are commonly used to purify these proteins [1]. The main protein of *Crataeva tapia* bark extract is a lectin of 20.2 kDa named CrataBL that has been previously purified and involved in various biological activities as anti-inflammatory, analgesic and antitumor [2]. Moreover, CrataBL acts in blocking coagulation, arterial thrombus and binds to heparin [3].

Heparin is known in the clinic for its anticoagulant activity, but is also capable of interacting with several other biological molecules because it has a unique structure, it is a highly sulfated and negatively charged linear GAG formed by residues of disaccharides consisting of α -D-glucosamine (GlcN) and α-1-iduronic acid (IdoA) or β-d-glucuronic acid (GlcA) [4]. Heparin-interacting proteins have a specific distribution of positively charged amino acid residues to which they are involved in the electrostatic interaction with negatively charged heparin [5]. Zhang et al. [6] reported that CrataBL shows to have high binding affinity with heparin (KD ~ 49 nM), and modified positions of sulfate groups on heparin alters the affinity of CrataBL for heparin indicating a specific interaction.

The magnetic particles are among the materials most used in separation techniques, with applications in immobilization of biomolecules (proteins, enzymes and others), systems of analysis in medicine and biotechnology [7]. Purification processes involving magnetic separation, the magnetic materials functionalized with a particular type of linker exhibit affinity for a specific target molecule present in a sample. Within an incubation period, the target molecules bind to the magnetic particles. The entire magnetic complex is subsequently separated from the sample from the application of an external magnetic field and, after washing, the target molecules are isolated [8].

In view of the potential biological applications presented by CrataBL, the present work aims to develop an alternative tool to purify this lectin by affinity to heparin immobilized on magnetic particles using a specifically, fast and easy method.

2. MATERIAL AND METHODS

2.1 SYNTHESIS OF MAGNETIC PARTICLES AND COVALENT IMMOBILIZATION OF HEPARIN.

Magnetic chitosan (MAG-CH) particles were used as a support for heparin immobilization and it were synthesized by co-precipitation method [9]. Briefly, solution 1:1 of $FeCl_3$ (1.1 M) and $FeCl_2$ (0.6 M) was added at the aqueous suspension of 2.0% (w/v) low molecular weight chitosan, then the pH was adjusted to 11 with ammonium hydroxide and that mixture remained for 30 minutes under stirring at 80 °C. At the end, the MAG-CH particles were recovered with a magnet and washed with distilled water until reaching a neutral pH.

The immobilization process was performed from the formation of a covalent bond between the heparin carboxyl groups and the amine groups present on the MAG-CH particles. Initially, 3 mg/mL of unfractionated sodium heparin (HEMOFOL®) was prepared in 10 mM phosphate buffer pH 7.4, followed by activation of the heparin carboxyl groups by the carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) according to the methodology of Oliveira et al. [10]. Thus, 1 mL of the activated heparin (EDC/NHS) solution was incubated in 30 mg of MAG-CH for 72 hours at 25°C. After this period, using a magnetic separation rack (6000 Oe), the magnetic chitosan particles with covalently immobilized heparin (MAG-CH-hep) were recovered and washed three times with 10 mM phosphate buffer pH 7.4 to remove the unfixed heparin. Quantification of immobilized heparin was performed according to the method of Mercês et al. [11].

2.2 PURIFICATION OF CRATABL BY AFFINITY TO HEPARIN IMMOBILIZED ON MAG-CH.

Crataeva tapia bark extracts was obtained according Araújo et al. [2]. Briefly, 10% (w/v) *Crataeva tapia* bark was mixed overnigh with 10 mM citrate-phosphate buffer pH 5.5 at 4 °C, after it was filtered and centrifuged to obtain the crude bark extract (supernatant). MAG-CH-hep composites (30 mg) was incubated with 10 mL (45.6 mg of proteins) of the crude bark extract for 30 minutes at 4°C under slight shaking. After incubation, using a magnetic separation rack (6000 Oe), the magnetic composites were recovered and then washed with 10 mM citrate-phosphate buffer pH 5.5. Therefore, the fixed proteins were eluted with the same buffer using

NaCl 0.25 M, 0.5, 1.0 and 2.0 M. Eluted proteins in these different ionic forces were monitored in spectrophotometer at 280 nm. The same crude bark extract and composites was reused three times and the peaks obtained in each elution were pooled. These eluted proteins were dialyzed (cut off 10 kDa) for 24 h in 0.01 M NaCl and then dried in speed vac.

2.3 IDENTIFICATION OF LECTIN PURIFIED.

The pool of eluted proteins obtained after the three reuses were resuspended in 500 μL (NaCl 0.01 M) for measurements of proteins by the method of Bradford [12]. SDS-PAGE (polyacrylamide gel - sodium dodecyl sulfate) 12.0% electrophoresis was performed to identify the purified proteins at the eluates samples obtained at NaCl 0.25, 0.5, 1.0 and 2.0 M. Electrophoresis gel were stained with a solution of coomassie brilliant blue (R250). Pool of the purified protein with NaCl 0.25 M NaCl was analyzed on the ÄKTA Purifier-FPLC to observe the protein profile by the size-exclusion chromatography using Superdex-G75 column (flow: 0.5 mL / min; elution: 10 mM citrate phosphate, pH 5.5)

3. RESULTS AND DISCUSSION

The magnetic affinity separation process is an efficient method used in the purification of proteins and is based on the formation of specific and reversible complexes between an immobilized molecule and its ligands to be purified. This method has some advantages, such as being carried out more quickly and by using separation processes based on magnetic field [13]. Ferromagnetic levan particles was used by Angeli et al. [14] for purification of Cramol 1 lectin from the seed of *Cratylia mollis*. The amount of immobilized heparin was 94.4 μg per mg of MAG-CH particles. In a study that has been conducted by Zhang et al. [6] the binding affinity between heparin and CrataBL was proven, and the studies reported here demonstrate the ability of this lectin to bind to heparin immobilized on MAG-CH particles. This suggests a possible development of a new tool to purify this lectin by affinity, magnetic field and separation by ionic force. The literature describes several applications and biological properties of CrataBL, for example, in studies conducted by Ferreira et al. [15], they have shown that addition of these lectin caused maximum growth inhibition in human prostate cancer cell lines. Moreover studies in mice demonstrated that CrataBL is a potential therapeutic tool in the

treatment of chronic obstructive pulmonary disease [16] and its plays a role in controlling asthma response [17].

Figure 1A shows the chromatogram of the proteins from the *Crataeva tapia* bark extract eluted with increasing ionic strength NaCl solutions from MAG-CH-hep composites. The same particles and extract were reused three times (reusability). The proteins eluted at 0.25 NaCl; 0.5 and 1.0 M to each elution were pooled and presented amounts of protein of 181, 57 and 36 μg, respectively. No proteins were observed in the elution with 2.0 M NaCl. Figure 1B shows the protein bands observed in the SDS-PAGE electrophoresis of the proteins present in each elution. In the fractions eluted with 0.25 and 0.5 M NaCl, there are bands around 20 and 40 kDa which represent the presence of CrataBL in its monomeric and dimeric form, respectively. Ferreira et al. [15] reported that CrataBL at high concentrations has a tendency to form dimers. There is an excess of CrataBL that has not bound to heparin because of saturation in addition to other proteins. Superdex-G75 (ÄKTA-Purifier FPLC) size-exclusion chromatography confirmed the presence of the lectin and can be seen in figure 2 which suggests the presence of CrataBL eluted in 0.25 M NaCl in its monomeric form which is present in the first peak collected in 8 mL and in its dimeric form collected in the second peak in 16 mL.

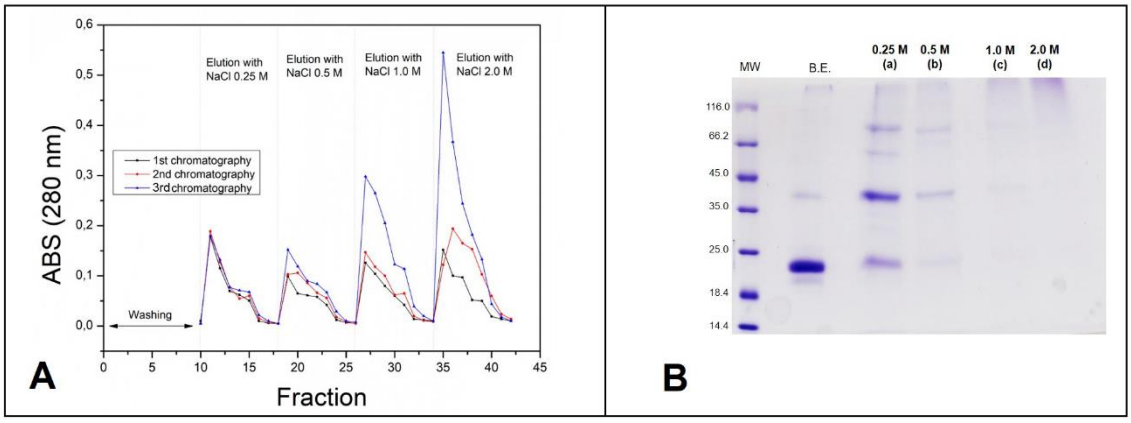

Figure 1. Purification of CrataBL by affinity to heparin immobilized onto MAG-CH particles. Elution of the proteins of *Crataeva tapia* bark extract using MAG-CH-hep a using NaCl concentrations (0.25, 0.5, 1.0 and 2.0 M) in 10 mM citrate-phosphate buffer pH 5.5 (A); SDS-PAGE 12% of the proteins eluted with NaCl 0.25 (a); 0.5 (b); 1.0 (c) and 2.0 (d) M in 10 mM citrate-phosphate buffer pH 5.5; stained with a solution Coomassie brilliant blue (R250). Samples not reduced. MW: molecular weight standard. B.E.: *Crataeva tapia* bark extract (B). ABS: absorbance.

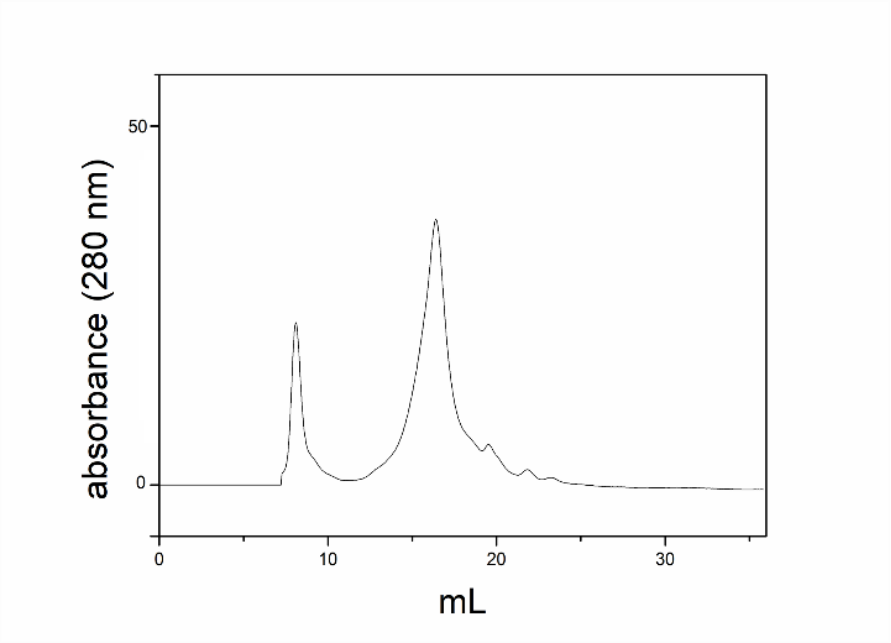

Figure 2. Profile of size-exclusion chromatography of CrataBL. The eluate obtained in 0.25 M NaCl was subjected to gel filtration on the Superdex G75 column and eluted with 10 mM citrate phosphate buffer pH 5.5 at a flow rate of 0.5 mL/min.

4. CONCLUSIONS

The results of this contribution showed that CrataBL, the main lectin present in the *Crataeva tapia* bark extract, is able to bind to heparin immobilized on the magnetic particles. Furthermore, it has been purified using simple methods: magnetic separation and ionic strength. Knowing the various interactions that heparin can perform with different proteins together with the versatility of magnetic particles with possibility of reuse, the tool presented here appears as a simple, specific and inexpensive alternative for the study and purification of proteins such as lectins. Furthermore, this method can also be used to investigate or purify other classes of molecules not yet known that can be found in several plant species.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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ANEXO A – PARECER DO COMITÊ DE ÉTICA

UNIVERSIDADE FEDERAL DE PERNAMBUCO CENTRO DE CIÊNCIAS DA SAÚDE / UFPE-

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Identificação e purificação das proteínas plasmáticas mediante o emprego de compósitos magnéticos com heparina

Pesquisador: Aurenice Arruda Dutra das Merces

Área Temática:

Versão: 2

CAAE: 62555516.8.0000.5208

Instituição Proponente: Universidade Federal de Pernambuco - UFPE

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.917.688

Apresentação do Projeto:

Projeto de Tese de Doutorado apresentado ao Programa de Pós-Graduação em Biologia Aplicada à Saúde da Universidade Federal de Pernambuco, pela estudante Aurenice Arruda Dutra das Mercês, sob orientação do Prof. Dr. Luiz Bezerra de Carvalho Júnior. A pesquisa tem como título: "Identificação e purificação das proteínas plasmáticas mediante o emprego de compósitos magnéticos com heparina". Trata -se de um estudo experimental que visa identificar proteínas do plasma de 10 indivíduos adultos saudáveis e 10 crianças com trombofilias. Serão sintetizadas partículas magnéticas para construção de colunas de afinidade para que as proteínas do plasma possam interagir por com a heparina imobilizada na coluna magnética. Em seguida as proteínas serão estudadas por eletroforese bidimensional e espectrometria de massa.

Objetivo da Pesquisa:

OBJETIVO GERAL

Investigar as proteínas plasmáticas de pacientes saudáveis e com trombofilias através de colunas magnéticas com heparina imobilizada.

Endereço: Av. da Engenharia s/nº - 1º andar, sala 4, Prédio do CCS
Bairro: Cidade Universitária **CEP:** 50.740-600
UF: PE **Município:** RECIFE
Telefone: (81)2126-8588 **E-mail:** cepccs@ufpe.br

OBJETIVOS ESPECÍFICOS

1.Sintetizar as particulas magnéticas de magnetita revestidas com polianilina (MAG-PANI);

2.Sintetizar as particulas de quitosana magnética (QS-MAG);

3.Imobilizar heparina nas particulas de MAG-PANI e QS-MAG;

4.Incubar o plasma sanguíneo de pacientes saudáveis nas particulas magnéticas (QS-MAG e MAG-PANI) com heparina imobilizada;

5.Incubar o plasma sanguíneo de pacientes com trombofilias nas particulas magnéticas com heparina imobilizada;

6.Identificar as proteínas plasmáticas dos pacientes saudáveis e dos pacientes com trombofilias;

Avaliação dos Riscos e Benefícios:

Riscos: os riscos serão mínimos, pois o sangue será coletado por uma médica hematologista com muita experiência de coleta. Porém, durante a coleta por punção venosa pode haver uma pequena dor ou incômodo que podem ser amenizados com uso de gelo sobre o local da coleta.

Benefícios: o benefício será a produção de uma nova e importante ferramenta para ser utilizada no monitoramento e purificação das proteínas plasmáticas. Além disso, será feito um estudo inicial do mapeamento das proteínas plasmáticas que poderá ser aplicado na área terapêutica e prognóstica para os pacientes com trombofilias, por exemplo.

Comentários e Considerações sobre a Pesquisa:

O projeto parece relevante, a tecnologia utilizada é arrojada, de ponta, especifica e de alto custo. Provavelmente os resultados serão significativos dentro da perspectiva de diagnosticar doenças causadas por alterações de proteínas plasmáticas. No entanto, o projeto estava escrito de forma resumida e com insuficiencia de informações. No entanto o projeto foi devidamente corrigido em todas as suas pendencias e reapresentado de forma completa e satisfatória.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos obrigatórios foram apresentados corretamente na segunda versão.

Recomendações:

Nenhuma

Conclusões ou Pendências e Lista de Inadequações:

Projeto apresentado com sucesso. Nenhuma pendencia.

Considerações Finais a critério do CEP:

As exigências foram atendidas e o protocolo está APROVADO, sendo liberado para o início da coleta de dados. Informamos que a APROVAÇÃO DEFINITIVA do projeto só será dada após o envio do Relatório Final da pesquisa. O pesquisador deverá fazer o download do modelo de Relatório Final para enviá-lo via "Notificação", pela Plataforma Brasil. Siga as instruções do link "Para enviar Relatório Final", disponível no site do CEP/CCS/UFPE. Após apreciação desse relatório, o CEP emitirá novo Parecer Consubstanciado definitivo pelo sistema Plataforma Brasil.

Informamos, ainda, que o (a) pesquisador (a) deve desenvolver a pesquisa conforme delineada neste protocolo aprovado, exceto quando perceber risco ou dano não previsto ao voluntário participante (item V.3., da Resolução CNS/MS Nº 466/12).

Eventuais modificações nesta pesquisa devem ser solicitadas através de EMENDA ao projeto, identificando a parte do protocolo a ser modificada e suas justificativas.

Para projetos com mais de um ano de execução, é obrigatório que o pesquisador responsável pelo Protocolo de Pesquisa apresente a este Comitê de Ética relatórios parciais das atividades desenvolvidas no período de 12 meses a contar da data de sua aprovação (item X.1.3.b., da Resolução CNS/MS Nº 466/12).

O CEP/CCS/UFPE deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (item V.5., da Resolução CNS/MS Nº 466/12). É papel do/a pesquisador/a assegurar todas as medidas imediatas e adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e ainda, enviar notificação à ANVISA – Agência Nacional de Vigilância Sanitária, junto com seu posicionamento.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_818465.pdf	06/02/2017 17:45:40		Aceito
Outros	Carta_de_resposta_as_pendencias.docx	06/02/2017 17:42:03	Aurenice Arruda Dutra das Merces	Aceito
Outros	Carta_anuencia_FISICA.pdf	06/02/2017 17:41:24	Aurenice Arruda Dutra das Merces	Aceito
Outros	Carta_anuencia_CETENE.pdf	06/02/2017 17:40:58	Aurenice Arruda Dutra das Merces	Aceito
Outros	TALE_adolescentes_de_12_a_18_Modificado.doc	06/02/2017 17:40:25	Aurenice Arruda Dutra das Merces	Aceito

Outros	TCLE_Responsaveis_menores_Modificado.doc	06/02/2017 17:40:08	Aurenice Arruda Dutra das Merces	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Maior_de_18_Modificado.docx	06/02/2017 17:39:20	Aurenice Arruda Dutra das Merces	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_Modificado.doc	06/02/2017 17:38:37	Aurenice Arruda Dutra das Merces	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.doc	01/12/2016 10:42:25	Aurenice Arruda Dutra das Merces	Aceito
Folha de Rosto	folha_de_rosto_assinada.pdf	01/12/2016 10:40:54	Aurenice Arruda Dutra das Merces	Aceito
Outros	TALE_adolescentes_de_12_a_18.doc	30/11/2016 12:09:02	Aurenice Arruda Dutra das Merces	Aceito
Outros	TCLE_Responsaveis_menores.doc	30/11/2016 12:08:35	Aurenice Arruda Dutra das Merces	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_Maior_de_18.docx	30/11/2016 12:07:44	Aurenice Arruda Dutra das Merces	Aceito
Outros	Curriculo_do_Sistema_de_Curriculos_Lattes_Luiz_Bezerra_de_Carvalho_Junior.pdf	30/11/2016 12:07:18	Aurenice Arruda Dutra das Merces	Aceito
Outros	Curriculo_do_Sistema_de_Curriculos_Lattes_Aurenice_Arruda_Dutra_das_Merces.pdf	30/11/2016 12:06:45	Aurenice Arruda Dutra das Merces	Aceito
Outros	termo_de_compromisso.pdf	30/11/2016 12:00:37	Aurenice Arruda Dutra das Merces	Aceito
Outros	Declaracao_de_vinculo_doutorado.pdf	30/11/2016 11:59:59	Aurenice Arruda Dutra das Merces	Aceito
Outros	Carta_de_Anuencia.pdf	30/11/2016 11:58:58	Aurenice Arruda Dutra das Merces	Aceito
Outros	Carta_de_Anuencia_LIKA.pdf	30/11/2016 11:58:17	Aurenice Arruda Dutra das Merces	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

RECIFE, 13 de Fevereiro de 2017

Assinado por:
LUCIANO TAVARES MONTENEGRO
(Coordenador)

ANEXO B – INSTRUÇÕES PARA AUTORES: CARBOHYDRATES POLYMERS

Introduction

Aims and scope- The Aims and Scope of Carbohydrate Polymers must be complied with in order for submissions to be considered for review and possible publication.

Carbohydrate Polymers is a major journal within the field of glycoscience, and covers the study and exploitation of polysaccharides which have current or potential application in areas such as bioenergy, bioplastics, biomaterials, biorefining, chemistry, drug delivery, food, health, nanotechnology, packaging, paper, pharmaceuticals, medicine, oil recovery, textiles, tissue engineering and wood, and other aspects of glycoscience.

The role of the well-characterized carbohydrate polymer must be the major proportion of the work reported, not a peripheral . At least one named carbohydrate polymer must be cited and be the main focus of the paper and its title. Research must be innovative and advance scientific knowledge.

Characterization? For all polysaccharides, including those obtained from a supplier, essential structural information which will affect their behavior in the subsequent work should be given, along with a description of how that information was ascertained. Examples of such essential information include molecular weight, mannuronate/guluronate ratio for alginates, degree of esterification for pectin, degree of deacetylation for chitosan. Editors are unlikely to send papers for formal review with a statement such as "sodium alginate was purchased from XXX Inc." unless additional information is supplied. For papers involving synthesis, polysaccharide derivatives must also be well-characterized. For papers describing identity or application of newly-discovered polysaccharides, purity and monosaccharide composition are essential; some molecular size and linkage information is highly desirable.

Hypothesis?Nearly all scientific papers benefit from inclusion of a statement of hypothesis. Such statements should be clear, concise, and declarative. The statement should describe the one or more key hypotheses that the work described in the manuscript was intended to confirm or refute. Inclusion of a hypothesis statement makes it simple to contrast the hypothesis with the most relevant previous literature and point out what the authors feel is distinct about the current hypothesis (novelty). It also permits the authors to describe why they feel it would be important to prove the hypothesis correct (significance).

Topics of interest to the journal:

- structure-property relationships
- analytical methods
- chemical, enzymatic and physical modifications
- biosynthesis
- natural functions
- interactions with other materials
- Glycogen

Topics not of interest to the journal:

- biological, physiological and pharmacological aspects of non-carbohydrate; molecules attached to, or mixed with, carbohydrate polymers, unless the polysaccharide has a relevant and specific role;
- materials science of biocomposites where there is no mention of any specific carbohydrate polymer, or the role of the carbohydrate polymer is not the major proportion of the study;
- polyalkanoates, polylactic acid, or lignin;
- routine studies of extraction yields without characterisation of the extracted polysaccharide under the different conditions;
- routine studies of complexation of a drug with a single cyclodextrin;
- studies of newly discovered natural polysaccharides or new polysaccharide derivatives where the structure of the polysaccharide (derivative) is unknown;
- production and isolation of enzymes which act on polysaccharides (studies on the mode of action of an enzyme on a polysaccharide are within the journal scope);

- carbohydrate oligomers where the degree of polymerization is less than four;
- treatments of cotton fabrics and cellulose-based paper where the research is largely not about the component cellulose itself;
- use of carbohydrate polymers as a support material (e.g. in enzyme immobilization, chromatography, etc.) where there is no specific involvement of the chemistry of the carbohydrate polymer.

Types of paper

Original full-length research papers should contain material that has not been previously published elsewhere, except in a preliminary form. These papers should not exceed 6000 words of text (including references) and generally not more than 10 figures/tables. The same information should not be repeated in a figure and a table. **Review papers** will be accepted in areas of topical interest and will normally emphasise literature published over the previous five years. They should not exceed 12,000 words (not including references) and should contain no more than 8 figures and 6 Tables. The same information should not be repeated in a figure and a table.

This journal (as do all high impact journals) places a very high bar on acceptance of reviews. This must be the case since a review is not the authors own work but is a representation from the author(s) of recent "high quality" work in an important field and is intended to provide an all-encompassing and in-depth presentation from the author(s) of the recent impactful developments, the opportunities, the failures, the challenges, the interfaces with other disciplines (and how these interfaces affect the science) in the field. Also, review manuscripts should be of the highest quality on initial submission and should not need considerable reworking or language improvements.

Contact details for submission

Contributors must submit their articles electronically via the Elsevier Editorial
System http://ees.elsevier.com/carbpol. This is the only method of submission, and facilitates processing of your article.

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1. on a topic outside the scope of the Journal,
2. lacking technical merit or lacking appropriate characterization,
3. missing a hypothesis,
4. containing data which are non-reproducible (another scientist from a third-party laboratory must be able to reproduce your work),
5. of narrow regional scope and significance,
6. lacking novelty, does not advance scientific knowledge

   or is

7. poorly written.

Previous publication of a paper on a particular topic does not guarantee publication of subsequent papers in that area, as the Aims and Scope of the journal are regularly updated. Please see the Current Aims and Scope before you submit.

Revisions
Any revised papers returned later than three months after being sent to authors with the reviewers' comments will be treated as a new submission. When submitting a revised paper authors must list all of the reviewer's comments and indicate how they have responded to the comment, and where in the paper they have made appropriate revisions. All modifications in the paper must be shown in red.

Resubmitted Manuscripts

It is the author's choice whether or not to resubmit a rejected manuscript to the journal. The expectation of the journal and its editors is that any resubmission will be the result of significant rewriting and perhaps additional experimentation as required to address all prior reviewer and editor concerns. Authors resubmitting previously rejected manuscripts are required to; 1) identify the manuscript as a resubmitted manuscript in the cover letter to

the editor, including identification of the prior title and manuscript number, 2) address all reviewer concerns from the final reviews of the previous, rejected manuscript, and 3) include a "Response to Reviewers" document that includes those reviews from the previous version and your responses to those reviews; clearly identify what are reviewer comments and what are your responses, often the use of color is a convenient way to do so. You will also be requested to indicate this status during the submission process in EES.

Before You Begin

Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

Studies in humans and animals

If the work involves the use of human subjects, the author should ensure that the work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The manuscript should be in line with the Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals and aim for the inclusion of representative human populations (sex, age and ethnicity) as per those recommendations. The terms sex and gender should be used correctly.

Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed. The sex of animals must be indicated, and where appropriate, the influence (or association) of sex on the results of the study.

• Plagiarism and Ethical Concerns
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Author contributions

For transparency, we encourage authors to submit an author statement file outlining their individual contributions to the paper using the relevant CRediT roles: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. Authorship statements should be formatted with the names of authors first and CRediT role(s) following. More details and an example

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Preparation

Peer review

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Use of word processing software
It is important that the file be saved in the native format of the word processor used. The text should be in single-column format. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words. However, do use bold face, italics, subscripts, superscripts etc. When preparing tables, if you are using a table grid, use only one grid for each individual table and not a grid for each row. If no grid is used, use tabs, not spaces, to align columns. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). Carbohydrate Polymers requires authors to include tables and figures in the body of the article at the appropriate position, not at the end of the article. See also the section on Electronic artwork.
To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Pages must be numbered, and lines must be numbered consecutively throughout the manuscript.

Article structure

(The abstract is not included in section numbering; see specific instructions below.)

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results. Focus on a number of key references; do not overlook the earlier, seminal work.

Hypotheses
Nearly all scientific papers benefit from inclusion of a statement of hypothesis. Such statements should be clear, concise, and declarative. The statement should describe the one or more key hypotheses that the work described in the manuscript was intended to confirm or refute. Inclusion of a hypothesis statement makes it simple to contrast the hypothesis with the most relevant previous literature and point out what the authors feel is distinct about the current hypothesis (novelty). It also permits the authors to describe why they feel it would be important to prove the hypothesis correct (significance). **Submissions must include a statement of hypothesis and authors will be asked to copy and paste this into the editorial system as part of the submission process.** The hypothesis shall be stated in the introductory section, and the conclusion section shall include your conclusion about whether the hypothesis was confirmed or refuted, as well as describing any new hypotheses generated by the work described. Here is an example of a famous, excellent hypothesis statement; declarative, concise, clear, and testable:
"Equal volumes of gases, at the same temperature and pressure, contain equal numbers of molecules."
Lorenzo Romano Amedeo Carlo Avogadro di Quareqa e di Carreto (Avogadro), 1811

Material and methods (or experimental)
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results
A combined Results and Discussion section is often appropriate. Avoid extensive citations and description of published literature.
Results should be clear and concise.

Discussion
This should explore the significance of the results of the work, not repeat them.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section. The Conclusion should not be a summary, but should illustrate the advances and claims of innovative aspects of the research work done.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

• ***Title.*** Concise, attractive and informative. The title should not exceed 120 characters excluding spaces and should make clear the focus of the paper and the fact that the focus is within the scope of the journal. Specifically name the carbohydrate polymer or group of carbohydrate polymers that is the main focus of the research. Because titles are used in information-retrieval systems, avoid abbreviations and formulae, avoid general terms when specific ones are available, avoid strings of names. Check for syntax and spelling. If your paper is a review paper, please include the word "review" somewhere in the title.
• ***Author names and affiliations.*** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and **the e-mail address of each author**. Authors must provide and use an email address unique to themselves and not shared with another author registered in EES, or a department. Institutional email addresses, rather than personal email addresses such as gmail, are strongly preferred for all authors who are affiliated to an institution; this is particularly important for the corresponding author.
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Highlights are mandatory for this journal as they help increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the examples here: example Highlights.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Abstract

A concise and factual abstract is required, and should be a maximum of 150 words in length. The abstract should state briefly the purpose of the research, the principal results and major conclusions. Numerical values for the most important findings should be reported. An abstract is often presented separately from the article in databases, so it must be able to stand alone. For this reason, vague terms and references should be avoided. Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

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Although a graphical abstract is optional, its use is encouraged as it draws more attention to the online article. The graphical abstract should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. You can view Example Graphical Abstracts on our information site.
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Keywords

Immediately after the abstract, provide a minimum of 3 and maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

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Define abbreviations that are not standard in this field or approved by learned societies in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article. Abbreviations, except for very common terms (e.g. DNA, NMR), should not be used in the title of the paper.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

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List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

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If no funding has been provided for the research, please include the following sentence:

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Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

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Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

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Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

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Electronic artwork
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• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
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Data used in tables and figures must be legible and relevant to the stated aims of your paper and related to a specific point. Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Please ensure that the data presented in tables do not duplicate results described elsewhere in the article. Do not exceed a total of 10 tables/figures; any additional figures or tables can be included in the supplementary data. **Please note that over half the papers submitted to Carbohydrate Polymers in 2017 were sent back to authors because of poor figure resolution or exceeding the number of figures permitted.**
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References

Citation in text
All citations in the text should refer to:
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ANEXO F – COMPROVAÇÃO DA SUBMISSÃO DO ARTIGO 1

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Manuscript Draft

Manuscript Number:

Title: IDENTIFICATION OF BLOOD PLASM PROTEINS USING HEPARIN-COATED MAGNETIC CHITOSAN PARTICLES

Article Type: Research Paper

Keywords: bioaffinity; ionic exchange; magnetic; prothrombin; serpin.

Corresponding Author: Professor Luiz Bezerra Carvalho Junior, PhD

Corresponding Author's Institution: Universidade Federal de Pernambuco

First Author: Aurenice A Dutra das Merces, MSc

Order of Authors: Aurenice A Dutra das Merces, MSc; Rodrigo S Ferreira, PhD; Karciano J Santos Silva, PhD; Bruno R Salu, PhD; Jackeline C Maciel, PhD; Jose Albino O Aguiar, PhD; Alexandre k Tashima, PhD; Maria Luiza V Oliva, PhD; Luiz Bezerra Carvalho Junior, PhD

Abstract: Heparin was immobilized onto magnetic chitosan particles to be used as a tool to human plasma protein identification. Chitosan was magnetized by co-precipitation with Fe2+/Fe3+ (MAG-CH). Heparin was activated by carbodiimide and N-hydroxysuccinimide and covalently linked to MAG-CH (MAG-CH-hep). X-ray diffraction confirmed the presence of chitosan and Fe3O4 in MAG-CH. This particle exhibited a superparamagnetism behaviour and 100-300 µm. Human plasma diluted with phosphate buffer 10 mM pH 5.5 or Tris-HCl buffer 50 mM pH 8.5 were incubated with MAG-CH-hep and the proteins fixed were eluted with these above buffers containing increasing concentrations of NaCl. The proteins obtained were investigated by SDS/PAGE, LC/MS and biological activity tests (PT, aPTT and enzymatic chromogenic assay). Inhibitors of the serpin family, prothrombin and human albumin were identified in this study. MAG-CH-hep can be used to purify these proteins and presents the following advantages: low-cost synthesis, magnet-based affinity, ionic exchange purification and reusability.

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biomaterials expert.

ANEXO G – COMPROVAÇÃO DA SUBMISSÃO DO ARTIGO 2

Manuscript Details

Manuscript number	SEPPUR_2020_547
Title	HUMAN PROTEINS BIOSEPARATION AND DEPLETION BY AFFINITY TO HEPARIN IMMOBILIZED ONTO MAGNETIC CHITOSAN PARTICLES.
Article type	Full Length Article

Abstract

Magnetic chitosan particles (MAG-CH) were prepared by co-precipitation method with Fe+3/Fe+2. Heparin functionalized with carbodiimide and N-hydroxysuccinimide was covalently immobilized onto MAG-CH yielding MAG-CH-hep composites. Differents volumes of a plasma pool (1 or 7 or 20 mL) were incubed for 40 minutes at 4 °C with each 30 mg of MAG-CH-hep. After, using an external magnet (0.6 T), washes and elution were carried out with 10 mM phosphate buffer pH 7.4 supplemented with 0.15, 0.25, 0.5, and 1.0 M NaCl. Eluted proteins were separated by gel filtration chromatography and investigated by SDS-PAGE. Inhibitory activity of the eluted proteins was analyzed by coagulation assays: aPTT and PT. Around 1.3 mg of proteins were depleted after incubation of 20 mL pool plasma, using MAG-CH-hep. These depleted proteins showed a molecular weight between 50 and 115 kDa, among them was human serum albumin (69 kDa). Proteins eluted with 0.25 M NaCl demonstrated inhibitory biological activity on the extrinsic pathway of blood coagulation, whereas proteins eluted with 1.0 M NaCl inhibited the coagulation factors of intrinsic pathway. Finally, the proteins eluted with 0.5 M NaCl showed inhibitory activity on coagulation factors of both intrinsic and extrinsic coagulation pathways. Therefore, using an easy and low cost methodology, MAG-CH-hep has proved to be an alternative tool in obtaining and depleting of inhibitory or high abundance proteins of human plasma.

Keywords	bioaffinity; chitosan; heparin; magnetite.
Manuscript category	Chromatographic separations in non-analytical applications
Corresponding Author	Luiz Bezerra Carvalho Junior
Corresponding Author's Institution	Universidade Federal de Pernambuco
Order of Authors	Aurenice Arruda Dutra das Mercês, Rodrigo da S Ferreira, Bruno Salu, Jackeline da Costa Maciel, Maria Luiza Oliva, Luiz Bezerra Carvalho Junior
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ANEXO H – COMPROVAÇÃO DA SUBMISSÃO DO ARTIGO 3

Manuscript Details

Manuscript number	MAGMA_2020_237
Title	INTERACTION BETWEEN HUMAN α-THROMBIN AND FACTOR Xa WITH HEPARIN IMMOBILIZED IN POLYANILINE-COATED MAGNETITE NANOPARTICLES.
Article type	Full Length Article

Abstract

This works aimed to immobilize heparin onto magnetite coated with polyaniline nanoparticles (MAG-PANI) and to use for interaction study or bioseparation of α-thrombin and Factor Xa. Magnetite (Fe_3O_4) was obtained by chemistry co-precipitation method (Fe^{2+}/Fe^{3+}). Magnetite coated with polyaniline was obtained by chemistry aniline oxidation. Heparin was activated by carbodiimide and N-hydroxysuccinimide and covalently linked to MAG-PANI (MAG-PANI-hep). SEM (scanning electron microscopy) and TEM (transmission electron microscopy) analysis showed that MAG-PANI has heterogeneus morphology and size of 11.7 ± 2.2 nm and XRD (X-ray diffraction) analysis suggests the presence of polyaniline and Fe_3O_4 in MAG-PANI that it exhibited superparamagnetism behavior (magnetic analysis). Human α-thrombin (10 nM) or Factor Xa (10 nM) was incubed with MAG-PANI-hep in 20 mM Tris-HCl pH 7.4 containing increasing concentrations of NaCl. After incubation, each supernatant was collected for the thrombin or Factor Xa enzyme activity test. Under conditions with 800 mM NaCl was observed a thrombin and Factor Xa supernatant enzymatic activity of around 85.0 ± 4 % and 55.4 ± 12 %, respectively. Under salt-free conditions the residual supernatant activity was 5.3 ± 0.4 and 10.9 ± 0.4% for thrombin and Factor Xa, respectively. These results suggest that the enzymes showed high affinity for immobilized heparin in MAG-PANI. This method can be applied to the blood products industry since it proved useful as a tool for the bioseparation of coagulation factors.

Keywords	Factor Xa; Fe_3O_4; heparin; polyaniline; thrombin.
Manuscript category	Outreach to the General Public
Corresponding Author	Luiz Bezerra Carvalho Junior
Corresponding Author's Institution	Universidade Federal de Pernambuco
Order of Authors	Aurenice Arruda Dutra das Mercês, Andre Lira, Rodrigo da S Ferreira, Karciano Santos, Jackeline da Costa Maciel, José Albino Aguiar, Maria Luiza Oliva, Luiz Bezerra Carvalho Junior
Suggested reviewers	Ming Ma, Junying Chen, Younggyun Choi, Marco Guerrini, Robert Linhardt

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ANEXO I – COMPROVAÇÃO DA SUBMISSÃO DO ARTIGO 4

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Manuscript Draft

Manuscript Number:

Title: MAGNETIC HEPARIN COATED PARTICLES FOR LECTIN PURIFICATION

Article Type: Research Paper

Section/Category: Biochemical Engineering/Bioprocess Engineering

Keywords: affinity chromatography; CrataBL; chitosan; magnetic separation.

Corresponding Author: Professor Luiz Bezerra Carvalho Junior, PhD

Corresponding Author's Institution: Universidade Federal de Pernambuco

First Author: Aurenice A Dutra das Merces

Order of Authors: Aurenice A Dutra das Merces; Rodrigo S Ferreira; Jackeline C Maciel; Maria Tereza S Correia; Patricia Maria G Paiva; Maria Luiza V Oliva; Luiz Bezerra Carvalho Junior, PhD

Abstract: Lectins can easily be purified by using magnetic particles-chitosan composite coated with heparin. Here, CrataBL, lectin obtained from bark of Crataeva tapia is purified according to this approach as a model. CrataBL has been showed potential biomedical application as anti-inflammatory and antitumor activities. Magnetic chitosan particles were synthesized by co-precipitation method of ferric and ferrous ions with chitosan (MAG-CH). Heparin activated with carbodiimide and N-hydroxysuccinimide was covalently coated the magnetic particle (MAG-CH-hep). Crataeva tapia bark extract was incubated with MAG-CH-hep for 30 minutes at 4° and then, using a magnetic separation rack, the complex MAG-CH-hep-CrataBL was recovered and washed with 10 mM citrate phosphate buffer pH 5.5. Afterwards, CrataBL was eluted with increasing ionic strength. SDS/PAGE showed 20 and 40 kDa bands suggesting the presence of CrataBL eluted with 0.25 M and 0.5 M NaCl, which were also confirmed by size-exclusion chromatography (Superdex G75). CrataBL presents high affinity for heparin immobilized on magnetic chitosan particles and it can be purified using ionic strength and magnetic separation by ease, simple and low-cost methods. MAG-CH-hep composites were reused three times extracting CrataBL from bark extract similarly.

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ANEXO J – PRODUÇÃO CIENTÍFICA DURANTE O DOUTORADO

Artigos completos publicados em periódicos:

MERCÊS, A A D; MACIEL, J.C. Bioquímica para estudantes da área da saúde: importância e alternativas de ensino. Health and Diversity (Online), v. 2, p. 52-56, 2018.

MACIEL, J. C. ; D. MERCÊS, A. A. ; CABRERA, M. ; SHIGEYOSI, W. T. ; DE SOUZA, S. D. ; OLZON-DIONYSIO, M. ; FABRIS, J. D. ; CARDOSO, C. A. ; M. NERI, D. F. ; C. SILVA, M. P. ; CARVALHO, L. B. . Magnetic nanoparticles coated with polyaniline to stabilize immobilized trypsin. Hyperfine Interactions, v. 237, p. 1-13, 2016.

MERCÊS, AURENICE ARRUDA DUTRA DAS; SILVA, RICARDO DE SOUZA ; SILVA, KARCIANO JOSÉ SANTOS ; MACIEL, JACKELINE DA COSTA ; OLIVEIRA, GIVANILDO BEZERRA ; BUITRAGO, DAVIAN MARTINEZ ; AGUIAR, JOSÉ ALBINO OLIVEIRA DE ; CARVALHO, LUIZ BEZERRA DE . SYNTHESIS AND CHARACTERISATION OF MAGNETISED DACRON-HEPARIN COMPOSITE EMPLOYED FOR ANTITHROMBIN AFFINITY PURIFICATION. Journal of Chromatography. B (Print), v. 1038, p. 73-79, 2016.

MERCÊS, A A D; MACIEL, J C; CARVALHO, L B . Magnetic particles as affinity matrix for purification of antithrombin. IOP Conference Series: Materials Science and Engineering (Print), v. 97, p. 012002, 2015.

Resumos publicados em anais de congressos:

MERCÊS, A A D; SILVA, K. J. S. ; NASCIMENTO, G. A. F. ; BUITRAGO, D. M. ; AGUIAR, J. A. O. ; CARVALHO JUNIOR, L. B. . Fractionation of Human Plasma Proteins Based on Heparin Affinity and Magnetic Separation. In: 45th Annual Meeting of the Brazilian Biochemistry and Molecular Biology Society (SBBq), 2016, Natal. Abstracts, 2016. v. J-019. p. 1-1.

Orientações, colaborações e atividades paralelas

2016 - 2019: Professora convidada pelo Prof. Dr Luiz Carvalho para ministrar aulas teóricas de Bioquímica para o segundo período do curso de medicina da Universidade Federal de

Pernambuco abordando tópicos relacionados aos módulos: cardio-respiratório, digestivo e urinário.

2017 - 2018: Co-orientação do projeto de iniciação científica (PIBIC/CNPq) da aluna do curso de ciências farmacêuticas (CCS/UFPE), Maria Luiza Cavalcanti Lucena: “Desenvolvimento de uma técnica para obtenção de trombina e fibrinogênio humanos a partir de uma única doação de plasma”.

2018: Realização do Doutorado Sanduíche (AMD/FACEPE) no laboratório de Bioquímica do Instituto de Farmacologia e Biologia Molecular da Escola Paulista de Medicina na Universidade Federal de São Paulo (INFAR/UNIFESP). Execução de atividades relacionadas a processos de purificação e caracterização de proteínas, com orientação da Profa. Dra. Maria Luiza Vilela Oliva.

2018: Revisora do periódico internacional PARTICULATE SCIENCE AND TECHNOLOGY (Print ISSN: 0272-6351 Online ISSN: 1548-0046).

2018 - 2019: Co-orientação do projeto de iniciação científica (PIBIC/CNPq) da aluna do curso de ciências farmacêuticas (CCS/UFPE), Maria Luiza Cavalcanti Lucena: “Padronização de metodologia analítica para caracterização de trombina e fibrinogênio humanos obtidos a partir de única doação de plasma”.

2019 - 2020: Co-orientação do projeto de iniciação científica (PIBIC/CNPq) da aluna do curso de ciências farmacêuticas (CCS/UFPE), Maria Luiza Cavalcanti Lucena: “Avaliação do perfil de qualidade de trombina humana obtida a partir de uma doação de plasma autóloga em sua forma liofilizada”.