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JACKELINE DA COSTA MACIEL

COMPÓSITOS DE PARTÍCULAS MAGNÉTICAS E POLÍMEROS PARA IMOBILIZAÇÃO DE TRIPSINA

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

Orientador: Prof. Dr. Luiz Bezerra de Carvalho Júnior

Co-orientadora: Profa. Dra. Maria da Paz Carvalho da Silva

Co-orientador: Prof. Dr. David Fernando Morais Neri

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Aprovada em 26 de julho de 2012 pela banca examinadora:

Prof. Dr. Luiz Bezerra de Carvalho Júnior
Departamento de Bioquímica/UFPE
Orientador e Presidente da banca

Profa. Dra. Adriana Fontes
Departamento de Biofísica e Radiobiologia/UFPE
Examinadora

Profa. Dra. Maria Danielly Lima de Oliveira
Departamento de Bioquímica/UFPE
Examinadora

Prof. Dr. José Luiz de Lima Filho
Departamento de Bioquímica/UFPE
Examinador

Dr. Ian Porto Gurgel do Amaral
Pesquisador do Departamento de Bioquímica/UFPE
Examinador

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"... Um trabalho científico não deve ser avaliado do ponto de vista de sua utilidade direta.

Ele precisa ser feito por si só, pela beleza da ciência."

Marie Curie (1867–1934)

Resumo

As enzimas são muito utilizadas em processos industriais; então, a busca por novas tecnologias, que permitam o uso otimizado dessas biomoléculas, confere redução dos custos e permite melhor rendimento da enzima. Uma das alternativas para isso consiste na imobilização dessas proteínas em suportes insolúveis. Dentre os diferentes tipos de suportes existentes, os compósitos de nanopartículas magnéticas e polímeros apresentam algumas vantagens, tais como: oferecem maior área superficial para ligação da enzima devido ao reduzido tamanho; permitem a rápida separação utilizando apenas um campo magnético externo e a presença do revestimento polimérico contribui com grande quantidade de grupos funcionalizáveis. Embora se conheçam todas as ferramentas, é preciso escolher as mais adequadas para a aplicação desejada. Neste trabalho, foram produzidos compósitos de partículas magnéticas e polímeros. Dois tipos de polímeros foram utilizados, o polissacarídeo levana e o polímero sintético polianilina (PANI). Nosso objetivo foi obter derivados enzimáticos com boa retenção de atividade. O compósito de partículas magnéticas e levana foi obtido pelo método de co-precipitação na presença do polissacarídeo. Para produção do compósito com PANI, o polímero só foi adicionado após a síntese das partículas magnéticas por co-precipitação. O processo de revestimento das partículas magnéticas com PANI foi otimizado por meio do uso de dois planejamentos fatoriais completos (2^3). Propriedades físicas, químicas e magnéticas de ambos os compósitos foram determinadas através de técnicas de microscopia eletrônica, FTIR, difração de raios-X, espectroscopia Mössbauer, área de superfície BET e porosidade e medidas de magnetização. Os resultados mostraram que as partículas magnéticas sintetizadas, na ausência do polímero, possuem menores tamanhos e estrutura cristalina bem definida. Ambos compósitos foram utilizados para imobilização de tripsina. No caso do compósito de partículas magnéticas e levana, após dez reutilizações, o derivado enzimático exibiu 84% de sua atividade inicial. Em relação, a tripsina imobilizada em compósito de partículas magnéticas e PANI, o derivado enzimático apresentou maior estabilidade térmica em temperaturas acima de 25C°; maior afinidade pelo substrato BAPNA (K_{map} 1,4 vezes menor que aquele para enzima solúvel); pequena redução na eficiência catalítica (1,1 vezes menor que aquele para enzima solúvel); retenção de 89% de sua atividade inicial após 48 dias de estocagem a 4°C e retenção de 81% de sua atividade inicial após vinte reutilizações. Tanto o compósito magnético com PANI quanto aquele com levana apresentaram bom resultado quanto à retenção da atividade da enzima imobilizada, sendo o derivado com PANI o de menor custo.

Palavras-chave: partículas magnéticas, levana, polianilina, imobilização, tripsina

Abstract

Enzymes are widely used in industrial processes, so the search for new technologies that allow the optimal use of these biomolecules, provides reduced costs and allow better use of the enzyme. An alternative to this is the immobilization of these proteins on insoluble supports. Among the different types of existing supports, the composite magnetic nanoparticles and polymers have certain advantages such as providing greater surface area for binding of the enzyme due to the reduced size; allow rapid separation using only an external magnetic field and the presence of the polymeric coating contributes large amounts of functional groups. Although you can learn all the tools you need to choose the most appropriate for the desired application. In this paper, were produced composite magnetic particles and polymers. Two types of polymers were used: the polyaniline (PANI) and the polysaccharide levan. Our goal was to obtain enzymatic derivatives with good retention of activity. The composite magnetic particles and levan was produced by co-precipitation in the presence of the polysaccharide. For production of the composite with PANI, the polymer was only added after synthesis of the magnetic particles by co-precipitation. The process of coating magnetic particles with PANI was optimized by using two full factorial design (2^3). Physical, chemical and magnetic properties of both composites were determined by electron microscopy, FTIR, X-ray diffraction, Mössbauer spectroscopy, BET surface area and porosity, and magnetization measurements. The results showed that the magnetic particles synthesized in the absence of polymer exhibit lower sizes and have well defined crystal structure. Both composites were used for immobilization of the trypsin. In the case of the composite of magnetic particles and levan, after ten reuses, the enzymatic derivative exhibited 84% of its initial activity. Regarding the immobilized trypsin on composite of magnetic particles and PANI, the enzymatic derivative had greater thermal stability at temperatures up to 25°C, greater affinity for the substrate BAPNA (K_{map} 1.4 times lower than that for the soluble enzyme), a slight decrease in catalytic efficiency (1.1 times lower than that for soluble enzyme), retaining 89% of its initial activity after 48 days of storage at 4°C and retaining 81% of its initial activity after twenty reuses. The two magnetic composites showed good results with regard to retention of activity of the immobilized trypsin, and the PANI derivative with the lowest cost.

Keywords: magnetic particles, levan, polyaniline, immobilization, trypsin

LISTA DE ILUSTRAÇÕES

Capítulo 1

Capítulo 2

Capítulo 3

Capítulo 4

LISTA DE TABELAS

Capítulo 1

Capítulo 2

Capítulo 3

LISTA DE ABREVIATURAS E SIGLAS

mPANI	compósito de nanopartículas magnéticas e polianilina
T	Temperatura
M	Magnetização
H	Campo magnético
T	tesla
nm	nanômetro
µm	micrômetro
°C	Grau celsius
pH	Potencial hidrogeniônico
Tris	Tris (hidroximetil) aminometano
DRX	Difração de raios-X
XDR	X-ray Diffractometry
MEV	Microscopia Eletrônica de Varredura
SEM	Scanning Electron Microscopy
IV	Infravermelho
FTIR	Fourier Transform Infrared
K_m	Constante de Michaelis-Menten
V_{max}	Velocidade máxima
2θ	Ângulo de espalhamento (graus)
λ	Comprimento de onda
K	kelvin
Oe	oersted
KOe	kilooersted
cm^{-1}	número de ondas
mg	miligrama
atm	atmosfera
emu	unidade eletromagnética
g	grama
min	minuto
h	hora
M	molar
mM	milimolar
mL	mililitro
µL	microlitro
µmol	micromol
U	unidade
Bhf	campo hiperfino
Δ	desdobramento quadrupolar
δ	deslocamento isomérico

SUMÁRIO

1. Introdução

A proteólise é uma das etapas mais importantes para o sequenciamento de proteínas em pesquisa proteômica (PARKER e TOMER, 2000; WANG, 2002), sendo a tripsina a enzima proteolítica mais utilizada nessa etapa. A imobilização dessas enzimas em suportes insolúveis visa diminuir o longo tempo de digestão quando comparado à enzima em sua forma solúvel. As vantagens das enzimas imobilizadas, em relação às solúveis, surgem de sua maior estabilidade e facilidade de separação do meio de reação, o que acarreta economia significativa no custo global do processo (BON et al., 2008).

As propriedades das preparações de enzimas imobilizadas são influenciadas pelas propriedades da enzima e do material do suporte (TISCHER e KASCHE, 1999). A interação entre esses dois componentes proporciona um derivado imobilizado com propriedades químicas, bioquímicas, mecânicas e cinéticas específicas. A imobilização da enzima tem um efeito benéfico na sua estabilidade, em função das interações físicas e químicas entre o suporte e as moléculas da enzima (DE CASTRO e ANDERSON, 1995; VILLENEUVE et al., 2000). Diferentes tipos de materiais têm sido usados como suportes para imobilização de enzimas, entre eles os suportes que possuem propriedade magnética. Além de serem utilizadas como adsorventes em separação magnética, essas partículas, dentro da escala nanométrica à micrométrica, estão sendo usadas em um número crescente de aplicações médicas devido a não toxicidade (ITO et al., 2005; TOKORO et al., 2009).

Óxidos de ferro com estrutura "core/shell" são os mais usados como fontes de materiais magnéticos (DRBOHLAVOVA et al., 2009). Este tipo de estrutura consiste em partículas magnéticas formadas por um núcleo magnético ("core") e um revestimento ("shell"). No entanto, somente maghemita e magnetita encontraram maior interesse em bioaplicações (TUCEK et al., 2006). Vários procedimentos sintéticos foram desenvolvidos para sintetizar nanopartículas de óxido de ferro (AH et al., 2007), sendo o método de co-precipitação o procedimento mais simples, barato e ecologicamente correto, que envolve a precipitação simultânea de íons Fe^{2+} e Fe^{3+} em meio aquoso básico (KANG et al., 1996; QU et al., 1999).

As nanopartículas de óxido de ferro apresentam alta sensibilidade magnética e baixa toxicidade, porém pequena estabilidade coloidal na fase aquosa. Modificações da superfície dessas partículas representam um ponto-chave para impedir a agregação e para torná-las altamente monodispersas (SAWANT et al., 2009). Para melhorar a estabilidade e biocompatibilidade, nanopartículas de óxido de ferro são frequentemente modificadas com alguns surfactantes ou polímeros (BERRY et al., 2003). O uso de um revestimento de superfície adequado permite que essas nanopartículas sejam dispersas dentro de fluidos homogêneos e melhorem sua estabilidade. Vários grupos de materiais de revestimento são usados para modificar a superfície química de

nanopartículas magnéticas (SHUBAYEV et al., 2009) tais como polímeros orgânicos, surfactantes orgânicos, metais inorgânicos, óxidos inorgânicos, moléculas e estruturas bioativas.

O objetivo deste trabalho foi produzir derivados enzimáticos magnéticos para serem utilizados em digestão de proteínas. Sabe-se que a presença do revestimento polimérico confere estabilidade às partículas magnéticas, diminuindo sua agregação e melhorando a biocompatibilidade; além de estabilizar a enzima imobilizada. A escolha dos polímeros foi baseada em alguns critérios. No caso da polianilina, o fato de ser um polímero de fácil síntese e de baixo custo foram fatores importantes; além de ser promissor para uso em biossensores. Já o polissacarídeo levana, foi escolhido por ser biocompatível, não tóxico e possuir propriedades biológicas interessantes para aplicações em sistemas de liberação de drogas e hipertermia magnética.

Uma vez que a presença do revestimento forneceria uma superfície quimicamente controlada, esses compósitos magnéticos poderiam ser usados como suportes para imobilizar tripsina. Com o objetivo inicial de aplicar estes derivados enzimáticos na etapa de proteólise, que antecede a identificação e o sequenciamento de uma proteína e, assim diminuindo custos, reduzindo o tempo de digestão e tornando o procedimento o mais simples possível. No entanto, outras possíveis aplicações para estes materiais também são visadas, como a montagem de reatores para produção de hidrolisados protéicos.

Capítulo 1

2. Revisão da literatura

2.1. Enzimas proteolíticas ou proteases

As proteases são amplamente utilizadas em aplicações industriais e biomédicas, entre as quais a tripsina (**Figura 1**) foi mais extensivamente empregada. A tripsina, uma serino protease, atrai considerável interesse devido a sua ampla variedade de aplicações, tais como na composição de detergentes, no amaciamento do couro, na produção de hidrolisados protéicos com valor nutricional (ROCHA et al., 2011), em cultura de células e tecidos (SOLEIMANI et al., 2009; BANUMATHI et al., 2009; YANG et al., 2009), na identificação de proteínas por meio de técnicas de sequenciamento (SCHUCHERT-SHI e HAUSER, 2009), em doenças pancreáticas, incluindo fibrose cística (TZETIS et al., 2007) e pancreatite crônica (CHEN et al., 2009). Contudo, suas aplicações são limitadas no que diz respeito a sua instabilidade e rápida perda de atividade catalítica durante os períodos operacionais e de estocagem resultantes da autólise, desnaturação e agregação dessas enzimas (VILLALONGA et al., 2000). A imobilização de enzimas pode superar essas limitações; a imobilização estabiliza a estrutura e, por conseguinte, a atividade da enzima. Os pontos-chave são o suporte e o método de imobilização adequados (NIKOLIC et al., 2010).

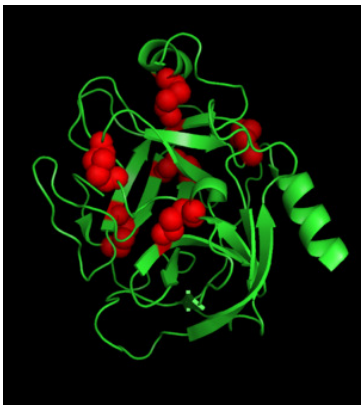


Figura 1. Molécula de tripsina. Em vermelho, ligações dissulfeto. *Fonte: http://prince.openwetware.org/FASP.html*

Dependendo das propriedades do suporte, a simples sedimentação, filtração ou centrifução podem ser suficientes. Se propriedades magnéticas podem ser adicionadas ao suporte, ele pode ser removido com maior facilidade e rapidez pela aplicação de um campo magnético (ROCHA et al., 2011). Por essa vantagem, a imobilização de tripsina em suporte magnético tem sido considerada como um procedimento potencialmente poderoso nos últimos anos (AMARAL et al., 2006; LIU et al., 2007; BAYRAMOĞLU et al., 2008; SUN et al., 2011; MACIEL et al., 2012). O interesse crescente na área de proteômica induz esforços intensivos para desenvolver metodologias analíticas automatizadas, sensíveis e com alto rendimento. Por exemplo, Krogh et al. (1999) demonstraram a

rápida digestão de proteínas usando enzimas imobilizadas em "beads" de vidro paramagnético. Wang et al. (2008) também ligaram tripsina a nanocómposito magnético para digestão de proteínas, resultando em boa sequência de conversão.

2.2. Imobilização de enzimas em suportes insolúveis

As vantagens das enzimas imobilizadas, em relação às solúveis, surgem de sua maior estabilidade e facilidade de separação do meio de reação, o que acarreta economia significativa no custo global do processo, desde que o procedimento de imobilização não seja muito dispendioso, haja boa recuperação da atividade enzimática e que a meia-vida operacional da enzima imobilizada seja suficientemente longa (BON et al., 2008).

De particular interesse é o aumento do uso de enzimas imobilizadas, fisicamente confinadas ou localizadas numa certa região do espaço com retenção de sua atividade catalítica, podendo ser usada repetida e continuadamente (WINGARD JR., 1972). Nesta técnica, a enzima fica retida no interior (poros) ou na superfície de um material que é utilizado como suporte. O complexo enzima-suporte mantém as características físicas do suporte e, ao mesmo tempo, retém a atividade biológica da enzima na forma solúvel (BON et al., 2008).

2.2.1. Classificação das enzimas imobilizadas

Zaborsky (1973, 1976) propôs uma classificação que leva em consideração – *o tipo de interação responsável pela imobilização* –, que pode ser conseguida por meios químicos (formação de ligação covalente) ou por meios físicos (envolvem somente forças físicas: adsorção, interações eletrostáticas e outras); – *a natureza dos suportes* –, que podem ser porosos ou não-porosos, orgânicos ou inorgânicos (VIETH, 1994; ZANIN e MORAES, 2004).

Do ponto de vista prático, o método de classificação não altera o sistema obtido, uma vez que as técnicas mais utilizadas para a preparação dos biocatalisadores de aplicação comercial envolvem uma combinação de métodos básicos (BON et al., 2008).

A classificação dos métodos de imobilização de enzimas, mais aceita na literatura, foi proposta por Kennedy e Roig (1995), que considera a combinação da natureza da interação responsável pela imobilização e o tipo de suporte utilizado. Assim:

1) Ligação em suportes – modificação da enzima, por meio de técnicas apropriadas, para torná-las imóveis no meio de reação e "cross-linking" – ligação cruzada intermolecular.
2) Enzimas solúveis sem derivatização – enzimas solúveis, utilizadas em reatores equipados com membranas semipermeáveis de ultrafiltração e fibras ocas que retêm a enzima no interior do reator.

3) Enzimas solúveis com derivatização – enzimas cuja mobilidade foi restringida pela ligação com outra macromolécula, porém permanecendo o complexo solúvel em água.

2.2.2. Métodos de imobilização de enzimas

É muito importante escolher um método de imobilização que conserve grande parte da atividade catalítica da enzima. Dentre as técnicas de imobilização (**Figura 2**) conhecidas podem citar-se: adsorção, onde há uma interação física não-específica entre a enzima e a superfície da matriz; oclusão ou enclausuramento em uma matriz semi-permeável ou no interior de polímeros; reações cruzadas ou cross-linking, consiste na ligação da enzima a uma outra proteína ou a grupos funcionais existentes na matriz; e ligação covalente, onde há a formação de ligações covalentes entre a enzima e a matriz (POWELL, 1990).

O método de ligação covalente é o mais estudado entre os métodos já citados. Este método pode ser classificado de acordo com o tipo de ligação (diazotização, amida, base de Schiff, etc). As condições para imobilização por este método são mais elaboradas que a técnica de adsorção. A ligação covalente, pode ainda, alterar a estrutura conformacional e o sítio ativo da enzima, resultando em maior perda da atividade e/ou mudanças do substrato. Contudo, a força de ligação entre a enzima e a matriz é tão forte que mesmo na presença do substrato ou de soluções de alta força iônica a ligação não é desfeita e a enzima continua imobilizada (HERMANSON et al., 1992; HERMANSON, 1996).

A imobilização covalente de biomoléculas em superfícies é de substancial interesse em aplicações biomédicas, tais como biosensores, cromatografia de afinidade, bioprocessamento, diagnósticos de fase sólida, terapia extracorpórea e liberação controlada de drogas (MALMSTEN et al., 1999).

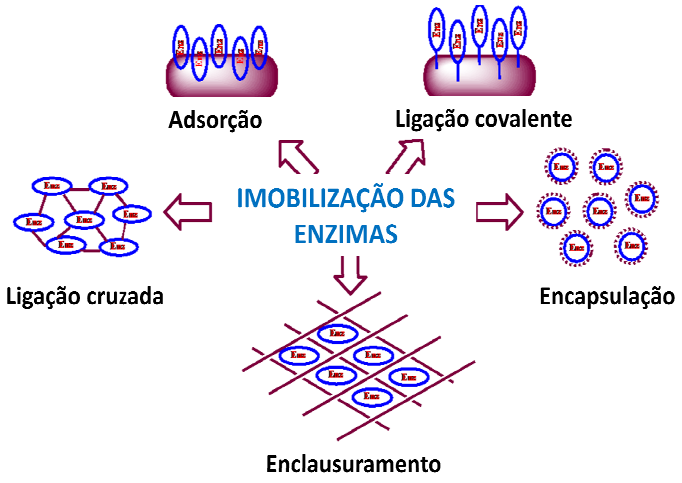


Figura 2. Métodos de imobilização de enzimas. Representação esquemática da enzima (em azul) e do suporte (em roxo). *Reproduzido de http://www.turqase.com/research/5/*

2.2.3. Suportes para a imobilização de enzimas

As propriedades das preparações de enzimas imobilizadas são influenciadas pelas propriedades da enzima e do material do suporte (TISCHER e KASCHE, 1999). A interação entre esses dois componentes proporciona um derivado imobilizado com propriedades químicas, bioquímicas, mecânicas e cinéticas específicas (**Figura 3**).

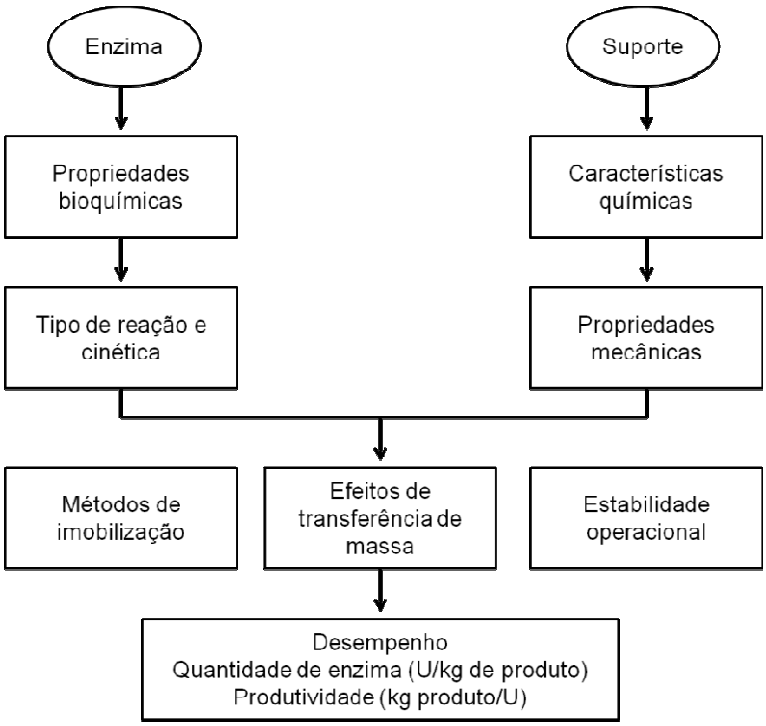


Figura 3. Interação entre suporte e enzima. *Reproduzido de BON et al., 2008.*

Dentre os vários parâmetros que devem ser considerados, no processo de imobilização de enzimas, os mais importantes são apresentados na **Tabela 1**, os quais incluem pH, temperatura, força iônica, pressão, agitação, liberação de co-fatores e do substrato com a remoção dos produtos. Estes fatores influem no desempenho do suporte, na conformação da enzima, na velocidade de transferência de massa e de reação intrínseca, e, portanto, afetam o comportamento da enzima imobilizada. De todos os fatores anteriormente citados, com exceção da enzima, a maior contribuição para o bom desempenho da enzima imobilizada é dada pelo suporte. Na escolha de um suporte para uma determinada aplicação, devem ser analisadas suas propriedades físicas e químicas, bem como as relativas à possibilidade de regeneração do material. O suporte deve ser quimicamente resistente nas condições de ativação, durante o processo de imobilização e nas condições em que se processa a reação. Em geral, os supores inorgânicos são mais apropriados para uso industrial por apresentarem elevada resistência mecânica, boa estabilidade térmica, resistência a solventes orgânicos e ao ataque por microrganismos (BON et al., 2008).

Tabela 1. Fatores que influenciam o desempenho de um sistema de enzima imobilizada. *Reproduzido de TISCHER e KASCHE, 1999.*

Enzima	**Propriedades Bioquímicas:** massa molar, grupos funcionais da superfície proteica, pureza (funções de inativação ou proteção das impurezas). **Parâmetros Cinéticos:** atividade específica, perfil de pH e temperatura, parâmetros cinéticos para ativação e inibição, estabilidade térmica, pH, solventes, contaminantes e impurezas.
Suporte	**Características Químicas:** composição e base química, grupos funcionais, estabilidade química, contribuições da superfície do suporte, tais como os microefeitos (pH, carga da superfície, natureza hidrofóbica e hidrofílica, efeito redutor e a presença de íons metálicos). **Características Mecânicas:** diâmetro do poro, comportamento de compressão, tamanho da partícula; área superficial; volume acessível da matriz, resistência à compactação em operações em altas vazões para reatores de leito fixo; abrasão para reatores agitados e velocidade de sedimentação para leitos fluidizados. **Características Morfológicas:** suportes não-porosos (baixa área superficial), porosos (grande área superficial), estrutura de gel.
Enzima imobilizada	**Método de Imobilização:** fixação de proteína, eficiência da enzima ativa, parâmetros cinéticos intrínsecos. **Efeitos de Transferência de Massa:** partição (diferente concentração do soluto dentro e fora das partículas do catalisador), difusão interna (poros) e externa. **Estabilidade:** operacional (expressa em tempo de meia-vida sob condições operacionais), estabilidade em estocagem. **Desempenho:** produtividade (produto formado por unidade de atividade ou massa de enzima), consumo de enzima (por exemplo, unidades por kg de produto).

2.2.4. Vantagens e limitações da imobilização de enzimas

A enzima imobilizada tem muitas vantagens sobre a enzima solúvel, tais como capacidade de reutilização, estabilidade melhorada, término da reação rápido, fácil separação entre biocatalisador e produto, redução dos custos operacionais, etc. (ELIF BOYUKBAYRAM et al., 2006). A imobilização também auxilia a dispersão homogênea da enzima no meio, essencial, para a condução de reações enzimáticas (DE CASTRO e ANDERSON, 1995; VILLENEUVE et al., 2000). Do ponto de vista comercial, as principais vantagens da utilização de enzimas imobilizadas, em relação às enzimas solúveis são, praticamente, as relativas à catálise.

O sucesso da tecnologia de imobilização mostra que, de modo geral, as vantagens superam as limitações. Porém, alguns fatores devem ser apontados, não como desvantagens do processo, mas

sim como pontos a serem evitados. Dentre estes fatores podem ser citados (ROSEVEAR et al., 1987): perda da atividade durante o processo de imobilização, os efeitos difusionais (transferência de massa), as características físicas do biocatalisador e do fluido e a estabilidade do biocatalisador.

Como os métodos de imobilização, de modo geral, envolvem interações fracas, ou fortes, entre a frágil estrutura da enzima e o suporte, ocorre invariavelmente alteração da estrutura tridimensional da proteína, resultando em menor atividade. Para se superar esse problema, busca-se a proteção do sítio ativo da enzima durante o processo de imobilização, o que pode ser conseguido empregando-se altas concentrações de substrato ou um inibidor de sítio ativo (BON et al., 2008).

A mobilidade restringida da enzima imobilizada consiste em outra desvantagem e provoca uma diminuição na acessibilidade ao substrato, o que leva à aparente redução da atividade, neste caso provocada por restrições difusionais (limitações do acesso do substrato ao sítio ativo devido à presença da matriz sólida). Este efeito deve ser evitado ou diminuído pela escolha criteriosa do suporte, ou pelas condições de operação. Normalmente, as enzimas imobilizadas devem ser utilizadas quando o substrato é solúvel. Quando as enzimas estão retidas no interior de matrizes porosas, os poros devem facilitar o livre acesso do substrato e reter ao mesmo tempo a molécula de enzima no seu interior. O custo da imobilização deve ser compensado pela longa vida do biocatalisador. O suporte deve manter suas propriedades físicas (resistência mecânica e ao ataque por reagentes químicos) durante o tempo de meia-vida estimado (BON et al., 2008).

2.3. Sistemas de imobilização de biomoléculas: partículas magnéticas

O emprego de partículas magnéticas ocorreu pela primeira vez na década de 1940 como uma nova tecnologia no tratamento de água poluída (ARIAS et al., 2001). Hoje em dia, a síntese e a aplicação de partículas magnéticas funcionalizadas tem despertado grande interesse em diferentes áreas (WU et al., 2008; HAO et al., 2010), sendo as técnicas de separação magnética as mais estudadas, uma vez que elas apresentam benefícios sobre a centrifugação, filtração e extração em fase sólida. No caso da extração em fase sólida usando materiais magnéticos, o adsorvente magnético é adicionado à solução ou à suspensão contendo o analito alvo. Esse analito é, então, adsorvido sobre o adsorvente magnético; capturado e recuperado da suspensão usando um separador magnético apropriado, sendo, em seguida, eluido do adsorvente e analisado (CHEN et al., 2011) como pode ser visto no esquema da **Figura 4**.

Esses sólidos magnéticos são amplamente usados em sistemas de detecção e analítico, pois oferecem algumas vantagens quando comparados aos sólidos similares que não possuem propriedades magnéticas. Tais sólidos podem ser usados na pré-concentração de analitos, na separação magnética, na identificação molecular de biomoléculas, de compostos orgânicos e inorgânicos (AGUILAR-ARTEAGA et al., 2010).

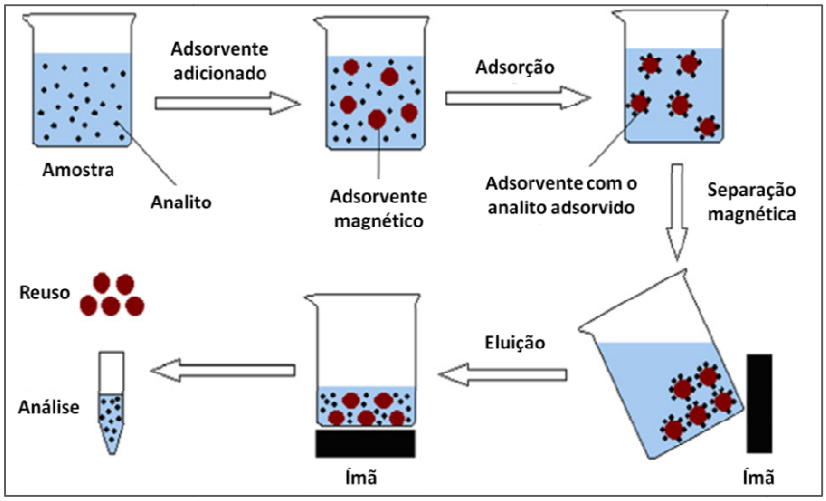


Figura 4. Procedimento de extração em fase sólida magnética. *Reproduzido de CHEN et al., 2011.*

Além de serem utilizadas como adsorventes em separação magnética, essas partículas, dentro da escala nanométrica à micrométrica, estão sendo usadas em um número crescente de aplicações médicas (hipertermia magnética, agentes de contraste para imagem por ressonância magnética e liberação de drogas, por exemplo), em especial, devido a suas importantes propriedades tais como não toxicidade, biocompatibilidade, injetabilidade e alto nível de acumulação no tecido ou órgão (ITO et al., 2005; TOKORO et al., 2009). Segundo Ito et al. (2005), dentre todas as propriedades mencionadas acima, a mais importante é a não toxicidade. De acordo com Kreuter (1983), em nanopartículas com um diâmetro geralmente menor que cerca de 100 nm, o revestimento é provavelmente o fator mais importante e determina muitas de suas propriedades. No caso de grandes microesferas magnéticas de diâmetros acima de 10 μm, o revestimento da microesfera tem maior influencia na reação tecidual (HÄFELI e PAUER, 1999).

Em geral, um único tipo de partículas não pode ser usado para todas as aplicações. O tamanho e o processo de produção das micropartículas ou nanopartículas magnéticas são determinados pelo tipo de aplicação. Partículas superparamagnéticas, ferromagnéticas e ferrimagnéticas podem ser usadas como carreadores magnéticos de drogas; enquanto que partículas superparamegnéticas são utilizadas para uso biomédico por que elas não exibem magnetismo quando não estão sob influencia de um campo magnético externo, prevenindo a aglomeração magnética indesejada (BRANDL et al., 2010).

2.3.1. Óxidos de ferro: os principais tipos de núcleo magnético

Os óxidos de ferro com estrutura “core/shell” são os mais usados como fontes de materiais magnéticos (DRBOHLAVOVA et al., 2009). Este tipo de estrutura consiste em partículas magnéticas formadas por um núcleo magnético (“core”) e um revestimento (“shell”). Há várias formas cristalinas conhecidas como α-Fe_2O_3 (hematita), β-Fe_2O_3, γ-Fe_2O_3 (maghemita), ε-Fe_2O_3,

Fe_3O_4 (magnetita) (ZBORIL et al., 2002) que podem ser utilizadas para composição do núcleo magnético. No entanto, somente maghemita e magnetita encontraram maior interesse em bioaplicações (TUCEK et al., 2006).

Em alguns trabalhos, metais puros, tais como Fe e Co foram utilizados como núcleo magnético por que eles têm várias vantagens sobre os óxidos de ferro, por ex., melhores propriedades magnéticas e alta magnetização de saturação (ARIAS et al., 2009). Contudo, Fe e Co apresentam menor estabilidade oxidativa, baixa compatibilidade em sistemas não aquosos e maior toxicidade que os óxidos de ferro (CHOMOUKA et al., 2010).

A estrutura básica de um óxido de ferro é de um octaedro, no qual cada átomo ferro (Fe^{2+} ou Fe^{3+}) é rodeado por seis O^{2-} ou por seis OH^-. A polimerização destes octaedros em arranjos compactos, em que essas unidades podem interagir por meio de seus ápices, arestas ou faces, forma a estrutura cristalina de todos os minerais deste grupo. A característica comum entre todos os óxidos de ferro é a presença de uma distância interatômica Fe-Fe, comuns entre dois octaedros compartilhados, que varia de 0,288 a 0,289 nm para ligações com ápices comuns (**Figura 5**) (PEREIRA, 2010).

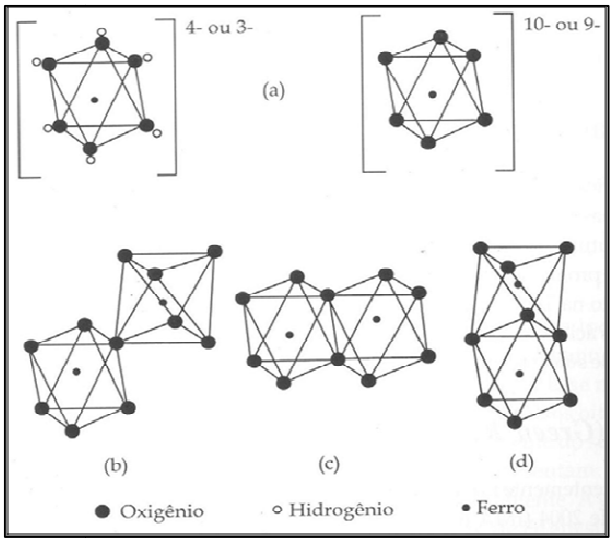


Figura 5. Representação do octaedro de ferro (a) e sua polimerização pelo ápice (b), aresta (c) e face (d). *Fonte: PEREIRA, 2010.*

Vários procedimentos sintéticos foram desenvolvidos para sintetizar nanopartículas de óxido de ferro (AH et al., 2007), sendo as técnicas de co-precipitação (WILLIS et al., 2005), decomposição térmica e/ou redução (PARK et al., 2004) e síntese hidrotermal (WANG et al., 2005) os métodos mais usados e com altos rendimentos.

O procedimento mais simples, barato e ecologicamente correto é o método de co-precipitação, que envolve a precipitação simultânea de íons Fe^{2+} e Fe^{3+} em meio aquoso básico

(KANG et al., 1996; QU et al., 1999). Durante ou após a sua preparação, as nanopartículas são usualmente revestidas com polímeros tais como dextrana, por exemplo, para garantir sua estabilidade sob condições fisiológicas (FIGUEROLA et al., 2010).

No método de co-precipitação, a temperatura de reação é limitada pelo ponto de ebulição da água, e nanopartículas de óxido de ferro sintetizadas, sob essas condições, exibem usualmente um baixo grau de cristalinidade e grande polidispersão. Uma melhoria neste sentido foi representada pelas rotas de síntese empregando microemulsões água em óleo (MUNSHI et al., 1997). Nesses tipos de sínteses, uma melhoria na dispersão das partículas foi relatada devido ao confinamento de tamanho oferecido pela água dentro de cada micela. Mesmo nesses casos, a limitada temperatura de reação leva a baixa cristalinidade do material e adicionalmente os produtos são obtidos com baixo rendimento (FIGUEROLA et al., 2010).

As técnicas de síntese hidrotérmicas são um método alternativo para a preparação de nanopartículas de óxido de ferro altamente cristalinas (WANG et al., 2005). Neste caso, uma mistura de sais de ferro dissolvidos em meio aquoso é introduzida em um recipiente de teflon selado e aquecida acima da temperatura de ebulição da água, e consequentemente, a pressão da reação é aumentada muito acima da pressão atmosférica. O efeito sinérgico de altas temperaturas e pressões melhora a qualidade dos nanocristais e por isso suas características magnéticas. Infelizmente, e em contraste à técnica de microemulsão, não há um caminho simples para controlar o tamanho e a forma das partículas resultantes e usualmente amostras polidispersas são obtidas (FIGUEROLA et al., 2010).

O tamanho e a forma das nanopartículas de óxido de ferro depende do tipo de sal usado (tais como cloretos, sulfatos, nitratos, percloratos, etc), da proporção entre os íons férrico e ferroso, da temperatura de reação, do valor do pH, da força iônica do meio e de outros parâmetros da reação (por ex., velocidade de agitação, velocidade de gotejamento da solução básica) (WU et al., 2008).

2.3.2. Magnetita e maghemita: os óxidos de ferro mais utilizados

A magnetita (Fe_3O_4) pertence à família dos óxidos de ferro, sendo um mineral magnético de cor preta encontrado em rochas. Já a maghemita (γ-Fe_2O_3) é um mineral magnético castanho avermelhado, estruturalmente similar à magnetita, mas com vacâncias (CORNELL e SCHWERTMANN, 2003). Suas propriedades globais são muito semelhantes, o que torna muito difícil distinguir entre elas.

A magnetita tem a particularidade de conter tanto o íon Fe^{2+} quanto Fe^{3+}, dentro de uma estrutura de espinélio inversa. Trinta e dois ânions oxigênio formam uma face centrada cúbica com um comprimento da aresta de 0.839 nm. Nesta célula unitária, os íons de ferro estão localizados em 8 sítios tetraédricos (cercado por 4 íons oxigênio) e 16 sítios octaédricos (cercado por 8 íons oxigênio). Os sítios tetraédricos são ocupados exclusivamente por íons Fe^{3+}, enquanto que os íons

Fe^{2+} e Fe^{3+} ocupam alternadamente sítios octaédricos (**Figura 6a**). Como existe o mesmo número de íons Fe^{3+} tetraédricos e octaédricos, o momento resultante em um cristal de magnetita surge apenas dos íons Fe^{2+} octaédricos não compensados (**Figura 6b**). As propriedades magnéticas da maghemita são devidas aos íons Fe^{3+} octaédricos não compensados (CORNELL e SCHWERTMANN, 2003).

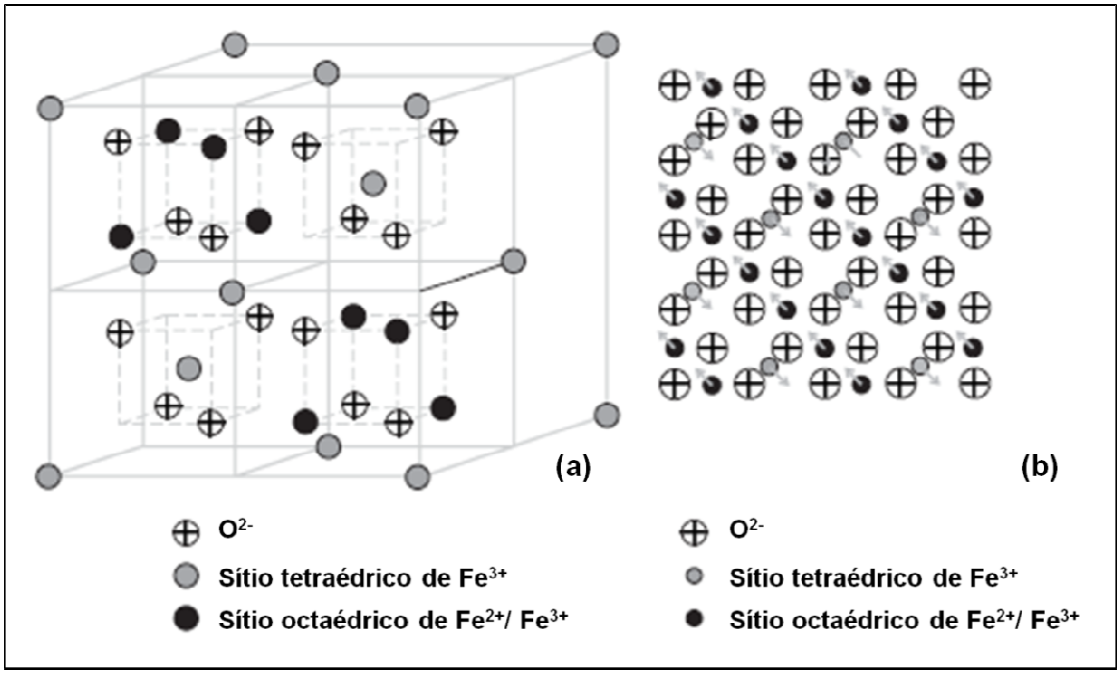


Figura 6. Estrutura de espinélio inversa da magnetita. (a) ilustração de célula unitária cúbica; (b) organização ferrimagnética na magnetita. *Reproduzido de CORNELL e SCHWERTMANN, 2003*.

Suspensões aquosas estáveis de nanopartículas de magnetita (ou maghemita) são ditas ser superparamagnéticas por que elas não mostram remanescência, ou seja, quando elas são submetidas ao campo magnético, seu momento magnético global alinha na direção do campo, mas quando o campo é zero, o momento magnético da amostra também retorna a zero. Este comportamento é o mesmo do paramagnético, mas com um momento magnético de partícula imenso (comparado aquele de um íon paramagnético, por exemplo): este fenômeno é chamado de superparamagnetismo (CORNELL e SCHWERTMANN, 2003).

Por sua biocompatibilidade, a magnetita, incluindo outras formas de nanopartículas de óxido de ferro, tem sido ressaltada para aplicações biomédicas (HUBER, 2005). O tamanho dessas partículas usualmente se refere ao diâmetro total destas incluindo o núcleo de ferro e o revestimento. Partículas com tamanhos maiores e/ou agregados de partículas pequenas podem ser aprisionadas, causando embolia dentro dos capilares pulmonares (KREUTER, 1983). Em nanopartículas com diâmetro menor que 100 nm, o revestimento é provavelmente o fator mais importante e determina muita das propriedades das partículas (KONCRACKÁ et al., 2002). O

tamanho da partícula é um fator importante no desempenho destas e uma redução neste tamanho assegura uma maior magnetização quando aplicado um campo magnético externo (O'HANDLEY, 2000).

2.4. Estabilidade de partículas magnéticas

As nanopartículas de óxido de ferro apresentam alta sensibilidade magnética e baixa toxicidade. No entanto, devido à sua baixa estabilidade coloidal na fase aquosa (tendência de se agregar), a modificação apropriada da superfície dessas partículas representa um ponto-chave para impedir a agregação e para torná-las altamente monodispersas (SAWANT et al., 2009).

Em geral, um revestimento polimérico pode reduzir a agregação e melhorar a estabilidade coloidal das nanopartículas magnéticas. Contudo, em alguns casos, a camada de polímero pode aumentar significativamente o tamanho total das nanopartículas, levando a limitadas distribuição tecidual, penetração e depuração metabólica das partículas. O desenvolvimento de nanopartículas magnéticas revestidas com pequenas moléculas pode ser promissor para obter nanopartículas revestidas de pequeno tamanho (CHENG et al., 2005; YANG et al., 2010).

Para melhorar a estabilidade e biocompatibilidade, nanopartículas de óxido de ferro superparamagnéticas são frequentemente modificadas com alguns surfactantes ou polímeros (BERRY et al., 2003). As nanopartículas de magnetita superparamagnéticas revestidas com polímeros são usualmente formadas por núcleos magnéticos responsáveis por uma resposta magnética forte e uma camada polimérica para fornecer grupos funcionalizáveis e característicos (WUNDERBALDINGER et al., 2002).

Alguns autores sugerem a substituição do ácido oléico por um polímero solúvel em água, revestimento individual das nanopartículas com polímeros anfifílicos e incorporação dessas nanopartículas em micelas lipídicas para obter nanopartículas solúveis em água monodispersas (TROMSDORF et al., 2007).

Revestimento de materiais poliméricos pode ser classificado em sintético e natural. Polímeros baseados em poli(etileno-co-vinil acetato), poli(vinilpirrolidona) (PVP), poli(ácido lático-co-glicólico) (PLGA), poli(etilenoglicol) (PEG), poli(álcool vinílico) (PVA), etc. São exemplos típicos de sistemas poliméricos sintéticos (NUNES et al., 2006). Sistemas de polímero natural incluem o uso de gelatina, dextrana, quitosana, pululana. A modificação de polímeros naturais oferece vantagens significativas em aplicações biomédicas devido a sua boa biocompatibilidade e degradabilidade (MASSIA et al., 2000).

Em muitos estudos, conduzidos principalmente sobre nanopartículas de maghemita, uma diminuição no valor da magnetização de saturação tem sido observado e atribuído ao efeito do tamanho finito, à desordem estrutural em toda a partícula e aos efeitos de superfície (DAOU et al.,

2008). É bem estabelecido que as propriedades das nanopartículas são fortemente dependentes do seu tamanho, mas diferentes rotas sintéticas podem fornecer nanopartículas de tamanhos similares com diferentes propriedades magnéticas. De fato, a distinção entre o tamanho finito e as contribuições do efeito de superfície não é fácil. Além disso, pode-se notar que a influencia específica da ligação orgânica sobre as propriedades magnéticas tem sido pouco estudada. As aplicações tecnológicas de nanopartículas magnéticas requerem a compreensão da infuencia de modificações químicas de superfície sobre as propriedades magnéticas (DAOU et al., 2008).

DAOU et al. (2008) observaram que a estrutura magnética da camada de superfície de nanopartículas de magnetita, na qual moléculas funcionais foram ligadas, depende da natureza do agente de ligação através do qual as moléculas são ligadas à superfície. O agente de ligação carboxilato induz um spin inclinado na camada oxidada, reduzindo a magnetização de saturação. Pelo contrário, não existe estrutura de superfície inclinada quando moléculas fosfonatadas são ligadas covalentemente às nanopartículas de óxido de ferro. Isto é de especial importância para manter as propriedades magnéticas inalteradas quando nanopartículas forem revestidas com uma camada orgânica (**Figura 7**).

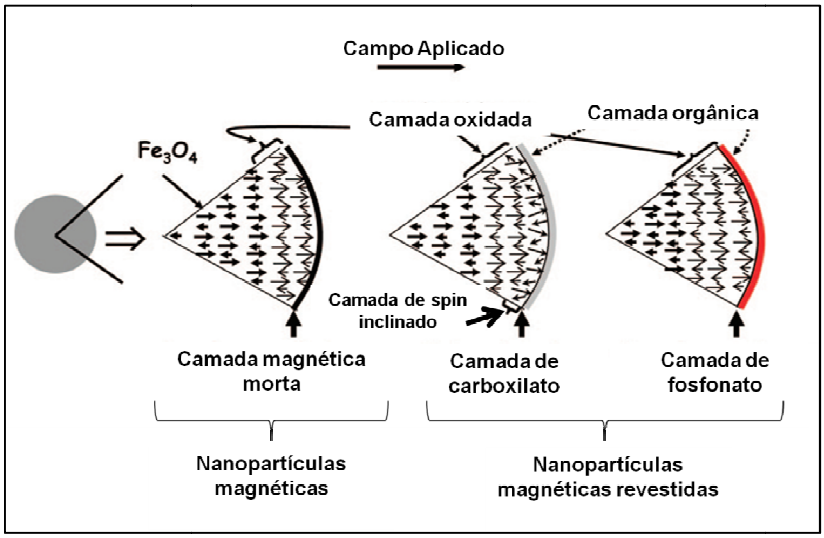


Figura 7. Representação esquemática da superfície de nanopartículas magnéticas (Fe_3O_4) com e sem revestimento orgânico na presença de um campo magnético aplicado. As setas representam o momento ferrimagnético resultante. *Fonte: DAOU et al., 2008.*

A área de superfície, o tamanho e a forma das nanopartículas são responsáveis pelas propriedades físicas e químicas dessas partículas em grande extensão, que por sua vez são responáveis pelo desempenho em várias aplicações (TARTAJ et al., 2003). O tamanho das partículas, e sua distribuição, as propriedades magnéticas e de fluxo do fluido influenciam a aplicação dos parâmetros especialmente em biomedicina. A forma esférica e monodispersibilidade de nanopartículas de óxido de ferro são frequentemente requeridas para aplicação em tecidos vivos

(PETRI-FINK e HOFMANN, 2007). Então, a otimização da síntese de nanopartículas e sua conjugação com moléculas orgânicas sobre a superfície torna-se muito mais essencial.

Nanopartículas magnéticas sem revestimento tem superfícies hidrofóbicas com uma grande área de superfície em relação ao volume e uma tendência a aglomerar (LU et al., 2007). O uso de um revestimento de superfície adequado permite que nanopartículas de óxido de ferro sejam dispersas dentro de fluidos homogêneos e melhorem sua estabilidade. Vários grupos de materiais de revestimento são usados para modificar a superfície química de nanopartículas magnéticas (SHUBAYEV et al., 2009):

a. Polímeros orgânicos, tais como dextrana, quitosana, polianilina
b. Surfactantes orgânicos, tais como oleato de sódio e dodecilamina
c. Metais inorgânicos, tais como ouro
d. Óxidos inorgânicos, tais como sílica e carbono
e. Moléculas e estruturas bioativas, tais como lipossomas, peptídeos e ligantes/receptores.

2.4.1. Polissacarídeos como revestimento: levana

A levana (**Figura 8**) é um polissacarídeo constituído por resíduos de D-frutose unidos por ligações β(2→6), podendo apresentar pontos de ramificação β(2→1) e ser produzida por diversas espécies de plantas e bactérias (KANG et al., 1998). Segundo alguns autores (VANDAMME e DERYCKE, 1983; FUCHS et al., 1985; FUCHS, 1991), as levanas de origem vegetal possuem uma glicose terminal, enquanto que as de origem bacteriana só possuem unidades de frutose (CALAZANS, 1997).

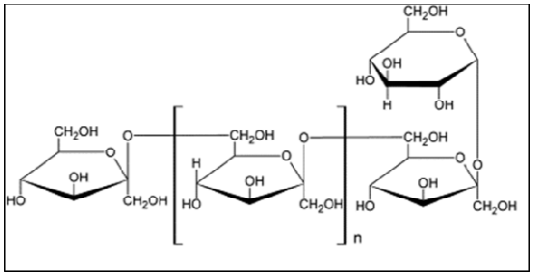


Figura 8. Estrutura química de uma levana de origem vegetal, mostrando a molécula de glicose terminal.

As levanas podem ser produzidas por diversas espécies de plantas e de bactérias, onde a distribuição do peso molecular e do grau de ramificação depende da fonte produtora deste biopolímero. Aquelas produzidas por vegetais apresentam baixo peso molecular e poucas ramificações; enquanto as produzidas por síntese microbiana ou enzimática exibem peso molecular elevado, da ordem de 2,5 x 10^6 daltons, e podem conter numerosas ramificações (BARROS, 2002; BEKERS et al., 2005). A hidrólise ácida parcial de levana produz uma série de oligossacarídeos e

levulanas (levanas degradadas) (FEINGOLD e GEHATIA, 1957). Levanas de pesos moleculares elevados são sorologicamente ativas e estimulam a formação de anticorpos; aquelas hidrolisadas ou purificadas não são antigênicas. A levana é eliminada do corpo lentamente e não é tóxica (HAN, 1990).

As levanas são um dos poucos polímeros naturais em que o carboidrato existe na forma de furanose. Esta característica estrutural tem um importante papel na conformação final das moléculas em solução (MARCHESSAULT et al., 1980). Além disso, a maior flexibilidade do anel de furanose, em comparação com o relativamente rígido anel de piranose da maioria dos polissacarídeos de reserva, confere uma flexibilidade adicional à molécula de frutana inteira.

As propriedades gerais das levanas assemelham-se aquelas das dextranas. As levanas são levorotatórias, amorfas ou microcristalinas, de solubilidade variada em água fria, muito solúvel em água quente e insolúvel em álcool etílico absoluto. As levanas são geralmente mais solúveis do que a inulina, a qual é quase insolúvel (< 0,5%) em água à temperatura ambiente (HAN, 1990). A elevada solubilidade das levanas pode ser uma característica da ligação β(2→ 6) comparada à ligação β(2→ 1). A ramificação deve ser apenas um fator secundário. As levanas são não-redutoras, não-hidrolisadas por invertase de levedura e amilase, mas são muito susceptíveis à hidrólise por ácidos. Elas não são coradas por iodo, mas cloreto de hidrogênio confere uma cor púrpura que distingue a levana de outros polissacarídeos que não contém frutose. O peso molecular e a viscosidade das levanas variam de acordo com a fonte produtora (HAN, 1990).

Certas propriedades da levana, tais como efeito antineoplásico (CALAZANS et al., 1997), aumento da permeabilidade celular para um agente citotóxico (LEIBOVICI e STARK, 1986), purificação de glicoproteínas (ANGELI et al., 2009) e imobilização de enzimas (VINA et al., 2001; MACIEL et al., 2012) têm atraído atenção. Maciel et al. (2012) estabilizaram partículas magnéticas produzidas por co-precipitação utilizando levana de *Zymomonas mobilis*, apresentando bons resultados ao imobilizarem a enzima tripsina.

2.4.2. Polímeros orgânicos sintéticos como revestimento: polianilina

Os polímeros que apresentam propriedade elétrica são chamados de “polímeros condutores” ou “metais sintéticos”, pois possuem propriedades elétricas, magnéticas e ópticas de metais e semicondutores. Entre os principais polímeros condutores, estão o poliacetileno, polianilina e polipirrol. A oxidação/redução da cadeia polimérica pode ser efetuada por agentes de transferência de carga (aceptores/doadores de elétrons), convertendo o polímero de isolante em condutor ou semicondutor. Esses agentes são chamados de dopantes. Na maioria dos polímeros condutores, como o polipirrol, o processo de dopagem ocorre simultaneamente com a oxidação da cadeia. Já a polianilina e seus derivados formam outra classe de polímeros condutores, pois ela pode ser dopada

por protonação, isto é, sem que ocorra alteração do número de elétrons associados à cadeia polimérica (FAEZ et al., 2000).

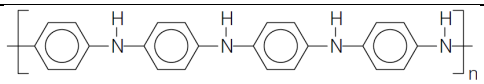
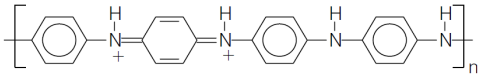
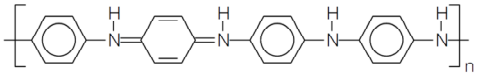
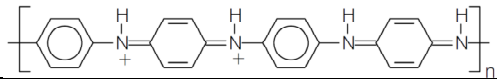
Os polímeros condutores são materiais extremamente versáteis e estão sendo amplamente utilizados em diversas aplicações, tais como tintas anticorrosivas, dispositivos eletrocrômicos e dispositivos emissores de luz (LEDs) (HEEGER, 2002). Como citado em Gizdavic-Nikolaidis et al. (2004), estes polímeros são utilizados no desenvolvimento de músculos artificiais, na liberação controlada de drogas e como estímulo para a regeneração de nervos. Considerando-se as aplicações na área biomédica, a eficiência dos dispositivos construídos a partir dos polímeros condutores é determinada através de duas características: a habilidade de o material desempenhar uma função específica e apropriada dentro do organismo e, a compatibilidade do material dentro do corpo.

Como a polianilina (PANI) pode ser utilizada em biomateriais, é necessário um melhor entendimento a respeito da toxicidade deste material. O monômero (anilina) e os subprodutos da síntese (compostos aromáticos, a maioria aminas) são altamente tóxicos (SOUZA et al., 2003). No entanto, estudos *in vivo* mostraram que ambas as formas de polianilina, condutora (sal de esmeraldina) e não-condutora (base esmeraldina), não provocaram respostas inflamatórias em modelos animais (roedores), sugerindo uma boa tolerância e bio/histocompatibilidade (WANG et al., 1999; KAMALESH et al., 2000). Yslas et al. (2012) verificaram que nanofibras de polianilina não exibiram qualquer efeito tóxico (morte ou teratogênese) em embriões de anfíbios. Resultados semelhantes foram observados por Villalba et al. (2012) ao realizarem estudos utilizando células embrionárias humanas (rim). Por outro lado, Oh et al. (2011) reportaram o efeito tóxico de nanomaterias de polianilina em células humanas (fibroblastos) e que essa toxicidade foi dependente da forma.

A polianilina (PANI) é um dos polímeros condutores mais estudados devido a suas propriedades eletrônica e óptica, excelente estabilidade ambiental (JARAMILLO-TABARES et al., 2012), fácil polimerização por síntese química (NERI et al., 2011), eletroquímica (TAKASHIMA et al., 1995), fotoquímica (ARAÚJO et al., 2010) e mais recentemente através de condições extremas ou não clássicas (AZEVEDO et al., 2008; BARROS e AZEVEDO, 2008) e baixo custo do monômero (UMARE et al., 2010). A PANI pode ocorrer em diferentes estados de oxidação (**Tabela 2**), dos quais a forma sal de esmeraldina, 50% oxidada, é a mais estável. A forma base esmeraldina (isolante) do polímero pode reagir com ácidos (ex: HCl) resultando na forma sal de esmeraldina (condutora). A reação de protonação ocorre principalmente nos nitrogênios imínicos da polianilina (–N=). Além da elevada condutividade elétrica, outra propriedade interessante da polianilina é exibir diferentes colorações quando se variam as condições de pH ou o potencial elétrico (FAEZ et al., 2000).

Devido as suas diferentes propriedades físico-químicas e a presença de grupos químicos funcionalizáveis, a PANI é utilizada como revestimento de partículas magnéticas. Um dos objetivos é unir as propriedades elétricas do polímero e a propriedade magnética da partícula magnética. A maioria dos trabalhos consiste no revestimento de nanopartículas de magnetita (APHESTEGUY e JACOBO, 2007; XIAO et al., 2007; ZHANG et al., 2010; GANDHI et al., 2011; SUN et al., 2011; BELAABED et al., 2012).

Tabela 2. Os três estados de oxidação mais importantes da polianilina: leucoesmeraldina, esmeraldina (isolante e condutora) e pernigranilina. *Fonte: FAEZ et al., 2000*

Estado de oxidação	Estrutura	Cor*	Característica
Leucoesmeraldina		Amarela 310	Isolante completamente reduzida
Sal de esmeraldina		Verde 320, 420, 800	Condutora parcialmente oxidada
Base esmeraldina		Azul 320, 620	Isolante parcialmente oxidada
Pernigranilina		Púrpura 320, 530	Isolante completamente oxidada

*Os valores numéricos referem-se ao comprimento de onda (em nanometros) onde a absorção é máxima

2.5. Aplicações de partículas magnéticas na área biomédica e biotecnológica

Em biomedicina, o revestimento polimérico garante a estabilidade das partículas magnéticas, em meio fisiológico, fornecendo não-toxicidade por evitar a ligação do ferro e permite a modificação química para ligar componentes biologicamente ativos. Não somente as características da superfície, mas primariamente o tamanho das partículas, são fatores cruciais que determinam o sucesso das partículas *in vivo* (HORÁK et al., 2007).

Em biotecnologia, essas partículas magnéticas têm encontrado aplicações em diagnósticos médicos como pesquisa genética (SCHERER et al., 2002) e tecnologias baseadas em separação magnética imune de células, proteínas, DNA/RNA, bactérias, vírus e outras biomoléculas, como adsorventes de bioafinidade, tratamento de água poluída via adsorção eletrostática, suportes para imobilização ou adsorção de enzimas e proteínas (HALLING e DUNNILL, 1980; MOFFAT et al., 1994; ANSELL e MOSBACH, 1998; DUNNILL e LILLY, 2000).

2.5.1. Imagem por ressonância magnética (IRM)

A imagem por ressonância magnética é baseada em sinal de ressonância magnética nuclear de prótons através do efeito combinado de um forte campo magnético e um campo de radiofreqüência (PANKHURST et al., 2003). As partículas magnéticas melhoram o contraste das imagens obtidas por ressonância magnética (WEISSLEDER et al., 1991; WEISSLEDER et al., 1992; KRESSE et al., 1998; BACHGANSMO, 1993).

Os agentes de contraste, constituídos por nanopartículas magnéticas, podem ser administrados por diferentes vias (aérea, oral, intersticial ou intravenosa) (SAIYED et al., 2003). Em comparação aos íons paramagnéticos, partículas de óxido de ferro superparamagnéticas têm relaxitividades maiores (SAINI et al., 1987; STARK et al., 1988).

Os ferrofluidos, usados como contraste para IRM, são formados por magnetita e estabilizados mais comumente por um polissacarídeo (ex: dextrana). O diâmetro médio desses agentes varia de 10 nm a 1 μm (WEISSLEDER et al., 1991; KRESSE et al., 1998; WEISSLEDE et al., 1992).

2.5.2. Separação magnética

As técnicas de separação magnética possibilitam a separação de compostos alvo biologicamente ativos e células de uma variedade de materiais (extratos brutos, meios de cultivo, sangue, fluidos corporais, amostras ambientais, etc.) (ŠAFAŘÍK, e ŠAFAŘÍKOVÁ, 1999; ŠAFAŘÍK e ŠAFAŘÍKOVÁ, 2000). Várias partículas magnéticas têm sido desenvolvidas como carreadores magnéticos em processos de separação incluindo purificação e imunoensaios (HUBER, 2005). Adsorventes de afinidade têm sido usados para a separação de proteínas (enzimas, anticorpos, antígenos, receptores, etc.), ácidos nucléicos (DNA, RNA, etc.), compostos biologicamente ativos de baixo peso molecular (drogas) e xenobióticos (corantes solúveis em água, metais pesados, radionuclídeos, etc.) (ŠAFAŘÍK e ŠAFAŘÍKOVÁ, 2000). O uso de colunas cromatográficas convencionais pode consumir muito tempo e é neste campo que o uso de adsorventes magnéticos ou magnetizáveis ganha importância.

2.5.3. Imobilização de enzimas

A suscetibilidade das enzimas à ação de fatores externos e às dificuldades de regeneração catalítica limita a aplicação das mesmas em larga escala. Alguns métodos de estabilização são comumente empregados para preservar a atividade da enzima, incluindo técnicas de imobilização física e/ou química, que abrem possibilidades para aplicações industriais. Entre estes, a ligação covalente de enzimas em matrizes insolúveis em água é o método mais atrativo para estabilização, reutilização e recuperação de enzimas (ENGASSER e COULET, 1977; MARTINEK et al., 1977; BRYJAK e KOLARZ, 1998; XI et al., 2005). As enzimas imobilizadas podem substituir as enzimas

nativas em vários processos, resultando em reações mais rápidas, menor contaminação da amostra e melhorando o interfaciamento para procedimentos modernos, como espectrometria de massa (KROGH et al., 1999).

A escolha da matriz (propriedades químicas e magnéticas, tamanho da partícula e distribuição, porosidade) é um fator chave que influencia na qualidade da imobilização e na conquista das aplicações finais (BÍLKOVÁ et al., 2002). Matrizes (suportes) convencionais podem ser magnetizadas pelo tratamento com materiais magnéticos sem qualquer perda de atividade da ligação subsequente. Uma variedade de materiais magnéticos (**Figura 9**) tem sido usada para produzir partículas magnéticas (AMARAL et al., 2006; ANGELI et al., 2009; NERI et al., 2009; NERI et al, 2011; MACIEL et al., 2012).

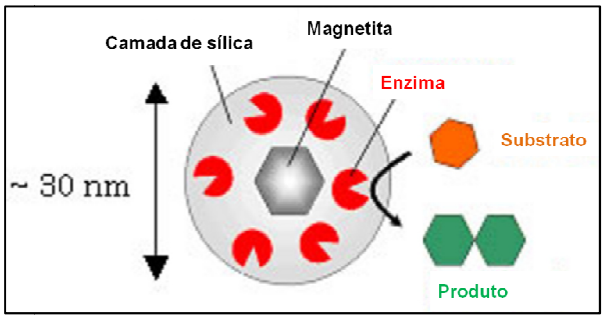


Figura 9. Desenho esquemático de um "nanobead" magnético que aprisiona moléculas de enzima em sua camada de sílica. *Reproduzido de http://seeds.kyushu-u.ac.jp/en/seed/d382.html*

2.5.4. Hipertermia magnética

A técnica de hipertermia magnética se baseia na teoria que qualquer objeto metálico, quando colocado em um campo magnético alternado, tem induzida uma corrente elétrica. A quantidade de corrente é proporcional à intensidade do campo magnético e ao tamanho do objeto. Como essas correntes fluem dentro do metal, o metal resiste ao fluxo da corrente e assim se aquece um processo denominado de aquecimento indutivo, se o metal é magnético, como o ferro, o fenômeno é muito ampliado. Portanto, quando um fluido magnético é exposto a um campo magnético alternado, as partículas tornam-se poderosas fontes de calor (**Figura 10**), destruindo as células tumorais (BABINCOVA et al., 2000).

Essa técnica destrói especificamente tumores sem danificar tecidos normais pelo aquecimento seletivo das células tumorais. Portanto, a hipertermia magnética é uma terapia promissora contra o câncer (HAYASHI et al., 2009). Um pré-requisito importante para a utilização clínica da hipertermia magnética é que as nanopartículas devem apresentar baixos níveis de toxicidade, assim como um elevado momento de saturação magnética que permita minimizar as doses eletromagnéticas requeridas quando a radioterapia é utilizada (CASTRO et al., 2010).

2.5.5. Carreador de fármacos

A tecnologia de liberação controlada caracteriza o sistema capaz de prover algum controle terapêutico, seja de natureza temporal, espacial ou ambos. Os sistemas de liberação de drogas ("drug delivery systems") oferecem inúmeras vantagens quando comparados a outros de dosagem convencional, tais como: maior eficácia terapêutica, liberação progressiva e controlada do fármaco, diminuição significativa da toxicidade e maior tempo de permanência na circulação, diminuição do número de doses devido à liberação progressiva e possibilidade de direcionamento a alvos específicos são alguns exemplos (DURÁN e AZEVEDO, 2003).

Muitos sistemas nanoparticulados estão sendo usados atualmente e, entre eles, os baseados nas nanopartículas magnéticas assumem um papel importante devido à propriedade de serem conduzidas e retidas em uma região específica do corpo por meio de gradiente de campo magnético externo. Com o objetivo de aumentar a especificidade, o conjugado nanopartícula magnética-droga pode ser associado à outra molécula capaz de reconhecer e se ligar especificamente ao sítio alvo. Tais moléculas podem ser anticorpos, proteínas, lectinas, hormônios, entre outros (**Figura 10**).

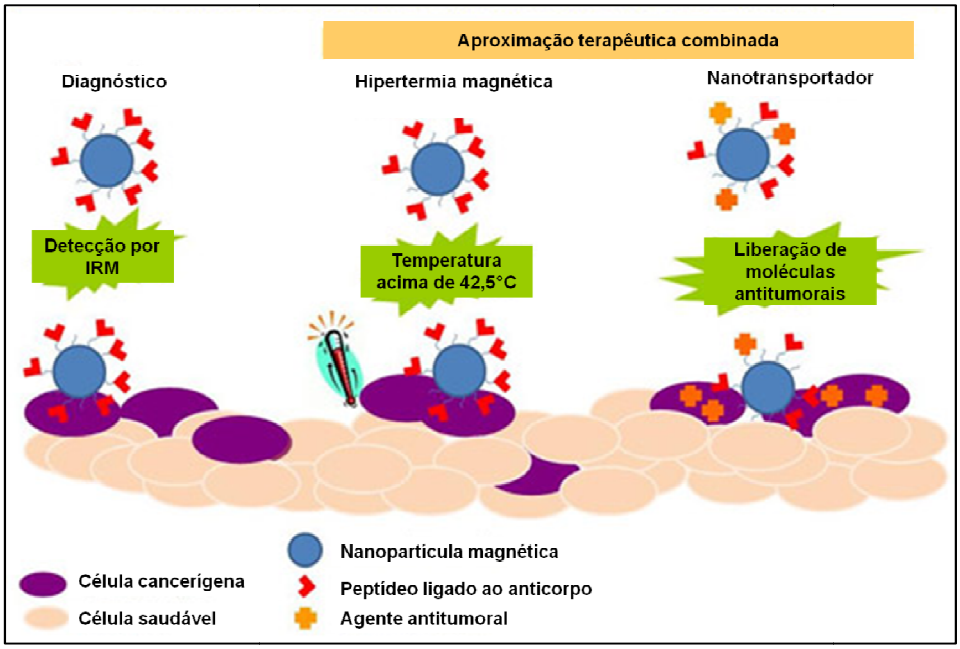


Figura 10. Aplicações médicas de nanopartículas magnéticas. *Reproduzido de http://www.madrimasd.org/*

2.5.6. *Chips* de células

Os sensores de células são úteis para estudar os efeitos de drogas e de interações entre os estímulos externos e as células (CHOI et al., 2005). A imobilização de células *in vitro* constitui um importante processo na fabricação de "chips de células", e a interação entre as células e a adesão dessas células à superfície do chip constitui uma carcaterística importante para a imobilização

celular sem perda de viabilidade. Os fatores que têm um papel essencial nos mecanismos de adesão celular são: a composição química da superfície e a morfologia do suporte e as interações deste com proteínas (LAKARD et al., 2004). Os polímeros condutores são amplamente utilizados no desenvolvimento de tecnologias de biosensores, sendo as enzimas, os anticorpos e as células os principais elementos de bioreconhecimento dentro desses polímeros condutores (MORRIN et al., 2005). Entre os polímeros condutores, a PANI é um dos mais empregados, em especial devido ao fácil processo de síntese, ao baixo custo e à estabilidade ambiental (CAMALET et al., 1998). El-Said et al. (2009) imobilizaram células HeLa em filme de PANI depositado em eletrodo de índio-estanho. Esses autores verificaram a viabilidade dessas células para diferentes concentrações de drogas anticâncer.

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Capítulo 2

4. Artigo submetido ao periódico *Analytica Chimica Acta*

Título: Magnetic particles coated with polyaniline: effect of the polymerization conditions on the activity of immobilized trypsin for protein digestion

Autores: Jackeline C. Maciel, Mariana Cabrera, David F.M. Neri, Maria da Paz C. Silva, Luiz B. Carvalho Jr.

Magnetic particles coated wtih polyaniline: effect of the polymerization conditions on the activity of immobilized trypsin for protein digestion

Jackeline C. Maciel[a], Mariana Cabrera[a], David F.M. Neri[b], Maria da Paz C. Silva[a], Luiz B. Carvalho Jr.[a]

[a]Departamento de Bioquímica and Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Cidade Universitária, Recife-PE CEP 50670-901, Brazil

[b]Universidade Federal do Vale do São Francisco, Centro, Petrolina-PE CEP 56304-205, Brazil

***Corresponding author:**

Luiz Bezerra de Carvalho Júnior

Laboratório de Imunopatologia Keizo Asami (LIKA)

Universidade Federal de Pernambuco

Cidade Universitária, Recife – PE CEP 50670–901, Brazil

Telephone number: + 55-81-21268485/ Fax: +55-81-21268485

E-mail address: lbcj@hotlink.com.br

Abstract

The main objective of this work was to produce an enzymatic derivative with higher retention activity to be used the protein digestion. The proteolytic enzyme trypsin was immobilized on composite of magnetic nanoparticles-polyaniline and the influence of coating on residual specific activity of the enzyme was studied using two 2^3 complete factorial designs. The 1st factorial design results showed that the best conditions for the $KMnO_4$ concentration, aniline concentration and polymerization time were 0.1 M; 0.5 M and 1 h, respectively, with a maximum residual specific activity of 70% of the initial activity of the free enzyme solution. In the sequence, the best result in the 2nd factorial design for the contact time of $KMnO_4$, polymerization temperature and offered protein were 1 h; 4°C and 50 μg mL^{-1}, respectively, with a maximum residual specific activity of 79%. Under the optimized conditions, the immobilized trypsin exhibited higher thermal stability (35°C–65°C); higher apparent affinity for the substrate (K_{mapp} was 1.4-fold lower than that of the soluble enzyme) and after 48 days of storage at 4°C retained 89% of its initial activity. The feasibility and performance of this enzymatic derivative offers a wide range of applications in the biomedical sciences including proteome research due the simple method and low cost of production of the enzymatic derivative.

Keywords: magnetic composite, magnetic nanoparticles, PANI, enzyme

1. Introduction

Immobilization of biomolecules onto insoluble supports is an important tool for the fabrication of a diverse range of functional materials or devices [1]. It provides many distinct advantages including enhanced stability, easy separation from reaction mixture, possible modulation of the catalytic properties, and easier prevention of microbial growth [2]. Numerous approaches had been taken to immobilize bioactive molecules onto solid substrates including adsorption, covalent coupling and tethering via an intermediate linker molecule [3].

The typical protocol for protein digestion is accomplished by enzymatic hydrolysis [4]. However, the traditional digestion (in solution) has drawbacks, because often produces autoproteolysis of the enzyme, leading to non-substrate related background peaks [5]. Different types of enzymes can be used in this step, depending of the purpose of the analysis [6]. Trypsin is by far the most common enzyme used, this enzyme cleaves peptide bonds predominantly at the carboxyl side of amino acids lysine and arginine [7].

Magnetite (Fe_3O_4) or maghemite (γ- Fe_2O_3) are usually used as magnetic materials [8], especially as supports for immobilization. The magnetite particles are preferred because of their greater saturation magnetization [9]. These magnetite beads are generally of core-shell type: the biological species cells, nucleic acids, and proteins are connected to the magnetite core through an organic or polymeric shell [10].

In our lab a composite has been proposed including polyaniline (PANI) to the magnetite [11,12]. PANI is one of the most investigated conducting polymers. It has been studied frequently because of its low cost, its ease in preparation, good thermal and environmental stabilities, its versatile applications [13–16], its diverse structures, good solution processability and high conductivity [17].

For the optimization of process, factorial designs are widely used to investigate the effects of experimental factors and the interactions between those factors, that is, how the effect of one factor varies with the level of the other factors in a response [18]. The advantages of factorial experiments include the relatively low cost, a reduced number of experiments, and increased possibilities to evaluate interactions among the variables [19].

In this work, were optimized the aniline polymerization conditions. Two factorial designs were used to investigate the effects of experimental factors and the interactions between those factors. The properties of immobilized trypsin were characterized and compared with those of the soluble trypsin. Finally, the applicability of this enzymatic derivative shows advantages due to its low cost and simple method, specially.

2. Materials and methods

2.1. Materials

Trypsin (E.C.3.4.21.4) from porcine pancreas, *N*-benzoyl-D-L-arginine-*p*-nitroanilide (BAPNA), aniline (ACS reagent), potassium permanganate (ACS reagent) and glutaraldehyde were all products from Sigma-Aldrich (USA). Dimethylsulfoxide (DMSO) and sodium hydroxide were from Vetec Chemical (Brazil). Ferric chloride hexahydrate and ferrous chloride tetrahydrate were from Merck (Germany). All other reagents were of analytical grade.

2.2. Magnetic particles preparation

Solutions of 1.1 M $FeCl_3 .6H_2O$ (5 mL) and of 0.6 M $FeCl_2 .4H_2O$ (5 mL) were added to 50 mL of distilled water, under magnetic agitation, then 5.0 M NaOH was added dropwise up to pH 10 when black particles precipitate were being produced. The mixture was heated at 50°C for 30 min with vigorous stirring. The magnetic particles were thoroughly washed with distilled water until pH was neutral. The material was dried up and kept at room temperature.

2.3. Polymerization of aniline

The oxidative polymerization of aniline was carried out in the presence of magnetic particles (0.01 g) by treating them with $KMnO_4$ solution at 25°C and washing with distilled water (in the 1st design, contact time of $KMnO_4$ with magnetic particles was overnight; whereas in the 2nd design it was in agreement with the Table 3). Then magnetic-$KMnO_4$ particles were immersed into aniline solution in 1.0 M HNO_3. After polymerization, the magnetic nanoparticles-polyaniline (mPANI) were successively washed with distilled water, 0.1 M citric acid and rewashed with distilled water, and finally the material was dried up and kept at 25°C.

2.4. Immobilization of trypsin

mPANI (0.01g) was incubated with 2.0% w/v glutaraldehyde (1.0 mL) at 25°C for 2 h under mild stirring, washed with distilled water and Tris-HCl buffer to remove the excess of glutaraldehyde. mPANI activated with glutaraldehyde (mPANIG) was kept in buffer at 4°C until use. mPANIG was incubated with trypsin solution ranging from 50–200 $\mu g\ mL^{-1}$ (Table 3) for 16 h at 4°C under mild stirring. The enzymatic derivative (mPANIG-Trypsin) was collected by a magnetic field (Ciba Corning; 0.6 T) and the supernatant and washings were used for protein determination.

2.5. Activity assay

Amidase activity of the trypsin was measured by using the artificial substrate BAPNA (4.0 mM BAPNA in DMSO). The mixture of reaction was incubated at 25°C for 15 min. The hydrolyzed p-nitroanilide was monitored in spectrophotometer at 440 nm and the activity of enzyme was calculated. All assays were carried out in replicates and the results are expressed as mean ± standard deviation. One unit of proteolytic activity of trypsin was defined as 1μmol of BAPNA hydrolyzed per minute using an absorption coefficient of 9.100 M^{-1} cm^{-1}. The specific activity was calculated by dividing the enzymatic activity (U) by the amount of protein (mg). For the residual specific activity was considered the specific activity of the free enzyme as 100%.

2.6. Protein determination

The retained protein was estimated by the difference between the offered protein and that found in the supernatant and washing solutions measured according to the method of Lowry et al. [20] using bovine serum albumin (BSA) as a standard.

2.7. Experimental design

According to literature data, the synthesis of PANI is influenced for experimental factors, such as aniline concentration and polymerization temperature for example. Therefore based in an experimental protocol routinely in our laboratory [11,12] were carried out two 2^3 complete factorial designs in this study, requiring 10 experiments for 1st factorial design and 20 experiments for 2nd factorial design. The central point was run in replicates to provide an estimate of the pure error variance in the experimental responses. Experimental errors of the effects were estimated and used to assess the significance of the effects and interactions of the independent variables. The variables and their levels selected for study were: $KMnO_4$ concentration (0.1–0.3 M), aniline concentration (0.5–1.0 M), polymerization time (1–3 h), contact time of $KMnO_4$ (1–5 h), polymerization temperature (4–50ºC), and offered protein amount (50–200 μg mL^{-1}). Table 1 and 3 show the independent factors, levels and experimental design. The results were statistically analyzed by variance analysis (ANOVA) at a significance level of $p \leq 0.05$. All statistical and graphical analyses were carried out with the "Statistical 8.0" software (StatSoft, Inc., 2004, USA).

2.8. Characterization of mPANIG-Trypsin

2.8.1. Thermal stability

The thermal stability was evaluated by incubating samples of soluble trypsin and mPANIG-Trypsin in Tris-HCl buffer for 30 min at temperatures ranging from 25°C to 65°C. The heated samples were

kept at 25°C for temperature equilibration and their activities measured. All activity assays were carried out as described in the item 2.5.

2.8.2. Kinetic constants

The K_m for soluble trypsin and mPANIG-Trypsin was calculated from initial rate data using BAPNA as substrate (0.15–3.75 mM). The Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) of the soluble trypsin and mPANIG-Trypsin were determined mathematically using the Michaelis-Menten equation.

2.8.3. Stability during storage

The mPANIG-Trypsin was stored in Tris-HCl buffer at 4°C. The remaining activity of immobilized trypsin was evaluated during 48 days, by measurements of its activity, followed by washing with Tris-HCl buffer and storage again in the buffer at 4°C.

2.9. Protein digestion

mPANIG-Trypsin was used for digestion of bovine serum albumin (BSA). The stock solution of BSA (2.0 μg mL^{-1}) was prepared in Tris-HCl buffer. Before tryptic digestion, it was denatured in a 95°C water bath for 5 min. Then, 100 μL of stock solution and 800 μL of Tris-HCl buffer were transferred into an tube containig mPANIG-Trypsin (10 μg of protein/mg of mPANI). After the mixture solution was incubated in 37°C water bath for 15 min, mPANIG-Trypsin was separated by an external field. The supernatant was transferred to an Eppendorf tube and lyophilized. For comparison, 0.2 mg mL^{-1} BSA was digested by conventional in-solution proteolysis in Tris-HCl buffer for 12 h at 37°C (trypsin/substrate ratio, 1:40 w/w).

2.10. MALDI-TOF MS procedure

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) measurements were performed in positive ion mode using an Autoflex III proteomics analyzer (Bruker Daltonics Inc., Billerica, MA, USA). The samples were prepared using the dried-droplet method [21]. They were dissolved in 100 μL of 0.1% TFA. A volume of 1.0 μL of matrix solution (10 μg mL^{-1} CHCA dissolved in 50% aqueous ACN containing 0.3% TFA) was mixed to 1.0 μL of the sample and 0.5 μL of mixture was deposited on a MALDI plate. The MS instrument was operated at an accelerating voltage of 19 kV. A 100 Hz pulsed Nd:YAG laser at 355 nm was used. "FlexAnalysis" software obtained from Bruker Daltonics was used to identify proteins using the peptide fingerprint mass spectra (PMF). For PMF data, 1000 laser shots were accumulated for each

spectrum. Data obtained were searched using MASCOT (Matrix Sciences, London, UK) as the search engine.

3. Results and discussion

3.1. $KMnO_4$ concentration, aniline concentration and polymerization time effect in the mPANIG-Trypsin activity (1st factorial design)

The main effects represent deviations of the average between the high and low levels for each factor. When the effect of a factor is positive, the response increase as the factor changes from low to high levels, for example [22]. The results of the 1st complete factorial design are presented in the Table 1.

Table 1. Matrix of experiments and results for 1st factorial design

Run				Response
	$KMnO_4$ concentration (M)	Aniline concentration (M)	Polymerization time (h)	Residual specific activity (%)
1	0.1	0.5	1	70.05
2	0.3	0.5	1	47.09
3	0.1	1.0	1	26.37
4	0.3	1.0	1	32.86
5	0.1	0.5	3	69.49
6	0.3	0.5	3	53.46
7	0.1	1.0	3	33.04
8	0.3	1.0	3	39.98
9	0.2	0.75	2	37.09
10	0.2	0.75	2	36.75

The $KMnO_4$ concentration and aniline concentration showed significant negative effect, suggesting that the decrease of the parameter values increase the residual specific activity of mPANIG-Trypsin (Table 2). Moreover, the polymerization time factor showed significant positive effect, suggesting that the increase of the parameter values increase the residual specific activity of enzyme. Alone the aniline and $KMnO_4$ concentrations interaction between was statistically significant have positive effect (Table 2).

Table 2. Main and interaction effects for the 1st complete factorial design

Factors	Effects on residual specific activity
(*a*) $KMnO_4$ concentration	–6.39
(*b*) Aniline concentration	–26.96
(*c*) Polymerization time	4.90
*a*b*	13.10
*a*c*	1.84*
*b*c*	1.99*
*a*b*c*	–1.61*

*Not significant ($p<0.05$)

According to the results shown in Table 1, the highest residual specific activity (70.05%) corresponded to lowest levels (0.5 M aniline, 0.1 M $KMnO_4$ and polymerization time of 1 h). These results are similar to the conditions used in our research group [11,12], except by reduction in the polymerization time of 2 h to 1 h, thus an advantage in this study.

As shown in Pareto chart (Figure 1), the main factors ($KMnO_4$ concentration, aniline concentration and polymerization time) and their interactions that extend beyond the reference line were significant at the level of the 0.05. These results can be corroborated by data showed in the Table 2. The aniline concentration represented the main effect most significant on residual specific activity of mPANIG-Trypsin, ie, it was what exerted a greater influence in the response. This 1st design allowed obtaining an enzymatic derivative with 70.05% residual specific activity.

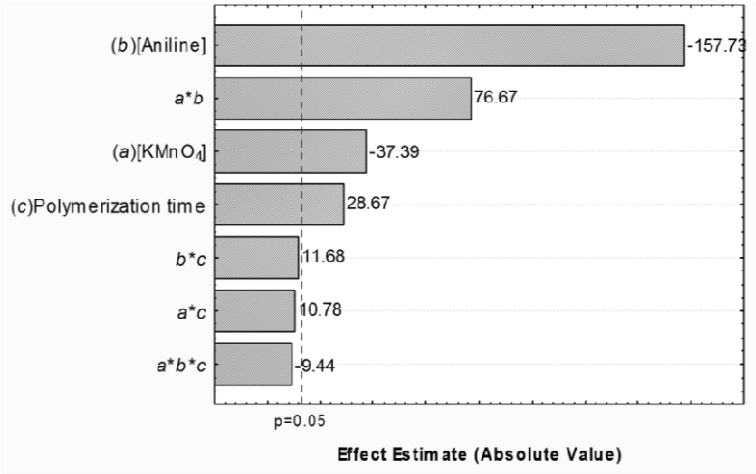

Figure 1. Pareto chart for the effects of the variables $KMnO_4$ concentration (*a*), aniline concentration (*b*) and polymerization time (*c*) on residual specific activity of mPANIG-Trypsin from the 1st factorial design.

3.2. Contact time of $KMnO_4$, polymerization temperature and offered protein effects in the mPANIG-Trypsin activity (2nd factorial design)

A 2nd complete factorial design was carried out to produce an enzymatic derivative with greater activity than that observed in the 1st design done in this work. The results of this 2nd design can be seen in the Table 3.

It is possible to see that the levels inferiors are the best conditions observed in this design also (76.01%). According to data from Table 3, when the contact time of $KMnO_4$ was elevated from 1 to 5 h, maintaining the polymerization temperature at 4°C and 50 μg mL^{-1} offered protein, the activity decreased about 15%. This result shows the advantage of the optimization of original protocol, that uses a contact time of $KMnO_4$ overnight [11,12].

Table 3. Matrix of experiments and results for 2nd factorial design

Run				Response
	Time of $KMnO_4$ (h)	Polymerization temperature (°C)	Offered protein amount (μg mL^{-1})	Residual specifica ctivity (%)
1 (1)	1	4	50	72.70
2 (1)	5	4	50	60.45
3 (1)	1	50	50	34.85
4 (1)	5	50	50	32.38
5 (1)	1	4	200	37.01
6 (1)	5	4	200	41.61
7 (1)	1	50	200	31.51
8 (1)	5	50	200	30.74
9 (1)	3	27	125	44.41
10 (1)	3	27	125	43.80
11 (2)	1	4	50	79.05
12 (2)	5	4	50	60.87
13 (2)	1	50	50	30.07
14 (2)	5	50	50	30.63
15 (2)	1	4	200	38.32
16 (2)	5	4	200	36.70
17 (2)	1	50	200	33.35
18 (2)	5	50	200	31.93
19 (2)	3	27	125	45.33
20 (2)	3	27	125	39.69

The Pareto chart represents the estimated effects of the variables, and their interactions on residual specific activity in decreasing order of magnitude. It can be seen in Figure 2 that all main effects (polymerization temperature, contact time of $KMnO_4$, offered protein) are significant at a confidence level of 95%. Thus the values of the effects indicate that, on average, higher activities were obtained when factors contact time of $KMnO_4$, polymerization temperature and offered protein were at their lowest levels.

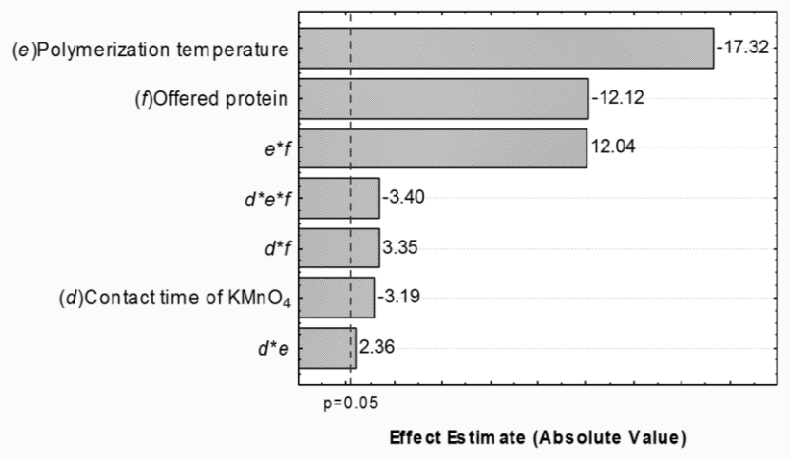

Figure 2. Pareto chart for the effects of the variables contact time of $KMnO_4$ (*d*), polymerization temperature (*e*) and offered protein (*f*) on residual specific activity of mPANIG-Trypsin from the 2nd factorial design.

Raising the polymerization temperature from 4 to 50°C with contact time of $KMnO_4$ of 1 h and 50 μg mL^{-1} offered protein, the activity was reduced about 44%. The polymerization of aniline showed best results when carried out at 4°C than at higher temperatures as in the original protocol [11,12]. Increasing offered protein from 50 to 200 μg mL^{-1} with contact time of $KMnO_4$ of 1 h and polymerization temperature of 4°C, the activity decreased about 38%. Among these, the polymerization temperature represented the main effect most significant on residual specific activity of mPANIG-Trypsin, it is corroborated by data showed in the Table 4. The results observed in these experiments show the influence of the conditions of coating on the catalytic performance of the mPANIG-Trypsin. In addition, the advantages of using a factorial design to obtain an enzymatic derivative with good retention of the activity when compared to the enzyme in its soluble form.

Table 4. Main and interaction effects for the 2nd complete factorial design

Factors	Effects on residual specific activity
(*d*) Contact time of $KMnO_4$	–3.94
(*e*) Polymerization temperature	–21.41
(*f*) Offered protein amount	–14.98
*d*e*	2.92
*d*f*	4.14
*e*f*	14.88
*d*e*f*	–4.21

*Not significant ($p<0.05$)

3.3. mPANIG-Trypsin characterization

3.3.1. Thermal stability

The second goal this study was the mPANIG-Trypsin characterization with respect to temperature, substrate concentration and stored stability. The presence of the coating has an important role in the properties of the immobilized enzyme, in particular their catalytic performance as observed by Maciel et al. [23]. Activity of retention on the support is one of the most important aspects in the enzyme immobilization process.

Fig. 3 shows the higher thermal stability of the immobilized trypsin at temperatures higher than 35°C. This results were similar to those obtained by Bryjak and Kolarz [24], using trypsin immobilized on acrylic carrier.

The link of a molecule of an enzyme to rigid support by several strong chemical bonds makes the structure of the protein molecule much more rigid, and hence unfolding and inactivation (for example, on heating) of such immobilized enzyme will be much more difficult occur than that of the starting, native enzyme [24]. At 45°C, the activity of mPANIG-Trypsin was 77%, compared to

soluble trypsin which was 44%. An increase in thermal stability was observed when trypsin was immobilized on vermiculite [26], on carboxymethylcellulose [27] and ferromagnetic Dacron [28]. These results suggest that the immobilized enzyme was more stable compared to the soluble enzyme.

Thermal deactivation of soluble enzyme is caused by disruption of weak intra-molecular forces and subsequent unfolding of the protein chain. Immobilization of trypsin on composite of magnetic nanoparticles-polyaniline increased the thermal stability by stabilizing the weak ionic forces and hydrogen bonds, there by increasing the range of operating temperatures. Increase in thermal stability of the mPANIG-Trypsin was also due to multipoint attachment of the enzyme to PANI via glutaraldehyde and prevention of autolysis.

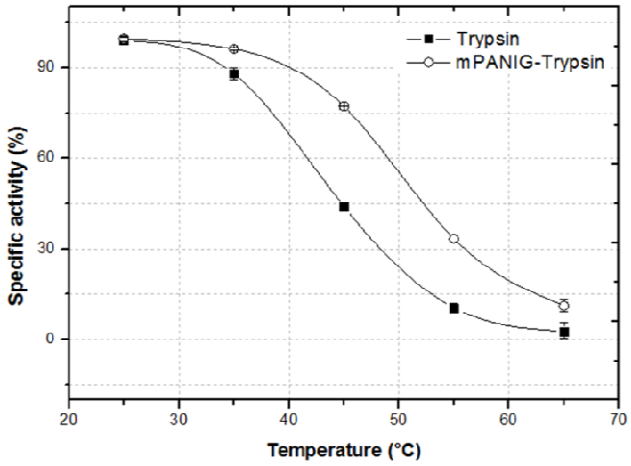

Figure 3. Thermal stability of the mPANIG-Trypsin (B). The obtained activities were referred to the highest activity (100%).

3.3.2. Kinetic constants

The affinity of the enzyme for substrate is characterized by the constant of Michaelis–Menten (K_m). K_m for soluble trypsin and mPANIG-Trypsin were determined using BAPNA. In comparison with K_m of soluble trypsin (2.96 ± 0.23 mM) K_m of mPANIG-Trypsin has decreased to the value of 2.13 ± 0.19 mM. In this amidolytic reaction catalyzed by immobilized trypsin, the value of saturation constant K_m was 1.4-fold lower than that of the soluble enzyme, a clear sign of higher "apparent" affinity of BAPNA to the bound enzyme. This indicates an alteration in the affinity of the enzyme towards to the substrate upon covalent immobilization on mPANIG. Amaral et al [28], Kumar and Gupta [29] and, Korecká et al. [30] reported similar results. The ratio K_{cat}/K_m defines a measure of the catalytic efficiency of an enzyme-substrate pair. In this study, the catalytic efficiencies (K_{cat}/K_m) of the soluble trypsin and mPANIG-Trypsin were found to be 645 and 580, respectively. The

catalytic efficiency of trypsin was decreased about 1.1-fold upon immobilization. These data lead to the conclusion that the immobilization apparently did not introduce mass-transfer and diffusion limitations. According to Amaral et al. [28] such phenomenon could also be attributed to eventual negatively charged matrix attraction for the positively charged substrate BAPNA. The results of the catalytic efficiency were similar to those obtained by Bayramoğlu et al. [31].

3.3.3. Stability during storage

Data of operational stability showed that after 5 cycles combining storage and reuse, mPANIG-Trypsin maintained 89% of its initial activity (Fig.4). An 11% decrease in activity was detected during this period. The immobilized trypsin on polyaniline supports retained 50% its original activity after 21days when stored at 4°C in phosphate buffer [32], 58% its original activity after 49 days when stored at 4°C in glycine buffer containing $CaCl_2$ [33] and 80% its original activity after 44 days (approximately) when stored in glycine buffer containing $CaCl_2$ [34]. In our study, we observed better results. Moreover, Chellapandian [26] reported that immobilized trypsin on vermiculite retained 84% its original activity after 60 days when stored in borate buffer. The enzymatic derivative obtained this study was more stable during the period of 48 days, however its activity after 55 days was 77% of initial value. This result indicates that the mPANIG-Trypsin may be stored in buffer only (no stabilizer, e.g. $CaCl_2$) for approximately 50 days, retaining about 90% of its initial activity.

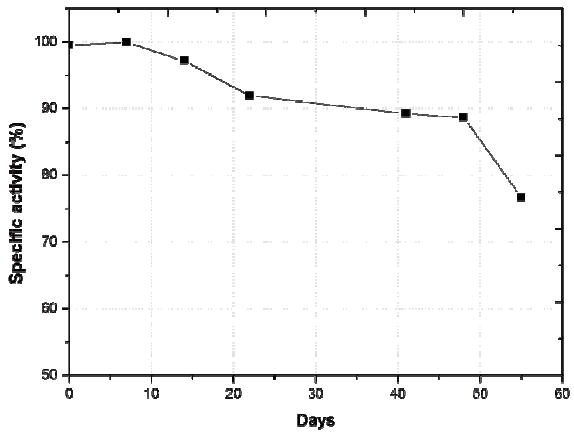

Figure 4. Shelf life of mPANIG-Trypsin at 4°C stored in Tris-HCl buffer.

3.4. Model protein substrate digestion

The main application of the mPANIG-Trypsin is residue-specific proteolysis to generate fragments for protein identification. The procedure included the digestion of BSA with mPANIG-Trypsin in

15 min digestion. The resulting fragments were determined using MALDI-TOF MS and the analyses of these MS spectra were made using a Mascot database search as showed in the Table 5.

Table 5. Identified peptide residues obtained from 15 min digestion using mPANIG-Trypsin

Peptide sequence	MW	Missed cleavages	Position
BSA, 28% sequence coverage			
DTHKSEIAHR	1193.66	1	25–34
SEIAHR	712.37	0	29–34
FKDLGEEHFK	1249.67	1	35–44
DLGEEHFK	974.49	0	37–44
LVNELTEFAK	1163.68	0	66–75
VASLR	545.25	0	101–105
QEPER	658.29	0	118–122
KFWGK	665.36	1	156–160
FWGK	537.19	0	156–160
GACLLPK	701.33	0	198–204
GACLLPKIETMR	1347.58	1	198–209
IETMR	664.28	0	205–209
QRLR	572.23	1	219–222
CASIQK	649.31	0	223–228
FGER	508.15	0	229–232
ALKAWSVAR	1001.63	1	233–241
AWSVAR	689.37	0	236–241
RHPEYAVSVLLR	1439.88	1	360–371
HPEYAVSVLLR	1283.77	0	361–371
HLVDEPQNLIK	1305.77	0	402–412
QNCDQFEK	1011.41	0	413–420
LGEYGFQNALIVR	1479.87	0	421–433
YTRK	568.07	1	434–437
KVPQVSTPTLVEVSR	1640.01	1	437–451
VPQVSTPTLVEVSR	1511.91	0	438–451
LCVLHEK	842.56	0	483–489
RPCFSALTPDETYVPK	1823.99	0	508–523
AFDEK	609.24	0	524–528
QIKK	515.23	1	545–548
EACFAVEGPK	1050.48	0	588–597

The peptide mass fingerprinting (PMF) spectra of the in-solution tryptic digest was also measured as control (22 peptides matched, sequence coverage 30%). As can be seen in the Table 5, a total of 29 tryptic peptides from BSA were identified with the corresponding amino-acid sequence coverage of 28% for BSA using mPANIG-Trypsin. Comparing the MALDI-TOF MS results obtained from 15 min digestion using mPANIG-Trypsin and 12 h in-solution digestion, the enzyme soluble exhibited higher sequence coverage (30%). The identification results of BSA are comparable to those obtained by using in-solution digestion that required a longer reaction time. The digestion time was significantly reduced from 12 h for in-solution digestion to 15 min for the digestion based on the immobilized enzyme. The results showed that mPANIG-Trypsin offers a proteolysis similar the in-solution digestion in relation to the sequence coverage, but the types of peptides obtained by the immobilized enzyme has a different profile from that of the soluble enzyme. According to Wang

et al. [35] the high surface area-to-volume ratio of trypsin-immobilized on magnetic particles can lead to the risks of the adsorption of proteins and peptides that might be the reason for the large missing parts in the coverage (e.g. 280–347 and 489–548 for BSA). Furthermore, the protein BSA contain several disulfide bridges and therefore more rigid and difficult to digest [36].

A total of 28, 30 and 28 tryptic peptides from BSA were identified with the corresponding amino-acid sequence coverage 46%, 44% and 43% for BSA after digestion with Trypsin-immobilized PA/Fe_3O_4/CNT composite [35], Trypsin-immobilized magnetic nanoparticles on chip [37] and Trypsin-immobilized on magnetic carbonaceous microspheres [38], respectively. In this study, the result found had a sequence coverage of 28%, much smaller than those observed by Wang et al. [35], Liu et al. [37] and Yao et al. [38]. A possible explanation for this low conversion (compared to the authors cited) may be due to the immobilization trypsin on microparticles and nanoparticles not, decreasing the surface area leading to a reduction in the enzyme-interaction. Another possible explanation may be due to a reduced contact, because the enzymatic derivative is not left under stirring proteolysis.

Moreover, the digestion of specific proteins tends to be incomplete without additional pre-treatments [39]. The reduced and alkylated BSA was generally used to obtain the digestive peptides with reproducibility [40], confirming the results of Sun et al. [41] that after pre-treatment of BSA exhibited sequence coverage above 90%.

4. Conclusions

The uses of the factorial design allowed obtaining an enzymatic derivative with residual activity of 79% of the initial activity of soluble enzyme, showing the advantages offered by the use of optimized experimental conditions. In these conditions, the enzymatic derivative mPANIG-Trypsin exhibited: increased stability at high temperatures, and retention of 89% of its initial activity after storage at 4°C for 48 days. Furthermore, mPANIG-Trypsin showed higher affinity for the substrate BAPNA and a lower catalytic efficiency of 580. The excellent catalytic performance of mPANIG-Trypsin is the major benefits of this enzymatic derivative. It can be used in proteins digestion to be simple method, rapid and low cost.

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Capítulo 3

5. Artigo submetido ao periódico *Composites Science and Technology*

Título: Synthesis, characterization and magnetic property of composite of magnetic nanoparticles-polyaniline for enzyme immobilization

Autores: Jackeline C. Maciel, Willian T. Shigeyosi, Maristela Olzon-Dionysio, Cláudio A. Cardoso, David F.M. Neri, Maria da Paz C. Silva, Luiz B. Carvalho Jr.

Synthesis, characterization and magnetic property of composite of magnetic nanoparticles-polyaniline for enzyme immobilization

Jackeline C. Maciel[a], Willian T. Shigeyosi[b], Maristela Olzon-Dionysio[b], Cláudio A. Cardoso[b], David F.M. Neri[c], Maria da Paz C. Silva[a], Luiz B. Carvalho Jr.[a]

[a]Departamento de Bioquímica and Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, 50670–901 Recife, PE, Brazil

[b]Departamento de Física, Universidade Federal de São Carlos, 13565–905 São Carlos, SP, Brazil

[c]Universidade Federal do Vale do São Francisco, 56304–205 Petrolina, PE, Brazil

***Corresponding author:**

Luiz Bezerra de Carvalho Júnior

Laboratório de Imunopatologia Keizo Asami (LIKA)

Universidade Federal de Pernambuco

Cidade Universitária, Recife – PE CEP 50670–901, Brazil

Telephone number: + 55-81-21268485/ Fax: +55-81-21268485

E-mail address: lbcj@hotlink.com.br

Abstract

In this paper, we report the preparation of magnetic nanoparticles via the chemical coprecipitation method. These magnetic nanoparticles were coated with polyaniline (PANI) in optimized conditions and characterized by different techniques. The electronic micrograph analysis showed nanoparticles with about 15 nm of average diameter and apparently regular morphology. A combination of FTIR, X-ray diffraction and Mössbauer spectroscopy techniques was used to understand the chemical composition of the nanoparticles, being identified as phases magnetite and maghemite. Both nanoparticles exhibited magnetic behavior and strong magnetization at room temperature. The coated magnetic nanoparticles (mPANI) presented ability to bind to biological molecules such as trypsin, forming the magnetic enzymatic derivative mPANIG-Trypsin. The protein amount and specific activity of the immobilized trypsin were 12.84 ± 4.95 μg of protein/mg of mPANI (49.27% of immobilized protein) and 24.06 ± 0.67 U/mg of protein, respectively. After twenty reuses, the activity of mPANIG-Trypsin was 81% of initial activity. These results suggest that the use of this enzymatic derivative in different biomedical applications due to its excellent operational stability, high activity, simple method and at low cost.

Keywords: magnetic nanoparticles, PANI, trypsin, biomedical applications.

1. Introduction

Functionalization of magnetic iron oxides nanoparticles by organic molecules gives rise to intensive research for biomedical applications such as magnetic drug targeting, hyperthermia and enzyme immobilization [1]. This attention is due to their unique catalytic, magnetic and electrical properties [2]. Within this scope, magnetite (Fe_3O_4) has become an important kind of magnetic material in different applications. The main aspects that have given a boost to the study of magnetite nanoparticles are mainly their chemical functionality of the active surface, biocompatibility and low cost [3], and its good magnetic property and a very high saturation magnetization [4].

The need for combining the magnetic properties with the conductive properties leads to processing new materials such as conductive polymers with magnetic behavior, or magnetic particles containing a conductive polymer [5].

Polyaniline (PANI) receives a special attention among other conducting polymers due to the simple synthetic methodology, good environmental stability, optical activity, controllable doping [6], an easy tunability of their electronic properties and high levels of electromagnetic shielding performances at microwave frequencies with a low mass by unit of surface [4]. The electromagnetic properties of polyaniline can be modified by the addition of inorganic fillers, such as the inclusion of magnetic particles may improve the magnetic and dielectric properties of host materials [7]. The production of monodisperse nanopowders and the control of their composition and size are therefore key points to address for applications of these materials [8].

Our main objective was to produce magnetic nanoparticles coated with polyaniline (PANI) to be used as a support for immobilization of trypsin. The catalytic performance of this enzymatic derivative was evaluated by its activity (reuses). For this, magnetic nanoparticles were prepared via coprecipitating ferrous and ferric ions in aqueous solution and then coated with PANI. These magnetic nanoparticles were characterized by electronic micrographic observations, FTIR, X-ray diffraction analysis, Mössbauer spectroscopy and magnetization measurements.

2. Materials and methods

2.1. Materials

Trypsin (E.C.3.4.21.4) from porcine pancreas, bovine serum albumin (BSA), *N*-benzoyl-D-L-arginine-*p*-nitroanilide (BAPNA), aniline (ACS reagent), potassium permanganate (ACS reagent), Folin-Ciocalteu's phenol reagent and, 25 % glutaraldehyde were purchased from Sigma-Aldrich (USA). Dimethylsulfoxide (DMSO) and sodium hydroxide were from Vetec Chemical (Brazil). Ferric chloride hexahydrate and ferrous chloride tetrahydrate were from Merck (Germany). All other reagents were of analytical grade.

2.2. Magnetic particles preparation

Solutions of 1.1 M $FeCl_3$.$6H_2O$ (5 mL) and of 0.6 M $FeCl_2$.$4H_2O$ (5 mL) were added to 50 mL of distilled water, under magnetic agitation, then 5.0 M NaOH was added dropwise up to pH 10 when black particles precipitate were produced. The mixture was heated at 50°C for 30 min with vigorous stirring. The magnetic nanoparticles were thoroughly washed with distilled water until pH was neutral. The material was dried up and kept at room temperature.

2.3. Coating with PANI

Oxidative polymerization of aniline was carried out in the presence of magnetic nanoparticles (0.5 g) by treating them with 0.1 M $KMnO_4$ (50 mL) solution at 25°C for 1 h and washed with distilled water. Then magnetic-$KMnO_4$ nanoparticles were immersed into 50 mL of 0.5 M aniline solution (in 1.0 M HNO_3). Polymerization was allowed to occur at 4°C for 1 h and after that the magnetic nanoparticles coated with polyaniline (mPANI) were successively washed with distilled water, 0.1 M citric acid and rewashed with distilled water, and finally the material was dried up and kept at 25°C. This process was performed using the optimum conditions obtained after the full factorial design (results not shown).

2.4. Trypsin immobilization

mPANI (0.01g) was incubated with 2.0% w/v glutaraldehyde (1.0 mL) at 25°C for 2 h under mild stirring, washed with distilled water and Tris-HCl buffer (0.1 M, pH 8.0) to remove the excess of glutaraldehyde. mPANI activated with glutaraldehyde (mPANIG) was kept in buffer at 4°C until use. mPANIG (0.01g) was incubated with trypsin for 16 h at 4°C under mild stirring. The enzymatic derivative (mPANIG-Trypsin) was collected by a magnetic field (Ciba corning; 0.6 T) and the supernatant and washings were used for protein determination.

2.5. Activity assay

Amidase activity of trypsin was measured by using the artificial substrate BAPNA (4.0 mM BAPNA in DMSO). The mixture of reaction was incubated at 25°C for 15 min. The hydrolyzed p-nitroanilide was monitored in spectrophotometer at 440 nm and the activity of enzyme was calculated. All assays were carried out in replicates and the results are expressed as mean ± SD. Proteolytic activity of trypsin (unit) was defined as μmol BAPNA hydrolyzed per minute using an absorption coefficient of 9.100 M^{-1} cm^{-1}. The specific activity was calculated by dividing the enzymatic activity (U) by the amount of protein (mg). For the residual specific activity, was considered that the specific activity of free enzyme as 100%.

2.6. Protein determination

The amount of immobilized protein was estimated by the difference between the offered protein and that found in the supernatant and washing solutions measured according to the method Lowry et al. [9] using bovine serum albumin (BSA) as a standard.

2.7. Magnetic nanoparticles characterization

The morphology and particle size of the nanoparticles were characterized using a FEI QUANTA 200F scanning electron microscope (SEM) and a Jeol 1200EXII 80 transmission electron microscope (TEM).

The polydispersity index (PDI) was obtained in a Delsa Nano C particle analyzer (Beckman Coulter, Krefeld, North Rhine-Wetphalia, Germany). Particles were suspended in 1.0 M HCl and placed in ultrasonic for 10 min to break aggregates. For each measurent, three analyzes were considered.

The Fourier transform infrared (FTIR) spectra were obtained on an IFS–66 FTIR spectrometer (Bruker, Billerica, MA, USA) in the range of 4000-400 cm^{-1} using KBr pellets. Typically, 100 scans were recorded with a 4 cm^{-1} resolution.

The BET surface area (Brunauer-Emmett-Teller) and pore volume was measured by nitrogen adsorption isotherm using an ASAP 2420 (Micromeritics, Atlanta, GA, USA).

The structural properties of the magnetic particles were characterized by X-ray powder diffraction, which was carried out in a ray-X diffractometer Shimadzu model XDR 7000, using Cu Kα radiation ($\lambda = 0.154$ nm) at a scanning speed of 0.1° s^{-1} in the range of $2\theta = 10–90°$ with a step 0.02°.

Mössbauer data collection was carried out at room temperature 298K using a ^{57}Co (Rh-matrix) source of initially 10 mCi strength. The cobalt source is oscillated longitudinally so that its velocity varies between –10.5 and 10.5 mm s^{-1}. Mössbauer spectrometer (model MB500) was calibrated using a thin α-Fe foil. We performed a Mössbauer set using three distributions. In the first and the second were used a magnetic hyperfine field distribution (0 T to 55 T and 1 T-by-step). The third was using a quadrupole electric distribution (0 mm s^{-1} to 1.2 mm s^{-1} and 0.1 mm s^{-1} -by-step). Data analysis was performed using the computer program WinNormos for IGOR Pro 6.2® commercialized by WaveMetrics©, assuming that all the peaks are Gaussian in shape.

Magnetization measurements were obtained at 298 K and 323 K in ± 50 KOe (50 T) applied field using a SQUID magnetometer (Quantum Design Model MPMS-5S).

2.8. Reuse of the enzymatic derivate

Two series of reuse was performed. The first reuse was carried out by assaying the same mPANIG-Trypsin preparation with BAPNA ten times intercalating each successive use with buffer washing

(three times). This preparation was kept at 4°C during eight days and a second reuse was carried out as above. All of the experiments were conducted in triplicate.

3. Results and discussion

3.1. Synthesis of magnetic nanoparticles and PANI coating

Magnetic particles were prepared in accordance with Carneiro Leão et al. [10] except by modifications in the incubation time (30 min), temperature (50°C), final pH of the mixture (10) and co-precipitation agent (NaOH) in order to obtain nanoparticles. According to Sun et al. [11], the synthesis of magnetite with sizes around 10 nm can be achieved using a reaction temperature of 50°C, a solution pH of 10–11 and a high stirring rate (above 800 rpm). According to these authors, temperatures above 50°C lead to production of larger particles when the temperature increase, i.e., the growth of magnetite nucleus is easier to happen when the temperature is higher than 50°C. Regarding the pH, the reason that may explain why the size of magnetite nanoparticles is reduced with increasing pH of the solution when the pH is lower than 11 is based on the mechanism of formation of magnetite [11]. According to previous studies, Fe^{2+} adsorbs at the ferric hydroxide surface, the increase of the pH gives rise to dehydration of hydroxides and leads to the formation of magnetite facilitated by electron hopping between Fe^{2+} and Fe^{3+} [8]. NaOH was chosen as the precipitating agent instead of $NH_3 \cdot H_2O$ used by Carneiro Leão et al. [10] because according to Hong et al. [12], the diameter of magnetite nanoparticles by co-precipitation with NaOH is smaller and with minor agglomeration then using $NH_3 \cdot H_2O$ in water. The magnetic particles produced in this work (around 15 nm) have dimensions much smaller than those obtained by Neri et al. [13] (10–100 μm) who followed the protocol of Carneiro Leão et al. [10].

The magnetic nanoparticles produced were coated with PANI (mPANI) by the oxidation of aniline with potassium permanganate. It is well known that, under certain synthesis conditions, PANI forms a sub-micrometer film on the surface of the objects immersed into a polymerization mixture. These films are formed on various types of substrates: conducting, insulating, hydrophobic, and hydrophilic. Thus, the polymer strongly adheres to the surface and covers even nanoparticles and nanostructure relief of the objects. The parameters of the film (its thickness, uniformity, and morphology) depend both on the chemical nature of the coated surface and on the synthesis conditions [14]. While hydrophobic materials are covered by a more or less uniform PANI film, the film relief on hydrophilic microparticles has often a discontinuous patchy character [15]. In addition to the film structure, the synthesis process results in a PANI precipitate which has no adhesion and is easily washed away from the surface. The ratio between the amounts of precipitate and film depends on the synthesis conditions [16]. Modifications of the experimental protocol [13] were made to obtain coated nanoparticles with good binding capacity for biomolecules. The optimal

conditions were obtained from an experimental design (results not shown) used for the PANI coating. The conditions were: 0.1 M $KMnO_4$, 0.5 M aniline, contact time of $KMnO_4$ of 1 h, polymerization time of 1 h and polymerization temperature of 4°C. The concentrations of the oxidizing agent and aniline were identical to the protocol; however, the contact time of $KMnO_4$ was reduced from overnight to 1 h, the polymerization time from 2 h to 1 h and, the polymerization temperature from 25°C to 4°C. As a result, it was possible to form a core-shell structural mixture, in which magnetic nanoparticles are the core and polyaniline the shell.

3.2. Transmission and scanning electron micrographs

The morphology, shape and size distribution of magnetic nanoparticles and mPANI were examined by electron micrographs observation. Typical transmission electron microscope (TEM) image of magnetic nanoparticles is shown in Fig. 1A.

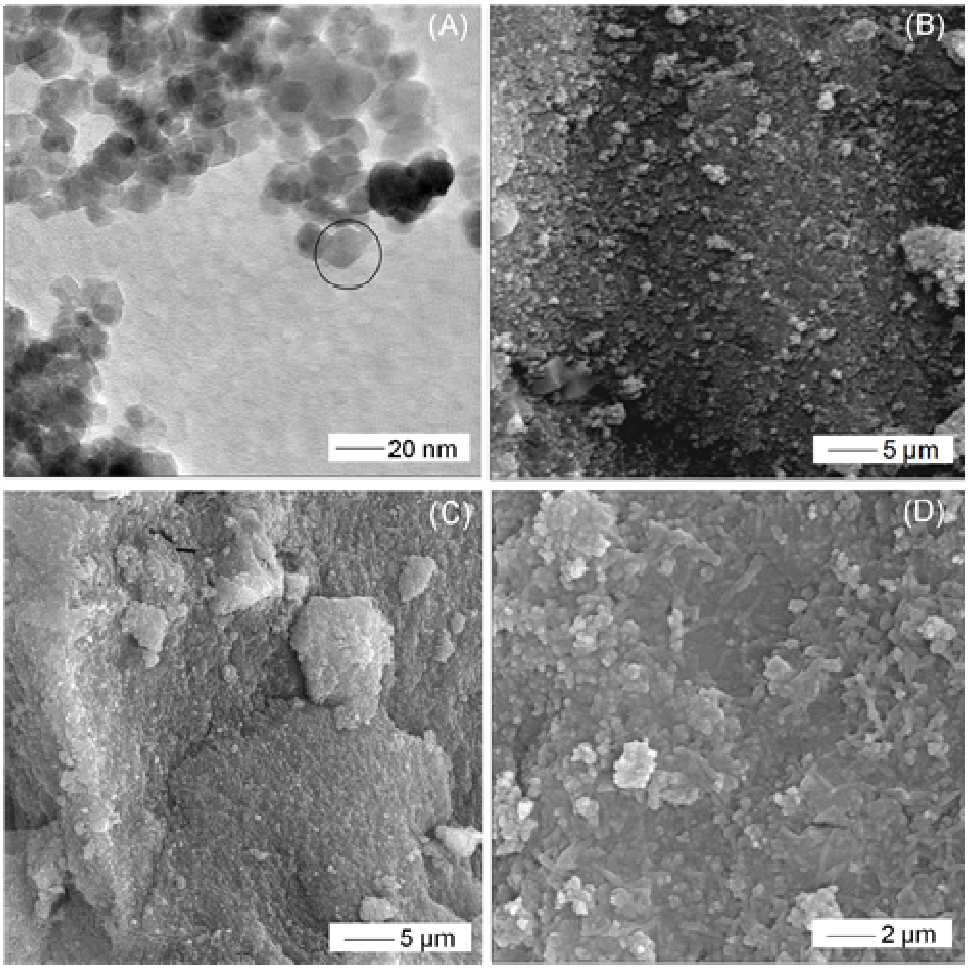

Figure 1. Electron microscopic images. TEM image of (A) magnetic nanoparticles and SEM images of (B) magnetic nanoparticles, (C) mPANI (lower resolution), (D) mPANI (higher resolution).

Some magnetic nanoparticles are partially superimposed onto the agglomerate core, i.e., the boundary limit of each nanoparticle is partially diffuse; thus a precise average particle size cannot be determined. However, according to Cótica et al. [17] a narrowed particle sizes distribution can be seen. It should be noted that generally, magnetite nanoparticles with no surface treatments tend to agglomerate with the neighboring ones, which could be due to the substantial built-up specific surface area of the nanosized particles [18]. The presence of theses agglomerates may be due also to the magnetic nanoparticles aggregated together to reduce their surface energy [19] and/or due to the magnetic dipole-dipole interactions and the van der Waals forces between the nanoparticles [20,21]. In our work, the magnetic nanoparticles exhibited agglomerates with apparently regular morphology, nearly spherical/ellipsoidal shape, dimensions around 15 nm, and narrow particle size distribution (Fig. 1A). The morphology of the magnetic nanoparticles and mPANI was also investigated using a scanning electron microscope (SEM). The micrographs were obtained on metalized films deposited on carbon strip and results shown in Fig. 1B–D. SEM images revealed that mPANI present similar shape to the magnetic nanoparticles, a homogeneous structure and look like small flakes, i.e., the unstructured analog of PANI were obtained [22]. The surface of mPANI becomes rougher, which is due to the polymerization that occurred on the surface of magnetite nanoparticles and the strong interfacial interaction between the polymer matrix and magnetic nanoparticles as observed by Gu et al. [2].

The polydispersity index (PDI) of magnetic nanoparticles and mPANI was 0.47 and 0.29, respectively. According to Pich et al. [23], the increase in polydispersity can be explained by a simultaneous formation of large composite microparticles and smaller particles with iron oxide core and polymeric shell which precipitate on the surface of larger particles during polymerization process leading also to a noticeable morphology variation.

3.3. FTIR spectra

The emeraldine salt is the partly oxidized form of polyaniline where six benzene rings and two quinoid rings are present in an eight-ring repeating unit. The conductivity of the emeraldine form of polyaniline can be changed by about 10 orders of magnitude by doping [24]. The FTIR spectra of magnetic nanoparticles and mPANI are shown in Figure 2. It was found that mPANI had characteristic peaks at around 1494 cm^{-1} (C=C stretching of benzenoid ring), 1398 cm^{-1} (Q=N–B stretching deformation, Q refers to the quinoid ring and B to the benzenoid ring), 1305 and 1260 cm^{-1} (C–N stretching benzenoid ring) and 805 cm^{-1} (out-of-plane deformation of C–H in the 1,4-disubstitued benzene ring), which was similar with polyaniline [13,25,26]. The low intensity of the bands of the FTIR spectra (Fig. 2A) is believed to be due to the fact that, because of the presence of magnetic nanoparticles in the reaction system, aniline gets adsorbed on the oxide particles.

Polymerization proceeds initially on the surface of these oxide particles in the presence of $KMnO_4$ in the reaction system. The adsorption of polymer to the magnetic nanoparticles results in constrained chain growth around the particles. Such adsorption and constrained motion of the chains will restrict the modes of vibration in polyaniline, which in turn lead to the reduction in intensity in the FTIR spectra [24,27]. The presence of Fe–O bond in magnetite particles can be seen by two strong absorption bands at around 635 and 590 cm^{-1}. These bands result from split of the ν_1 band at 570 cm^{-1}, which corresponds to the Fe–O bond of bulk magnetite [28–31]. In our work, absorption bands of Fe–O bond were observed around 631 and 588 cm^{-1} for magnetic nanoparticles and 630 and 580 cm^{-1} for mPANI. A principal effect of finite size of nanoparticles is the breaking of large number of bonds for surface atoms, resulting in the rearrangement of electrons on the particle surface [28]. The FTIR spectra in the range 750–450 cm^{-1} for magnetic nanoparticles and mPANI are shown in the Fig. 2B. These spectra are compared with the spectrum of maghemite (not shown) confirming the oxidation and its increase after coating (Figure 2B). The spectra exhibit absorption bands around the 570 cm^{-1} characteristic of magnetite [8]. Although, the bandwidth increases, and small shoulders in the 600–750 cm^{-1} range as well as a band at 450 cm^{-1} appear with greater intensity after coating of the magnetic particles. The appearance of the characteristic maghemite peaks shows that the relative quantify of maghemite increases the coating.

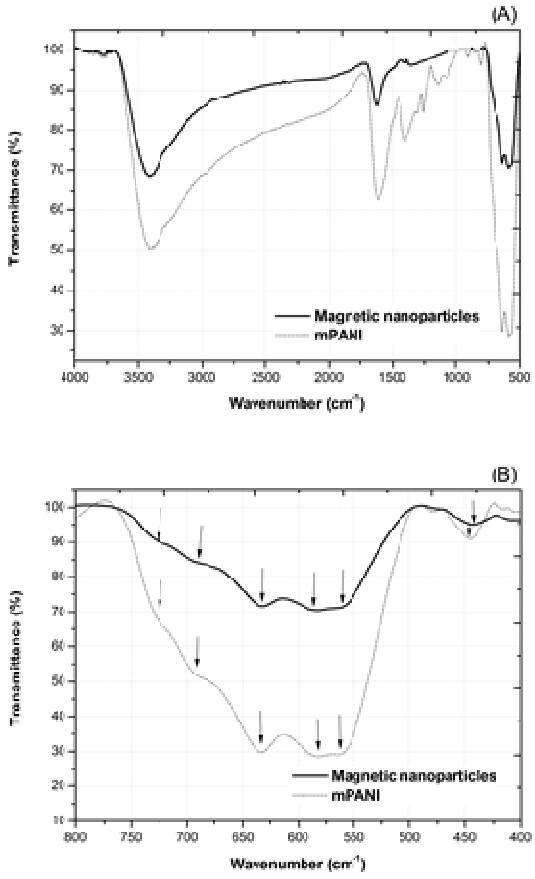

Figure 2. FTIR spectra of magnetic nanoparticles and mPANI.

3.4. Characterization of the surface areas of magnetic nanoparticles and mPANI

Nitrogen sorption isotherms for magnetic nanoparticles and mPANI can be seen in Fig. 3. The nitrogen adsorption–desorption isotherms of both magnetic materials produced belong to the type IV [32] with H4 hysteresis loops, according to IUPAC–classification [33]. In the Fig. 3, the curves represent the adsorption of N_2 gas on the surfaces of the magnetic nanoparticles and mPANI, while the upper curve represents the progressive withdrawal; desorption, of the adsorbed N_2 gas as observed by El-kharrag et al. [34]. The magnetic nanoparticles and mPANI are mesoporous materials (pores of widths between 2 nm and 50 nm) and their hysteresis loops are associated with the filling and emptying of the mesopores by capillary condensation [35].

For magnetic nanoparticles, the mesopores should be interparticle pores among magnetic nanoparticles. After coating such magnetic nanoparticles with polyaniline, there was a slight increase of 154.60 $m^2\ g^{-1}$ to 162.93 $m^2\ g^{-1}$ in BET surface area. On the other hand, pore volume and average pore diameter (Barrett–Joyner–Halena, BJH method) were slightly reduced when compared with uncoated magnetic nanoparticles. For magnetic nanoparticles, pore volume was of 0.36 $cm^3\ g^{-1}$ and average pore diameter of 9.46 nm, and for mPANI, pore volume was of 0.35 $cm^3\ g^{-1}$ and average pore diameter of 8.72 nm, respectively. No pronounced difference in the pore size was observed between samples. Both samples show a broader pore distribution (results not shown) in which pores with different sizes coexist [35].

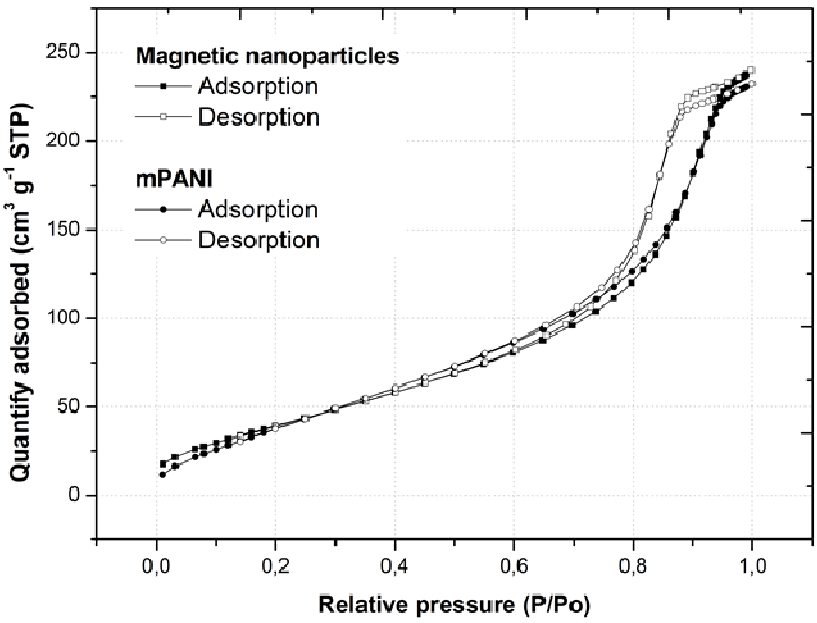

Figure 3. Nitrogen adsorption/desorption isotherms of magnetic nanoparticles and mPANI.

3.5. XRD analysis

Colors of iron oxide may be very dependent on grain size and chemical composition and this feature is a first auxiliary attribute in their identification [36]. In our work, magnetic nanoparticles and mPANI are black and magnetic, suggesting that magnetite may be the dominant phase, although maghemite may not be completely excluded. There are typically small differences between typical XRD patterns for magnetite and maghemite. As observed by Andrade et al. [36] no clear reflection peak due to other crystalline phase, which could occur as impurity, was observed, indicating that final products were pure enough, from this point of view, consisting essentially of binary mixture of the two spinel magnetic iron oxides, meaning magnetite and maghemite. The differences in the crystalline behavior of magnetic nanoparticles and mPANI are analyzed by XRD measurements. Fig. 4 shows XRD patterns for magnetic nanoparticles and mPANI. The 2θ peaks at 18.44°, 30.30°, 35.67°, 43.37°, 53.80°, 57.35°, 62.97°, 71.43° and 74.48° [2,6,37,38] are attributed to the crystal planes of magnetite at (111), (220), (311), (400), (422), (511), (440), (620) and (533) [39]. The index of magnetite nanoparticles is in accordance with the International Center for Diffraction Data (reference code: ICDD 01–071–636). In this study, XRD results confirmed the presence of unmodified magnetite in the mPANI as can be seen in Fig. 4. Furthermore, the results showed broadened lines that can be due to the magnetization relaxation from superparamagnetism and matrix constrictions for nanosized particles [40].

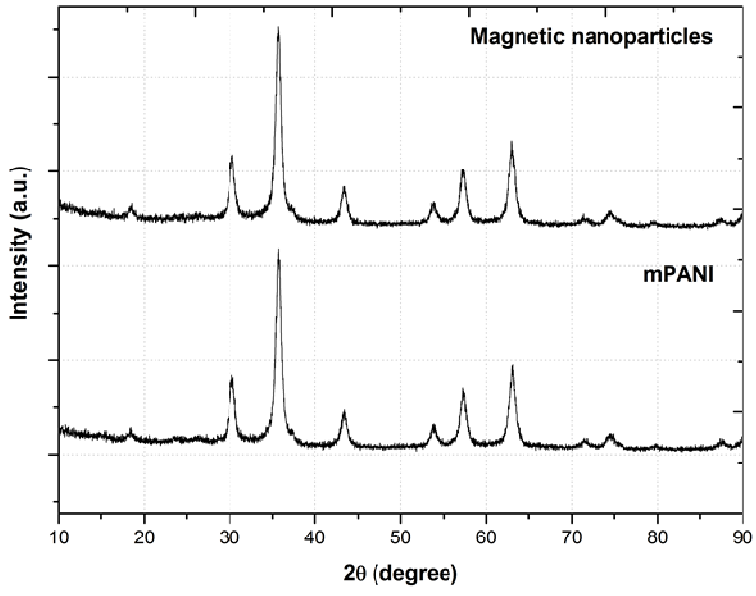

Figure 4. XRD patterns of magnetic nanoparticles and mPANI.

According to Yu et al. [37], the absence of the characteristic reflection of (221) plane, corresponding to maghemite, suggests the magnetite as phase predominant. In our work, we also observed the absence of this peak. However, we cannot rule out the presence of maghemite in the

samples produced, since the FTIR results, for example, suggest otherwise (Fig. 4). Vandenberghe et al. [41] and Gorski and Scherer [42] reported that the differentiation between non-stoichiometric magnetite and magnetite–maghemite mixture was claimed to be almost impossible. However, more recent studies report the differentiation between magnetite and maghemite by XRD analysis of high angle peaks as (511) or (440) and its resolution through the deconvolution [43].

3.6. Mössbauer spectroscopy (MS)

Because magnetite and maghemite have very similar XRD patterns, we performed further characterization by Mössbauer spectroscopy to distinguish the phases of iron oxide nanoparticles. The spectra were fitted using a two sextet model (sextet 1 and 2) for magnetite. Fig. 5 shows the adjusted Mössbauer spectra at 298 K for magnetic nanoparticles and mPANI, where the contribution of two magnetic subspectra corresponds to Fe^{3+} in the tetrahedral position and [Fe^{3+}/Fe^{2+}] in octahedral coordination in the spinel structure [38,44].

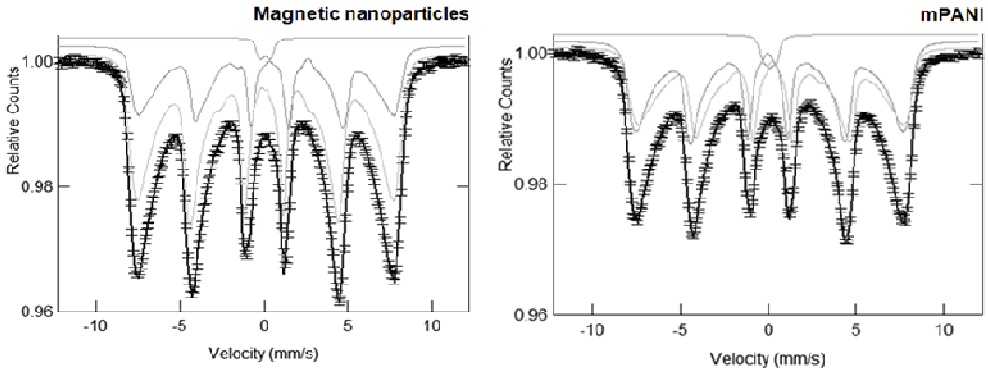

Figure 5. Mössbauer spectra of (A) magnetic nanoparticles and (B) mPANI.

The Table 1 shows the hyperfine parameters calculated from Fig. 5. The resonant lines of both sextets present an important broadening, the hyperfine magnetic fields for magnetic nanoparticles are sextet 1 equal to 48.2 T and sextet 2 equal to 44.9 T (Fig. 5A and Table 1). The hyperfine magnetic fields for mPANI are sextet 1 equal to 48.3 T and sextet 2 equal to 45.4 T (Fig. 5B and Table 1). These values are similar to the bulk material (sextet 1: 49.0 T and sextet 2: 46.0 T), but are slightly inferior to the ones corresponding to the bulk material [45], this deviation in the ideal ratio (1:2) of the iron in tetrahedral and octahedral position obtained from the subspectra area can be in accordance with to the small particle size [44]. When the size is lower than 20 nm, the magnetic hyperfine structure is not resolved, consisting of broadened, overlapped, and asymmetrical lines originating from the occurrence of superparamagnetic relaxation phenomena [8,47]. Previous work

reported on magnetite nanoparticles of 39 ± 5 nm in size have shown that the nanoparticles consist of a magnetite core surrounded by an oxidized layer close to maghemite [48].

Table 1. Analyzed results of Mössbauer spectra for magnetic nanoparticles and mPANI. Bhf: hyperfine field; Δ: quadrupole splitting; δ: isomer shift relative to the isomer shift of α-iron at room temperature

Sample	Component	Bhf (T)	Δ (mm s^{-1})	δ (mm s^{-1})	Area (%)
Magnetic nanoparticles	Doublet		0.00	0.21 ± 0.03	1
	Sextet 1	48.2 ± 0.56	0.08 ± 0.03	0.17 ± 0.02	67
	Sextet 2	44.9 ± 1.83	-0.21 ± 0.04	0.36 ± 0.04	32
mPANI	Doublet		0.00	0.21 ± 0.04	2
	Sextet 1	48.3 ± 0.49	-0.13 ± 0.03	0.31 ± 0.04	49
	Sextet 2	45.4 ± 0.56	0.11 ± 0.03	0.14 ± 0.02	48

In addition to the signals relating to magnetite, the Fig. 5 also shows a doublet having an isomer shift equal to 0.21 mm s^{-1} for both samples and an area equal to 1% and 2% for magnetic nanoparticles and mPANI, respectively. In Fig. 5A, it is possible to observe the presence of a doublet at the center of the spectrum. Moreover, in Fig. 5B, the doublet is not well defined, possibly due to the presence of the coating. This doublet emanates from ferric iron in a non-spherical local surrounding, maybe coming from the rim of the iron oxide core, i.e., the magnetic relaxation effect which is attributed to the presence of superparamagnetic as well as the ferromagnetic nanoparticles [44]. Thus the Mössbauer spectrum could not be fitted with two discrete tetrahedral and octahedral sites along with a doublet because of the superposition of relaxing sextet and doublet patterns. To block the superparamagnetic relaxation effect, the Mössbauer spectrum should be recorded at low temperature [49]. According to Mössbauer spectra and the hyperfine parameters, it is evident that the process for obtaining the mPANI does not interfere significantly with the nature of the oxide [38]. However, a small percentage of maghemite must be present in the samples due to the oxidation process as can be corroborated by Fig. 2B.

3.7. Magnetization measurements

The magnetic properties of the magnetic nanoparticles and mPANI were investigated in the applied magnetic field sweeping from –60 to 60 kOe at 298 K and 323 K as shown in Fig. 6. It is well-known that the size [50], structure and shape may affect the magnetic properties of the products [51]. Under applied magnetic field, magnetic nanoparticles and mPANI showed the positive magnetizations and there is no hysteresis (Fig. 6). It indicates the superparamagnetic behavior [30]. The difference between the magnetic behavior of small and medium sized particles can also be observed in their hysteresis loops [8].

Fig. 6 shows the field dependent magnetization curves of magnetic nanoparticles and mPANI. The saturation magnetization (M_s) for the magnetic nanoparticles has value equal to 60.51 emu g^{-1}, which is lower than that of bulk magnetite (M_s = 92 emu g^{-1}) [52], and mPANI has value equal to 59.05 emu g^{-1} (Fig. 6). The reduction in the saturation magnetization values signifies the decrease in the particles size [53] and can be attributed to surface effects such as magnetically inactive layer containing spins that not collinear with the magnetic field as observed by Gu et al. (2009) [30]. These discrepancies in the saturation values of magnetization may be explained by variations in the methods employed to synthesize magnetite, which can generate different particle sizes, magnetite surfaces and chemical compositions [54].

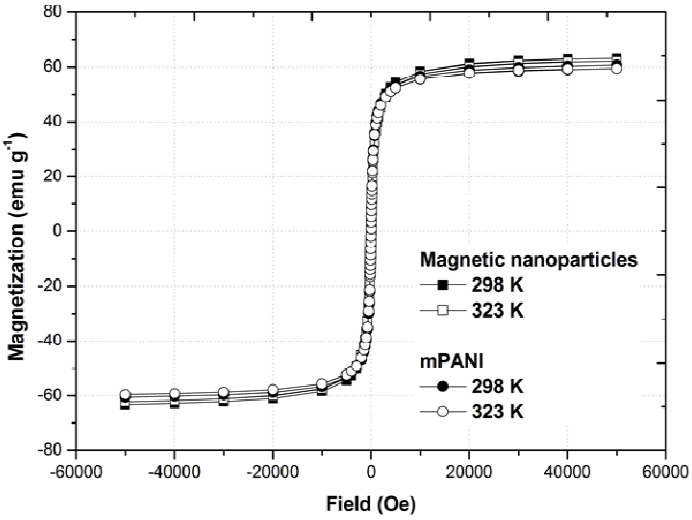

Figure 6. Magnetization curves of magnetic nanoparticles and mPANI at 298 K and 323 K.

3.8. Reuse of the magnetic enzymatic derivative

The trypsin was immobilized on mPANIG yielding a preparation containing protein amount of 12.84 ± 0.49 µg of protein/mg of mPANI (49.27% of immobilized protein). The immobilized trypsin was reused ten times and presented a mean specific activity of 93.63 ± 4.78% of the initial activity (22.44 ± 1.25 U/mg of protein), corresponding to 32.46 ± 3.11% of the activity of the free enzyme. After ten reuses, immobilized trypsin on mPANI was stored at 4°C for one week and then ten other reuses were performed. This immobilized enzyme presented the end of these reuses, a mean specific activity of 81.26 ± 1.51% of the initial activity (Fig. 7). The decrease in the trypsin derivative activity during these twenty assays was approximately 12% of the initial activity. Previously, immobilized trypsin on ferromagnetic Dacron presented after eight reuses a specific activity equal to 92.1 ± 7.3% of the initial activity [56] and immobilized trypsin magnetic levan particles after ten reuses, a specific activity equal to 84.43 ± 5.42% of the initial activity [57].

Moreover, Neri et al. [13] showed that after ten reuses the β-galactosidase immobilized on magnetic particles coated with PANI retained approximately 85% of the initial activity. Thus produced enzymatic derivative in this work exhibited excellent activity retention and high operational stability. Our goal is to use it in different applications in the biomedical field, especially in proteomics research, in the stage of protein digestion.

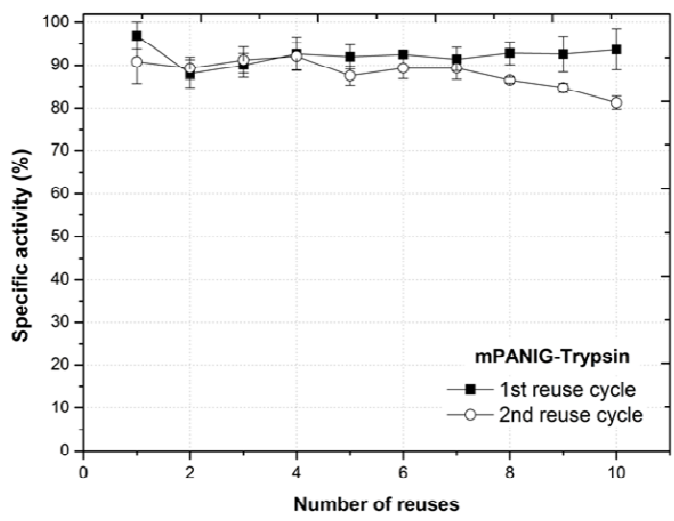

Figure 7. Specific activity of mPANIG-Trypsin. The immobilized trypsin on mPANIG was incubated with a 4 mM initial concentration of BAPNA and Tris-HCl buffer. After each cycle, the derivative were washed before new step.

4. Conclusions

In conclusion, magnetic nanoparticles were obtained from modifications of the experimental protocol used routinely by our research group. These nanoparticles showed dimensions smaller than those obtained by original the protocol, corroborating the advantages of the optimization process. The FTIR, XRD and Mössbauer spectroscopy analysis of the nanoparticles confirmed the presence of magnetite, but suggested the presence of another phase. Therefore, the nanoparticles produced consist of a mixture of magnetite and maghemite, where maghemite is a result of oxidation of the magnetite. Furthermore, after PANI coating, nanoparticles exhibited good magnetization, an increase in the surface area and showed to be an excellent support for trypsin immobilization. The major benefits of the magnetic enzymatic derivative (mPANIG-Trypsin) are its operational stability, high activity, simple method and low cost of production.

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Preparation and characterization of magnetic levan particles as matrix for trypsin immobilization

J.C. Maciel [a], P.L. Andrad [b], D.F.M. Neri [c,*], L.B. Carvalho Jr. [d], C.A. Cardoso [e], G.M.T. Calazans [f], J. Albino Aguiar [g], M.P.C. Silva [d]

[a] Programa de Pós-Graduação em Ciências Biológicas, Universidade Federal de Pernambuco, Cidade Universitária, 50670-901 Recife, PE, Brazil
[b] Programa de Pós-Graduação em Ciência de Materiais, Universidade Federal de Pernambuco, Cidade Universitária, 50679-901 Recife, PE, Brazil
[c] Universidade Federal do Vale do São Francisco, 56304-205 Petrolina, PE, Brazil
[d] Departamento de Bioquímica, Universidade Federal de Pernambuco, Cidade Universitária, 50679-901 Recife, PE, Brazil
[e] Departamento de Física, Universidade Federal de São Carlos, 13565-905 São Carlos, PE, Brazil
[f] Departamento de Antibióticos, Universidade Federal de Pernambuco, Cidade Universitária, 50670-901 Recife, PE, Brazil
[g] Departamento de Física, Universidade Federal de Pernambuco, Cidade Universitária, 50679-901 Recife, PE, Brazil

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ABSTRACT

Magnetic levan was synthesized by co-precipitating D-fructofuranosyl homopolysaccharide with a solution containing Fe^{2+} and Fe^{3+} in alkaline conditions at 100 °C. The magnetic levan particles were characterized by scanning electron microscopy (SEM), magnetization measurements, X-ray diffractometry (XRD) and infrared spectroscopy (IR). Afterwards, magnetic levan particles were functionalized by $NaIO_4$ oxidation and used as matrices for trypsin covalent immobilization. Magnetite and magnetic levan particles were both heterogeneous in shape and levan–magnetite presented bigger sizes compared to magnetite according to SEM images. Magnetic levan particles exhibited a magnetization 10 times lower as compared to magnetite ones, probably, due to the coating layer. XRD diffractogram showed that magnetite is the dominant phase in the magnetic levan. Infrared spectroscopy showed characteristics absorption bands of levan and magnetite (O–H, C–O–C and Fe–O bonds). The immobilized trypsin derivative was reused 10 times and lost 16% of its initial specific activity only. Therefore, these magnetic levan particles can be proposed as an alternative matrices for enzyme immobilization.

1. Introduction

Magnetic carrier technology has been applied as bioaffinity adsorbents for selective recovery from liquors containing other suspended solids; wastewater treatment via electrostatic adsorption; protein (enzyme) immobilization and in the preparation of immunological assays [1]. The magnetic properties of these particles play an important role in the effectiveness of the application and affect the behavior of the particles and ferrofluids in applied fields [2].

In biomedicine, the polymer shell ensures stability of the magnetic particles in physiological media providing non-toxicity by avoiding leakage of iron and enabling chemical modification for attachment of biologically active compounds [3]. The shells are biocompatible such as dextran, xylan, chitosan, PEG, etc. and possesses active groups, which can be conjugated to biomolecules such as proteins [4]. Magnetic particles are an attractive and efficient support for bioconversions using immobilized enzymes due to the following advantages: simplicity of the matrix synthesis and immobilization protocol and easy removal from the reaction medium by applying a magnetic field [5,6].

Magnetic carriers can be manufactured using inorganic materials or polymers. However, those based on polymers offers a variety of surface functional groups than can be tailored to specific applications [7]. The utilization of polysaccharides presents advantages due to a large number of derivable groups, wide range of molecular weights, low toxicity, biodegradability and high stability [8]. Most magnetic materials such as maghemite and magnetite are employed as the core of the supports [9]. The magnetite particles (Fe_3O_4) are preferred because of their greater saturation magnetization and no toxicity [10].

The application for biomolecules immobilization mainly based on the solid-phase magnetic feature which is able to achieve a rapidly easy separation and recovery from the reaction medium in an external magnetic field [11]. There are basically two main ways to immobilize protein on supports: physical adsorption and covalent immobilization. Comparatively, covalent immobilization presents the benefits of eliminating or reducing protein leakage (a stronger linkage is formed) and usually increases protein tertiary structure stability [12–16]. Immobilization of biomolecules onto

* Corresponding author. Tel.: +55 87 38629354.
E-mail address: davidfmneri@yahoo.com.br (D.F.M. Neri).

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insoluble supports is an important tool for the fabrication of a diverse range of functional materials or devices [17]. It provides many distinct advantages including enhanced stability, easy separation from reaction mixture, possible modulation of the catalytic properties, and easier prevention of microbial growth [18].

In our lab magnetic levan composite was previously used to purify using affinity binding lectins from *Canavalia ensiformis* (Con A) and *Cratylia mollis* seeds (Cramoll 1 and Cramoll 1, 4) [19]. Cramoll 1 was purified using this procedure in two steps instead of a preceding three-step protocol employing ammonium sulfate fractionation, affinity chromatography on Sephadex G-75, and ion exchange chromatography through a CM-cellulose column [19]. Here, these magnetic particles were characterized regarding structural, microstructural and magnetic properties and further used as matrix to immobilize trypsin.

2. Experimental

2.1. Materials

Levan from *Zymomonas mobilis* strain ZAG–12 (Molecular weight average equal to 300 kDa) was precipitated by addition of ethanol to 70% (v/v) at low temperature according to Calazans et al. [20]. Ferric chloride hexahydrate and ferrous chloride tetrahydrate were purchased from Merck (Germany) whereas ammonium hydroxide was from Vetec Chemical (Brazil). All other reagents were of analytical grade.

2.2. Preparation of magnetic particles of levan

An aqueous mixture with 5 mL of 1.1 M $FeCl_3.6\,H_2O$ and 5 mL of 0.6 M $FeCl_2.4\,H_2O$ were added to 50 mL of 2.0% w/v levan (prepared in distilled water). Ammonium hydroxide was then added to achieve a pH of 11. The mixture was then heated up to 85 ± 3 °C and kept for 30 min with vigorous stirring. The magnetic levan particles synthesized were thoroughly washed with distilled water to a neutral pH. The material was dried and kept at 25 °C. This procedure was according to Carneiro Leão et al. [21], except by incubation time (30 min), temperature (85 °C) and final pH of the mixture (11).

2.3. Matrix characterization

The particle size and morphology of the samples were established by scanning electronic microscopy (SEM), utilizing a JEOL Model JSN—5900 electron microscope. Magnetization measurements were obtained at 293 K and 313 K in magnetic fields from 0 to 5.0 T using a SQUID magnetometer (Quantum Design Model MPMS-5). The structural properties of the magnetic particles were characterized by X-ray powder diffraction, which was carried out in an X-ray diffractometer Siemens D5000. Representative powder samples were analyzed in the range $10° < 2\theta < 90°$ using CuKα radiation ($\lambda = 1.5406$ Å), in steps of 0.02°, and with a counting time of 1.0 s per step. Fourier transform infrared (FTIR) spectrum from the KBr pellet method in the range of 4000–400 cm^{-1} with particles-coated was recorded in a BRUKER instrument model IFS 66. Magnetite, magnetic levan and levan (2 mg each) were mixed with KBr (200 mg) and disks obtained under pressure at 490 atm. Scan (100 scans) was recorded with a 4 cm^{-1} resolution.

2.4. Magnetic levan particles Functionalization, trypsin immobilization and reuse of the enzymatic derivative

Magnetic levan particles (10 mg) and sodium metaperiodate (10 mg) were mixed in 3 ml distilled water [22]. This mixture was constantly stirred in the dark at 25° C for 7 h. The magnetic particles containing the partially oxidized levan were collected by a magnetic field of 0.6 T and washed with 0.1 M Tris-HCl buffer pH 8.0 (1.0 mL, 10 times). After this procedure, it was incubated with trypsin (0.2 mg/mL, 1.0 mL) for 16 h at 4 °C under mild stirring. The enzymatic derivative was collected by magnetic field of 0.6 T and supernatant and washings were collected for protein determination [23] using trypsin as standard. Sodium borohydride (0.03 M, 1 mL) was added to trypsin–magnetic levan particles and slightly mixed for 2 h at 4 °C. Then they were washed 10 times with the aforementioned buffer and kept in the buffer at 4 °C until use. The retained protein was estimated by the difference between the offered protein (200 μg) and that found in the supernatant and washings. The reuse was carried out by assaying of the same trypsin–magnetic levan particles preparation with BAPNA for 10 times intercalating each successive use by washing the immobilized enzymatic derivative 10 times with 0.1 M Tris-HCl buffer, pH 8. The activity of trypsin for the free and immobilized enzyme was measured as described by Amaral et al. [24]. The activity (unit) was defined as μmol BAPNA hydrolyzed during 1 min using an absorption coefficient of ε_{405} 9100 $M^{-1} \cdot cm^{-1}$. All of the experiments were carried out in duplicate.

3. Results and discussion

3.1. Preparation, size and morphology of the magnetic particles

The co-precipitation process to obtain magnetic levan was carried out in an alkaline aqueous medium and the final product obtained from this process yielded a dense, black and magnetic powder. These particles exhibited a magnetization in the presence of a magnetic field 0.6 T. SEM images shown in Fig. 1 reveal

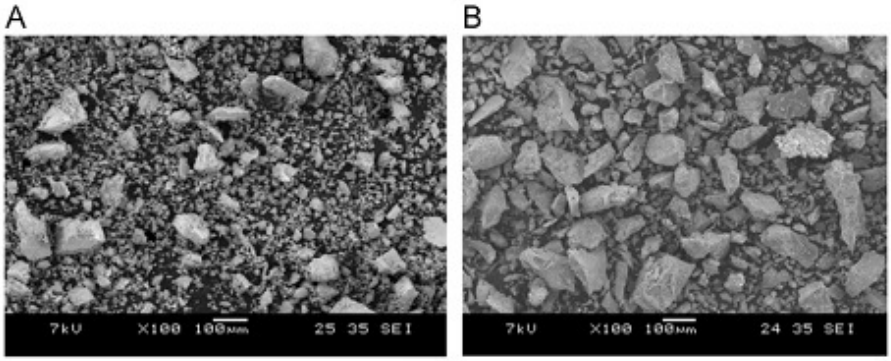

Fig. 1. Scanning electron microscopy images of magnetite (A) and magnetic levan (B) particles.

a heterogeneous morphology without porosity for both magnetite and magnetic levan particles. However, magnetic levan particles presented a bigger mean size than the magnetite ones. The particles can be considered as microparticles with sizes between 20–60 μm for magnetite and 100–200 μm for magnetic levan approximately. The coating produced changes in the size particle and according to Silva et al. [25] one can attribute to multi-core/shell structures.

3.2. Magnetization measurements

The magnetization of magnetite and magnetic levan particles (Fig. 2) indicated that there was neither remnant magnetization (Magnetization = 0 for Magnetic field = 0) nor coercivity. The saturation magnetization was found to be 10 times lesser for the magnetic levan as compared to magnetite. Neri et al. [26] using magnetic composite of polysiloxane coated with polyaniline also found the saturation magnetization lesser than magnetite and suggested that the presence of cations vacancy in maghemite could be responsible for this decrease. The coating decreases the force exercised by applied magnetic field because of the difficult alignment of magnetic dominions in the material, producing a smaller magnetization than that exhibited by particles-uncoated as well as observed by Xu et al. [27] and Ramanujam and Yeow [28]. Besides the presence of the polymer, the smaller amount of magnetic material (iron oxide core) and reduction in size of the particles (core-shell) should be considered, since magnetism arises because electrons have a property called spin. As dimensionality, and hence coordination, is reduced, more electrons are available to lead to magnetism [29].

3.3. X-ray analysis

Fig. 3 shows the XRD diffractograms for magnetite and magnetic levan particles. The magnetite was the dominant phase in the particles of magnetic levan with a primary scattering peak at around $2\theta = 35°$. This result is according to Chen and Hu [30]. Magnetic levan particles showed small peak dislocations as compared to magnetite patterns. They probably happened due to tensions in the crystalline structure from magnetite because of modifications occurred during synthesis of the material. Furthermore, Fig. 3 shows that magnetic levan do not present sharp diffraction peaks. Instead, a broad band appears in each spectrum, which is characteristic for amorphous materials and also of ultrafine crystalline particles [27]. It suggests the presence of levan polysaccharide in the produced magnetic particles. Characteristic peaks of goethite (at $2\theta = 21.38°$), hematite (at $2\theta = 33.15°$), ferric hydroxide (at $2\theta = 26.38°$) as well as other phases of iron oxide hydroxides were not detected [25,31]. However, another iron oxide (maghemite), difficult to distinguish from magnetite due to the proximity of the peaks, probably could have been formed as contaminant during the synthesis process [32].

3.4. Functional groups

Infrared spectroscopy (Fig. 4) showed that O–H groups are present in the levan polysaccharide, magnetite and magnetic levan near wavenumber of 3500 cm^{-1} with similar intensities. These O–H groups correspond to that present in organic compounds and to the OH^- groups adsorbed on the particle surface. The magnetic levan presented absorption bands in 2935.1 and 2878.5 cm^{-1} due to stretching vibration of C–H bond band in 1061.0 cm^{-1} and due to stretching vibration of C–O–C bond. These bonds are also present in the levan polysaccharide with bands in 2940.2 and 2886.2 cm^{-1} (stretching vibration of C–H bond), band in 1059.0 cm^{-1} (stretching vibration of C–O–C) indicative of the presence of polysaccharide in the magnetic particles. Previous studies [33] reported that the characteristic absorption bands of the Fe–O bond of bulk magnetite were in 570 and 375 cm^{-1}. However, Ma et al. [4] observed that these two bands shift of about 600 and 440 cm^{-1} respectively, and the band near 600 cm^{-1} is split into two peaks of 631.4 and 582.9 cm^{-1}. Here, (Fig. 4) is also shows a band near 600 cm^{-1} split in two peaks of 631.2 and 565.6 cm^{-1} for magnetite. However, levan–magnetite particles presented a single broad band at 583.6 cm^{-1}. This little difference can indicate that interactions

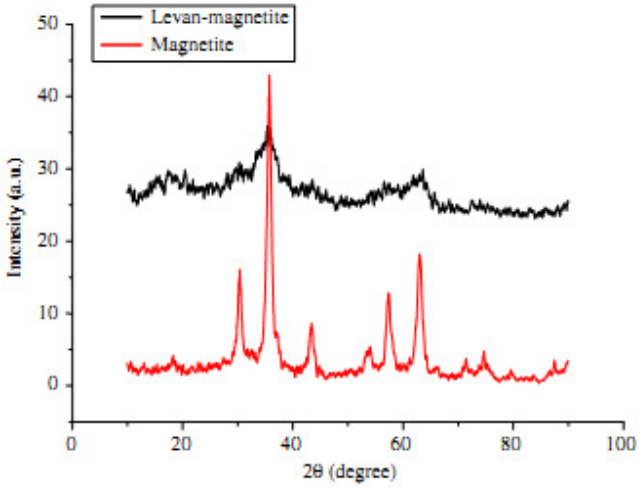

Fig. 3. X-ray powder diffraction patterns of magnetite and magnetic levan particles.

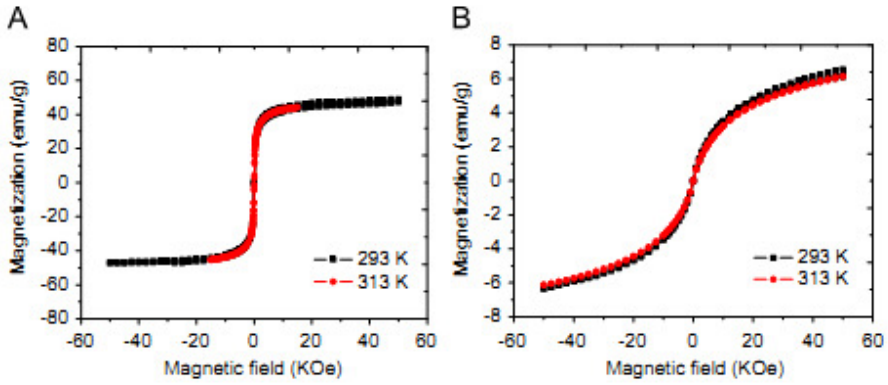

Fig. 2. Magnetization curves of magnetite (A) and magnetic levan (B) particles at 293 K and 313 K.

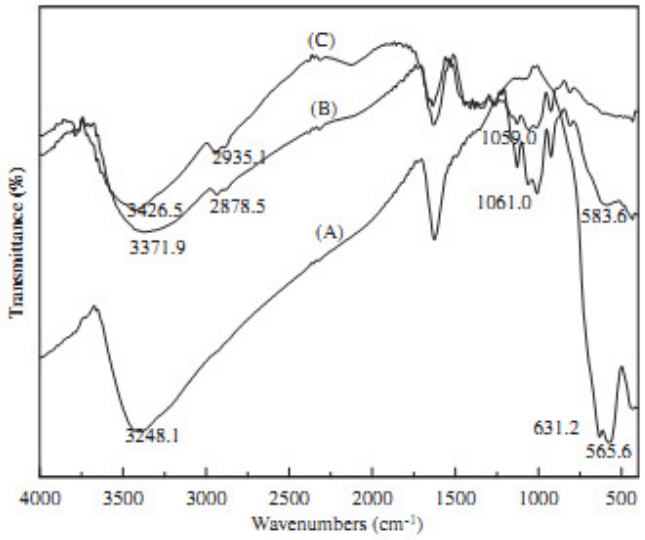

Fig. 4. FTIR spectra of the levan polysaccharide (A), magnetic levan (B) and magnetite (C).

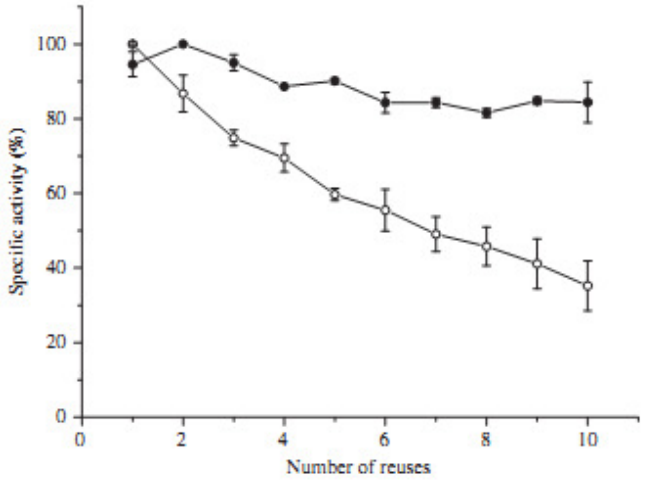

Fig. 5. Re-utilization of immobilized trypsin on magnetite (○) and magnetic levan (●) particles. The maximum specific activity of enzyme (100%) was 20.65 ± 0.69 mU/mg for trypsin immobilized on magnetic levan and 18.59 ± 0.08 mU/mg for trypsin immobilized on magnetite.

between coating (levan polysaccharide) and magnetite were intermolecular origins.

3.5. *Application of the particles of magnetic levan for Bioprocessing*

Trypsin was covalently immobilized on magnetic levan particles after partial oxidation of immobilized levan by the sodium periodate method (functionalization). Afterwards the trypsin was covalently bound to the oxidized levan [34]. The amount of immobilized trypsin was found to be 16.0 ± 2.8 μg/mg of matrix and the specific activity was 19.54 ± 1.34 mU/mg of protein when assayed under standard conditions using a low molecular weight substrate (BAPNA). The immobilized trypsin was reutilized 10 times and presented a mean activity equal to 84.43 ± 5.42% of the initial specific activity (Fig. 5). Meanwhile the adsorbed trypsin on magnetite (not coated) lost 64.75% of its initial specific activity after 10 reuses (Fig. 5). Therefore, coating [22] is important for immobilized enzyme performance providing higher stability. Neri et al. [35] using a different immobilized enzyme (β-galactosidase) and magnetic support (seminterpenetrating network of polysiloxane–polyvinyl alcohol composite) reported comparable retention after 10 reuses (84%). Similar results were also obtained by Amaral et al. [24] reusing immobilized trypsin on ferromagnetic Dacron (about 90%).

4. Conclusions

From the above displayed results one can conclude that magnetic levan particles presented larger size variation than magnetite particles due to the changes produced by coating. The presence of levan polysaccharide in the magnetic levan particles is suggested by FTIR characteristic absorption bands and by a broad band in each spectrum obtained from XRD diffractograms. The utilization of magnetic levan particles was shown to be efficacious for covalent enzyme immobilization as trypsin that can be reused several times without marked activity lost. Therefore, these magnetic levan particles can be proposed as alternative matrices for enzyme immobilization.

Acknowledgments

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6. Conclusões

- Foram produzidas partículas de levana magnética (seguindo o protocolo de rotina em nosso laboratório), por coprecipitação química de sais de ferro (II) e (III) em meio aquoso alcalino, na presença do polissacarídeo levana;
- As partículas de levana magnética apresentaram como fase cristalina predominante a magnetita; exibiram bandas de absorção no infravermelho características de ligações químicas presentes no polissacarídeo; com formação de maiores agregados quando comparada ao material sem a levana; redução de dez vezes nas medidas de saturação da magnetização, provavelmente devido a presença do polímero durante o processo de coprecipitação. A tripsina imobilizada em levana magnética apresentou boa capacidade de reutilização, retendo 84% de sua atividade inicial após dez reutilizações.
- Nanopartículas magnéticas foram obtidas a partir de modificações (temperatura, pH e agente de precipitação) do protocolo utilizado rotineiramente em nosso grupo de pesquisa;
- As nanopartículas magnéticas são constituídas principalmente por magnetita, porém maghemita também foi detectada nas análises realizadas, indicando possível oxidação da magnetita à maghemita;
- O processo de revestimento com polianilina não interferiu de forma significativa nas propriedades físicas, químicas e magnéticas do material magnético sintetizado;
- O compósito mPANI foi utilizado para imobilização de tripsina de forma eficaz;
- Foi observado que as condições de polimerização da anilina exercem influência na atividade da tripsina imobilizada, dessa forma o processo foi otimizado;
- O derivado enzimático imobilizado mostrou melhores atividades em temperaturas mais elevadas quando comparado à enzima solúvel, indicando que a presença do revestimento confere estabilidade à enzima;
- Foi verificado que o derivado enzimático apresenta excelente estabilidade operacional (reusos) e de estocagem;
- A performance do derivado enzimático foi demonstrada pela digestão tríptica de albumina de soro bovino dentro de 15 min, apresentando uma sequência de conversão de 28%. A vantagem desta conversão em relação àquela com a enzima solúvel é o tempo de digestão que é bem menor;
- O derivado enzimático produzido (mPANIG-Trypsina) apresenta boa capacidade de reutilização, baixo custo de obtenção, metodologia simples e grande número de possíveis aplicações.

7. ANEXOS

7.1. Instruções para autores

ANALYTICA CHIMICA ACTA

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Preparation

Use of wordprocessing software

It is important that the file be saved in the native format of the wordprocessor 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 wordprocessor'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: http://www.elsevier.com/guidepublication). Note that source files of figures, tables and text graphics will be required whether or not you embed your figures in the text. 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 wordprocessor.

Please use line numbering and double spacing for the manuscript at the time of submission.

Article structure

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.

Materials and methods

Experimental The experimental methods should be described after the introductory material. Detailed experimental descriptions should be restricted to one section of the paper. Sufficient detail should be given to allow any experienced worker to implement the procedures described. Procedural steps should not be numbered.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

This should include key findings of the research, quantitative analytical performance figures (if appropriate) and their significance to real sample matrices.

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 and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

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Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords

Immediately after the abstract, provide a 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.

Abbreviations

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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.).

Nomenclature, abbreviations and symbols

Please use L for litres. Always leave a space between units and use superscripts rather than /. For instance: use **mg mL^{-1}** and not **mg/ml.** Do not use ppm or ppb to denote solid/liquid concentrations. Do not use abbreviations in the title or keywords. Define abbreviations that are not standard in this field at their first occurrence in both the abstract and the main text. Ensure consistency of abbreviations throughout the remainder of the manuscript.

In all other cases, the recommendations of the International Union of Pure and Applied Chemistry (IUPAC) should be followed, and attention should be given to the recommendations of the Analytical Chemistry Division in the journal *Pure and Applied Chemistry* (see also IUPAC Compendium of Analytical Nomenclature, Definitive Rules, 1987).

Math formulae

Present simple formulae in the line of 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).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

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[3] G.R. Mettam, L.B. Adams, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing, Inc. New York, 2009, pp. 281–304.

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Article Type: Full Length Article

Section/Category: Materials science including physical and mechanical properties

Keywords: Magnetic Nanoparticles, Polyaniline, Trypsin, Biomedical Applications

7.3. Artigos publicados em periódicos

ANGELI, R.; DA PAZ, N.V.N.; MACIEL, J.C.; ARAÚJO, F.F.B.; PAIVA, P.M.G.; CALAZANS, G.M.T.; VALENTE, A.P.; ALMEIDA, F.C.L.; COELHO, L.C.B.B.; CARVALHO, L.B.; SILVA, M.P.C.; CORREIA, M.T.S. Ferromagnetic Levan Composite: An Affinity Matrix to Purify Lectin. ***Journal of Biomedicine and Biotechnology***, 2009, 1-7, **2009**.

MACIEL, J.C.; ANDRADE, P.L.; NERI, D.F.M.; CARVALHO Jr, L.B.; CARDOSO, C. A.; CALAZANS, G.M.T.; AGUIAR, J. ALBINO; SILVA, M.P.C. Preparation and characterization of magnetic levan particles as matrix for trypsin immobilization. ***Journal of Magnetism and Magnetic Materials***, 324, 1312-1316, **2012**.

7.4. Artigos a serem submetidos

Investigation of the molecular weight influence of levans from *Zymomonas mobilis* in the production of magnetic levan particles
Jackeline C. Maciel, Mariana P. Cabrera, Cláudio A. Cardoso, David F.M. Neri, Maria da Paz C. Silva, Luiz B. Carvalho Jr.
Periódico: Carbohydrate Polymers **FI**: 3.628

Properties of immobilized trypsin on magnetic levan particles
Jackeline C. Maciel, Ian P.G. Amaral, Givanildo B. Oliveira, Glícia M.T. Calazans, Luíz B. Carvalho Jr., Maria da Paz C. Silva
Periódico: Applied Catalysis A: General **FI**: 3.903

7.5. Trabalhos apresentados em congressos

MACIEL, J.C.; ANDRADE, P.L.; CALAZANS, G.M.T.; CARDOSO, C.A.; AGUIAR, J.A.; SILVA, M.P.C. Synthesis and characterization of levan-coated magnetite particles. In: ***International Conference on Science and Technology of synthetic metals***, **2008**, Porto de Galinhas.

ANDRADE, P.L.; MACIEL, J.C.; SILVA, V.A.J.; CALAZANS, G.M.T.; SILVA, M.P.C.; AGUIAR, J.A. Synthesis and structural studies of polysaccharides coated magnetite particles. In: ***II Simpósio Nacional em Diagnóstico e Terapêutica Experimental, V Jornada Científica do LIKA e II Fórum Brasileiro de Genética em Neuropsiquiatria***, **2009**, Recife.

ANDRADE, P.L.; SILVA, V.A.J.; MACIEL, J.C.; CALAZANS, G.M.T.; SILVA, M.P.C.; AGUIAR, J.A. Síntese e caracterização da magnetita revestida por levana e fucana como suporte para imobilização de enzimas. In: ***32a Reunião Anual da Sociedade Brasileira de Química***, **2009**, Fortaleza.

SILVA, V.A.J.; ANDRADE, P.L.; MACIEL, J.C.; SOARES, M.T.C.V.; SILVA, M.P.C.; AGUIAR, J.A. Síntese e caracterização de partículas de magnetita recobertas pelo polissacarídeo fucana. In: ***32a Reunião Anual da Sociedade Brasileira de Química***, **2009**, Fortaleza.

MACIEL, J. C.; SILVA, V.A.J.; ANDRADE, P.L.; AGUIAR, J.A.; NERI, D.F.M.; SILVA, M.P.C.; CARVALHO Jr, L.B. Magnetic iron particles coated with polyaniline (PANI). In: ***IX Brazilian MRS Meeting***, **2010**, Ouro Preto.

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ANDRADE, P.L.; SILVA, V.A.J.; MACIEL, J.C.; SILVA, M.P.C.; AGUIAR, J.A. Study of fucan-coated cobalt ferrite nanoparticles for immobilization of enzyme. In: ***III Simpósio Internacional em Diagnóstico e Terapêutica e VI Jornada Científica do LIKA***, **2011**, Recife.

MOURA, E.M.; MACIEL, J.C.; ANDRADE, P.L.; SILVA, V.A.J.; CARVALHO JR, L.B.; SILVA, M.P.C. Magnetic particles coated with PANI and Fucan for enzyme immobilization. In: ***III Simpósio Internacional em Diagnóstico e Terapêutica e VI Jornada Científica do LIKA***, **2011**, Recife.

MACIEL, J. C.; MOURA, E.M.; ANDRADE, P.L.; NERI, D.F.M.; AGUIAR, J.A.; SILVA, M.P.C.; CARVALHO JR, L.B. Preparation of magnetic microparticles magnetite coated with polyaniline. In: ***III Simpósio Internacional em Diagnóstico e Terapêutica e VI Jornada Científica do LIKA***, **2011**, Recife.

MACIEL, J.C.; MOURA, E.M.; ANDRADE, P.L.; SILVA, V.A.J.; AGUIAR, J.A.; SILVA, M.P.C.; CARVALHO Jr, L.B. Characterization of magnetic particles coated with polyaniline for enzyme immobilization. In: ***X Encontro da Sociedade Brasileira de Pesquisa em Materiais - SBPMat***, **2011**, Gramado.

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7.6. Participação em bancas examinadoras

Helena Paula Oliveira do Nascimento. Imobilização de proteases coaguladoras do leite em quitosana magnetizada. **2008**. Trabalho de Conclusão de Curso (**Graduação em Biologia**) – Universidade Federal de Pernambuco. (**Suplente**).

Larissa Parra de Araújo. Monitorização terapêutica de fármacos. **2009**. Trabalho de Conclusão de Curso (**Graduação em Farmácia**) – Universidade Federal de Pernambuco. (**Titular**).

Anna Carolina Araujo Ferreira da Silva. Protocolo quimioterápico baseado em fluorouracil e leucovorin para tratamento de câncer colorretal: uma revisão de suas variações. **2010**. Trabalho de Conclusão de Curso (**Graduação em Farmácia**) – Universidade Federal de Pernambuco. (**Titular**).

Sílvia Guedes Braga. Terra de diatomáceas magnética revestida com polianilina para imobilização de invertase. **2011**. Trabalho de Conclusão de Curso (**Graduação em Biomedicina**) – Universidade Federal de Pernambuco. (**Titular**).

7.7. Orientações e colaborações

Elaine Martins de Moura. Viabilização de partículas magnéticas revestidas com polímeros para a imobilização de enzimas. **2012**. Dissertação (**Mestrado em Bioquímica e Fisiologia**) – Universidade Federal de Pernambuco. (**Colaboradora**).

Albert Rocha de Oliveira. A importância do diagnóstico precoce e do tratamento adequado para gestantes com sífilis. **2012**. Trabalho de Conclusão de Curso (**Graduação em Farmácia**) - Universidade Federal de Pernambuco. (**Orientadora**).