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Desenvolvimento e Caracterização de Membranas de Quitosana para Recobrimento de Feridas e Liberação Controlada de Fármacos

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Tese de Doutorado apresentada à Faculdade de Engenharia Química como parte dos requisitos exigidos para a obtenção do título de Doutor em Engenharia Química.

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Profa. Dra. Lucia Helena Innocentini-Mei

Dedicatória

Dedico este trabalho a todas as pessoas que amo e que me apoiaram durante esta longa jornada:

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RESUMO

O tratamento de feridas de pele (queimaduras, úlceras, feridas cirúrgicas e de diabetes) é uma problemática mundial que vem sendo amplamente discutida e estudada devido às complicações decorrentes do processo de cicatrização, tais como infecções, septicemia e até óbito.

A pele é a interface do organismo humano com o meio externo e quando esta barreira é ferida, a infecção por microorganismos, bem como a perda de fluidos e desidratação são conseqüências temíveis.

Portanto, um recobrimento de ferida deve ser utilizado afim de proteger a mesma da invasão de microorganismos e da perda de fluidos, além de substituir as funções da pele.

Os recobrimentos de ferida disponíveis comercialmente são principalmente à base de colágeno. Entretanto, devido ao alto custo desta matéria-prima, a busca de novos materiais para a confecção de recobrimentos de ferida vem sendo constante.

A quitosana é um biopolímero derivado da quitina, o polissacarídeo mais abundante na natureza depois da celulose, encontrada principalmente no exoesqueleto de insetos e crustáceos. Além de biocompatível e biodegradável, a quitosana apresenta interessantes propriedades biológicas, tais como atividades bactericida, fungicida, hemostática, antitumoral e imuno-adjuvante.

O processo de produção de quitosana é econômica e ambientalmente viável, pois utiliza subprodutos da indústria pesqueira como matéria-prima. Sendo assim, o preço da quitosana purificada é cerca de 20 vezes menor que o do colágeno na mesma condição.

Por estas razões, a quitosana vem sendo amplamente estudada e empregada no tratamento de feridas, nas formas de filmes, membranas, matrizes porosas, géis e até mesmo soluções.

No sentido de melhorar suas propriedades, plastificantes, reticulantes e outros polímeros também são utilizados em associação com a quitosana afim de se obter um material viável para utilização.

No presente trabalho, membranas de quitosana para recobrimento de feridas foram desenvolvidas, caracterizadas e testadas *in vitro* e *in vivo*. O efeito do D-sorbitol como plastificante e o do hexametileno diisocianato como agente reticulante também foi estudado. Além disso, sistemas de liberação controlada de drogas à base de quitosana foram desenvolvidos e a liberação de gentamicina e sulfadiazina de prata foi avaliada *in vitro*.

O D-sorbitol mostrou-se um bom plastificante para as membranas de quitosana, pois melhorou as propriedades mecânicas e aumentou a permeabilidade ao vapor de água. Além disso, não apresentou citotoxicidades direta e indireta.

O hexametileno diisocianato foi utilizado como agente reticulante da quitosana e foi previamente bloqueado com bissulfito de sódio, afim de proteger os grupos reativos e aumentar o rendimento da reação de reticulação.

As membranas reticuladas de quitosana não apresentaram citotoxicidade e mostraram-se eficientes sistemas de liberação controlada de gentamicina e sulfadiazina de prata.

ABSTRACT

Skin wounds treatment (burns, ulcers, surgical and diabetes wounds) is a world-wide problem that has been widely argued and studied due to the healing process complications, such as infections, septicemia and death.

The skin is the interface of the human organism with the external environment and when this barrier is wounded, microorganism infections, as well as the loss of fluids and dehydration are possible consequences. Therefore, a wound dressing must be used to protect the wound from microorganisms' invasion and loss of fluids, besides substituting functions of the lost skin. Commercially available wound dressings are mainly based on collagen. However, because of the high cost of this raw material, the search for new materials for wound dressings has been continuous. Chitosan is a biopolymer derived from chitin, the most abundant polysaccharide found in nature after cellulose, found mainly in insects and crustaceans exoskeletons. Beyond biocompatible and biodegradable, chitosan also presents interesting biological properties, such as bactericidal, fungicidal, hemostatic, immune-adjuvant and anti-tumor activities. The process of chitosan production is economic and environmentally viable; since it uses fishing industry by-products as raw material. Thus, pure chitosan price is about 20 times cheaper than collagen one. For these reasons, chitosan has been widely studied and employed in the treatment of wounds like film, membrane, porous matrix, gel and solution. Moreover, plasticizers cross linkers and other polymers are also used in association with chitosan to improve the obtained material properties. In this present work, chitosan membranes for wound dressing had been developed and characterized. The use of D-sorbitol as plasticizer and hexamethylene diisocyanate as cross-linker also was investigated. Moreover, chitosan based drug release systems based was developed and the releases of gentamicin and silver sulfadiazine were *in vitro* evaluated.

D-sorbitol revealed a good plasticizer for chitosan membranes: it improved mechanical properties and increased water vapor permeability. Moreover, it did not show direct and indirect cytotoxicity.

Hexamethylene diisocyanate was previously blocked with sodium bisulfite to protect reactive groups and to increase chitosan cross linking reaction yield. Cross linked membranes had not shown cytotoxicity and had revealed to be efficient systems for controlled release of gentamicin and silver sulfadiazine.

SUMÁRIO

1. APRESENTAÇÃO

Esta tese de Doutorado é um dos requisitos exigidos para a obtenção do Título de Doutor em Engenharia Química e será apresentada com os capítulos no formato de trabalhos científicos.

A primeira parte do conteúdo apresentado (Desenvolvimento e Caracterização de Membranas de Quitosana para Recobrimento de Feridas) foi realizada na Universidade Estadual de Campinas, na Faculdade de Engenharia Química, Departamento de Tecnologia de Polímeros, com apoio de outras unidades, tais como a Faculdade de Engenharia de Alimentos e os Institutos de Biologia e Química.

A segunda parte, Desenvolvimento e Caracterização de Membranas de Quitosana Reticulada para Liberação Controlada de Fármacos, foi realizada na University of Texas Health Science Center at San Antonio, na Divisão de Biomateriais da Escola de Odontologia, apoiada pelo Programa de Doutorado com Estágio no Exterior – PDEE/CAPES, sob orientação do Prof. Dr. Ralph Rawls e da Profa. Dra. Neera Satsangi.

2. INTRODUÇÃO

2.1 Problemática

A existência de uma variedade de tipos de feridas com diversos modos e fases de cicatrização contribuiu para a evolução dos recobrimentos atualmente existentes.

Até aos anos 60 (século XX), os recobrimentos de ferida eram considerados produtos passivos, com mínima influência no processo de cicatrização. Porém, a pesquisa pioneira de Winter (1960) iniciou uma nova fase, onde foi considerado o envolvimento ativo do recobrimento com a ferida, com a finalidade de estabelecer e manter um ambiente ótimo para a cicatrização da mesma. Assim, foi dado início a um novo conceito de recobrimento, que resultou na evolução dos materiais passivos para os funcionalmente ativos, onde a interação do recobrimento com a ferida a ser coberta e cicatrizada foi enfatizada (PURNA e BABU, 2000).

O recobrimento de feridas continua sendo uma área de interesse de pesquisadores do mundo todo devido a sua grande importância no tratamento de traumas, queimaduras, ferida de diabetes, úlceras de pele e outros danos teciduais, tais como as feridas pós-cirúrgicas.

Nos Estados Unidos, cerca de 70.000 pessoas são hospitalizadas a cada ano, com ferimentos graves causados por trauma térmico (BLACK, 1996). No Brasil, não se têm dados estatísticos precisos, mas estima-se que quase um milhão de acidentes com queimaduras ocorre por ano, e destes, aproximadamente 200 mil são notificados pelos hospitais e cerca de 10 mil causam falecimento das vítimas (Cidade Sustentável, 2004). De acordo com os dados do Ministério da Saúde, o Sistema Único de Saúde (SUS) atendeu 27.906 pacientes queimados em 1998 e 27.694 em 1999. No ano seguinte, o governo brasileiro gastou 55 milhões de reais no tratamento de queimaduras (Portal Saúde.Gov, 2005).

O diabetes atinge cerca de dez milhões de pessoas no Brasil, e a incidência desta doença só tende a aumentar devido aos fatores de risco da população, tais como: sedentarismo, estresse, tabagismo e obesidade

(LAVA, 2005). Dentre os possíveis distúrbios, o diabetes pode causar úlceras no pé (feridas) e infecções que podem evoluir para o caso extremo de amputação.

No caso das úlceras de pele, estas podem ser causadas por diversos fatores: varizes, hipertensão arterial, anemia falciforme, diabetes, isquemia e infecções. Devido à grande incidência de anemia e infecções nas populações mais carentes, este tipo de ferida apresenta grande incidência no Brasil e nos demais países subdesenvolvidos.

No âmbito da saúde mundial, as feridas pós-cirúrgicas apresentam grande preocupação pelo fato de serem consideradas alvos fáceis para a instalação de infecção, principalmente da temida infecção hospitalar. Nos Estados Unidos, estima-se que das 23 milhões de cirurgias realizadas por ano, 920 mil desenvolvem infecções da ferida operatória, o que está associado ao aumento do tempo de internação, das taxas de letalidade e dos gastos hospitalares (KLUYTMANS, 1997). No Brasil, de acordo com o estudo prospectivo efetuado em um hospital universitário entre 1995 e 1999 por FERRAZ et al. (2001) aproximadamente 10% das feridas cirúrgicas apresentam infecção.

O tratamento da ferida infectada exige, além de um maior tempo de internação do paciente, o uso de antibióticos de terceira geração que, no Brasil, custam em média R$ 600/dose (InventaBrasilNet, 2001).

Neste contexto, o desenvolvimento de um recobrimento de feridas eficiente tem um papel fundamental no tratamento das lesões de pele, na prevenção de infecções, bem como na melhoria da qualidade de vida dos pacientes e na redução dos gastos, especialmente do governo, com internações hospitalares e tratamentos.

Para tanto, um recobrimento de ferida ideal deve substituir as funções da pele perdida: proteger a ferida da perda de fluidos e proteínas; prevenir a invasão de bactérias; dissipar o estresse mecânico externo; e, bem como melhorar e estimular a cicatrização da ferida. Além disso, o material deve

ser biocompatível para evitar rejeição e apresentar um custo acessível, para que sua utilização seja economicamente viável.

2.2 A Quitosana

A quitosana é um polímero derivado da quitina, o segundo polissacarídeo mais abundante na natureza, encontrado principalmente no exoesqueleto de insetos e nas carapaças de crustáceos. Pode ser encontrada na parede celular de algumas espécies de fungos, porém sua maior fonte de obtenção é através da desacetilação da quitina. No Brasil, foi criada, em 1997, uma empresa de base tecnológica, incubada no Parque de Desenvolvimento Tecnológico da Universidade Federal do Ceará e especializada na extração de quitina e produção de quitosana, obtidas a partir de carapaças de camarão, lagosta e caranguejo extraídos da costa brasileira (Polymar, 2004). O baixo custo de produção, a abundância de matéria-prima (fonte renovável) e o aproveitamento dos subprodutos da pesca de crustáceos tornam o processo de produção de quitosana ecologicamente interessante e economicamente viável.

Além de ser biocompatível, a quitosana é biodegradável e apresenta baixa toxicidade. Também apresenta atividades antimicrobiana, antitumoral e imuno-adjuvante (KURITA, 1998). Ela é capaz de acelerar o processo de cicatrização, através da ativação de macrófagos e fibroblastos, aumentando a síntese de ácido hialurônico e sua deposição no tecido conjuntivo regenerativo, bem como influenciando no depósito de colágeno nas matrizes extra-celulares durante a reconstrução do tecido invalidado (SYNOWIECKI e AL-KHATEEB, 2003).

Por todas estas razões, a quitosana foi escolhida como material de estudo do presente projeto, que envolve a caracterização deste material, bem como o desenvolvimento de um recobrimento de ferida baseado no mesmo.

Nas Figuras (1) e (2) têm-se as fórmulas estruturais da quitina e da quitosana, apresentando como principal diferença entre elas, a presença de grupos acetil em maior número na cadeia de quitina.

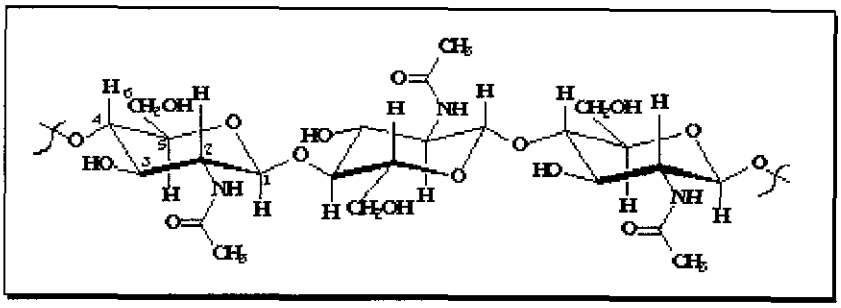


Figura 1: Estrutura da quitina.

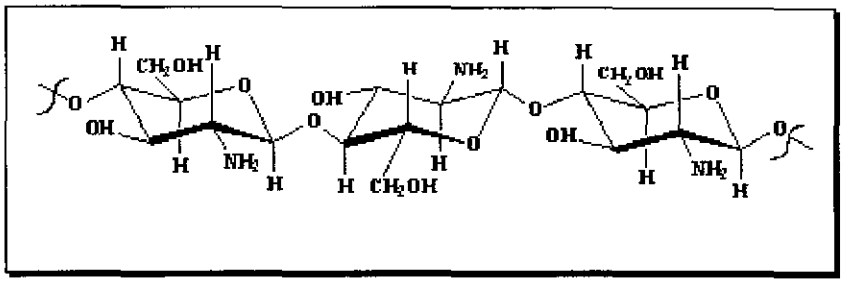


Figura 2: Estrutura da quitosana.

O grau de desacetilação, ou seja, a quantidade de grupos acetil removidos na desacetilação da quitina, é um importante parâmetro de classificação da quitosana, assim como o peso molecular.

Estes parâmetros têm influência direta nas atividades antibacteriana, antitumoral e imuno-adjuvante, bem nas propriedades mecânicas da quitosana.

MAEDA e KIMURA (2004) avaliaram o efeito do peso molecular da quitosana na sua atividade antitumoral e observaram que as moléculas de menor peso molecular promoveram uma diminuição no crescimento dos tumores.

KURITA (1998) avaliou o efeito do peso molecular na atividade antibacteriana da quitosana contra algumas espécies de *Streptococcus* e constatou que quanto maior o peso molecular, maior a repressão do crescimento desta bactéria.

WELING e colaboradores (2005) estudaram o efeito do grau de desacetilação da quitosana na cristalinidade, tração e compatibilidade com células Schawnn e concluíram que, para um mesmo peso molecular, o

aumento do grau de desacetilação promove um aumento na cristalinidade, tração e afinidade com células Schawnn.

A quitosana é solúvel em meio ácido e insolúvel em meio alcalino e na água. Em pH fisiológico, apresenta carga positiva, o que promove a bioadesão da mesma. Devido à protonação em meio ácido dos grupos amino presentes em sua cadeia carbônica, além das hidroxilas provenientes dos grupos álcool, a quitosana apresenta caráter higroscópico e é capaz de formar gel, podendo absorver até 400% da sua massa inicial em meio aquoso.

Dessa forma, o desenvolvimento de sistemas de liberação controlada de fármacos por difusão à base de quitosana seria inviável sem a utilização de um agente reticulador, que manteria as moléculas interligadas e diminuiria o grau de inchamento da mesma.

2.3 O Agente Reticulador

A reticulação entre cadeias poliméricas é obtida através da reação química entre os grupos funcionais das mesma. Ela pode ocorrer através da adição de um iniciador, quando a própria cadeia polimérica apresenta grupos funcionais reativos entre si; ou pela a adição de moléculas reativas e multifuncionais (reticulantes ou agentes reticuladores). Quando introduzidos no meio polimérico, os agentes reticulantes reagem em suas extremidades funcionais com determinados grupos funcionais presentes nas cadeias poliméricas, fazendo a ligação entre as mesmas. Dessa forma, uma rede polimérica é formada, promovendo uma maior interação entre as moléculas e significativas mudanças nas propriedades físicas e químicas do polímero inicial.

No caso da quitosana, as reações de reticulação podem ocorrer em dois grupos funcionais da molécula, isto é, nos grupos amino e nas hidroxilas. Além disso, modificações químicas também podem ser feitas a partir do grupo N-acetil, de acordo com o grau de desacetilação da quitosana. Diferentes propriedades são obtidas de acordo com o grupo funcional envolvido, bem como com o agente reticulador utilizado e com o grau de reticulação da reação.

A epicloridrina é um exemplo de agente reticulador que se interliga às hidroxilas da quitosana. Já o glutaraldeído, um dos reticulantes mais usados, é responsável pela reação entre os grupos amino da quitosana e os grupos aldeídos do glutaraldeído. Entretanto, esta reação, apesar de melhorar as propriedades químicas do material, promove uma diminuição das propriedades mecânicas, deixando o material mais quebradiço.

Dessa forma, o principal desafio deste projeto foi encontrar um agente reticulante capaz de interligar as cadeias poliméricas sem interferir negativamente nas propriedades do polímero. Além disso, o material resultante da reticulação deveria manter ou aumentar a biocompatibilidade do polímero inicial.

Os diisocianatos (Figura 3) são moléculas bifuncionais e de elevada reatividade, amplamente utilizadas como agentes reticulantes, principalmente na produção de poliuretanos, incluindo os utilizados na área médica. Na Figura 3, o radical R pode ser um grupo alifático ou aromático e tem uma forte influência nas propriedades do material resultante da reação. Dessa forma, é possível controlar as características do produto final, variando-se o radical R do diisocianato utilizado.

Figura 3. Fórmula molecular geral dos diisocianatos.

Neste presente trabalho, o 1,6-Diisocianato hexano ou Hexametileno diisocianato (Figura 4) foi escolhido por ser uma molécula linear, flexível, devido à presença dos grupos $-CH_2$, e simétrica, com os dois grupos cianatos (- N = C = O) de igual reatividade. Além disso, a escolha de um radical alifático insaturado aumenta a estabilidade do produto final, evitando o amarelamento do mesmo com o tempo de estocagem.

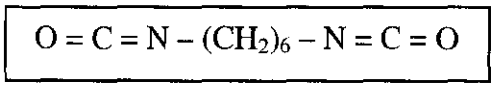


Figura 4. Fórmula molecular do hexametileno diisocianato.

O hexametileno diisocianato, bem como a maioria dos isocianatos, é insolúvel em água, além de reagir com a mesma. Dessa forma, é necessária uma reação prévia de proteção dos grupos reativos (cianatos) para evitar a reação com a água e, ao mesmo tempo, promover a dissolução do mesmo em meio aquoso, uma vez que a reação com quitosana ocorreria em solução aquosa de ácido acético.

Os bissulfitos são exemplos de sais utilizados nas reações de proteção dos diisocianatos. O bissulfito de sódio ($Na_2S_2O_5$), um sal bastante reativo e solúvel em água, foi utilizado na reação de bloqueio dos grupos cianatos do hexametileno diisocianato, obtendo-se o hexametileno -1,6 – di – (aminocarboxisulfonato) como produto principal da reação (Figura 5) (WELSH et al., 2002).

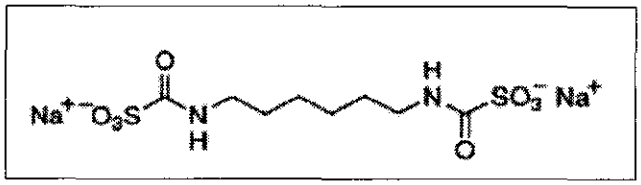


Figura 5. Fórmula estrutural do hexametileno -1,6 -di - (aminocarboxisulfonato).

O hexametileno - 1,6 - di - (aminocarboxisulfonato) é solúvel em água e além disso, estável em soluções aquosas ácidas. Dessa forma, a reação de reticulação da quitosana ocorre a partir da formação de ligações uréia, já que a taxa de reação do hexametileno - 1,6 -di - (aminocarboxisulfonato) com o grupo amina em meio ácido é muito maior do que a taxa de reação competitiva com os grupos álcool ou água.

2.4 Os Fármacos

O tratamento de feridas de pele, na maioria dos casos, exige a aplicação de um medicamento anti-microbiano para evitar ou combater a infecção. Devido à grande exposição da pele ao meio externo, o contato com os microorganismos é constante e inevitável. Os microorganismos mais comumente encontrados em feridas de pele são o *Staphylococcus aureus* e a *Pseudomonas aeruginosa*, espécies gram positivo e gram negativo, respectivamente. Dessa forma, seria interessante encontrar um agente antimicrobiano capaz de matar ou inibir o crescimento de pelo menos essas duas espécies de bactéria.

Dentre os agentes antimicrobianos disponíveis, a gentamicina é um antibiótico pertencente à família dos aminoglicosídeos, isolada em 1963, a partir do fungo de solo *Micromonospora purpura,* por Weinstein e seus colaboradores. Foi introduzida nos Estados Unidos da América em 1969 e, desde então, tem sido utilizada muito no tratamento de diversas infecções, devido ao seu baixo custo e ampla faixa de atividade antibacteriana.

A gentamicina tem dois aminoglicoses ligados a um núcleo hexose através de uma ligação glicosídea e tem sua fórmula estrutural apresentada na Figura 6:

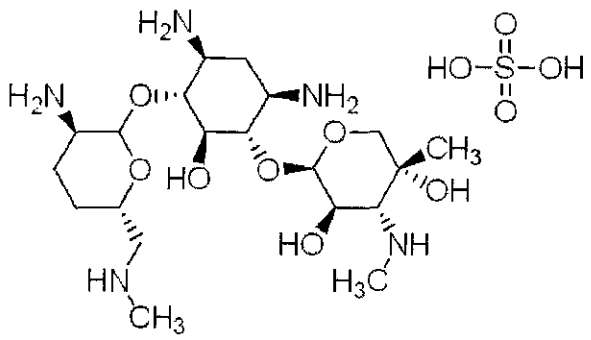
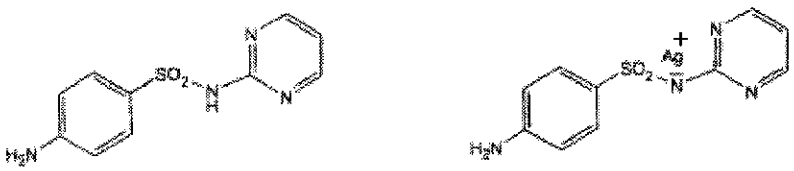


Figura 6. Fórmula estrutural do sulfato de gentamicina.

O sulfato de gentamicina é altamente solúvel em água; moderadamente solúvel em etanol, metanol e acetona e praticamente insolúvel em benzeno e hidrocarbonetos halogenados. Uma solução a 4% em água apresenta pH entre 3,5 e 5,5.

As concentrações mínimas (CMI) de gentamicina para a inibição do crescimento de *Pseudomonas aeruginosa* e *Escherichia coli* variam de 0,06 a 8 µg/mL e de 0,12 a 1,0 µg/mL para o *Staphylococcus aureus.* (SKUNDRIC et al., 2002)

Outro agente antibacteriano testado no presente trabalho foi a sulfadiazina de prata. A sulfadiazina (Figura 7a) é um antibiótico que elimina as bactérias através da inibição da produção de ácido fólico no interior de suas células. Ela é comumente usada em associação com prata (Figura 7b) no tratamento de feridas de pele, sendo um tratamento padrão para queimaduras, úlceras e feridas infectadas devido ao seu amplo espectro de atividade bactericida contra as espécies gram positivo e negativo.



(a) (b)

Figura 7. Fórmula estrutural da (a) sulfadiazina e (b) da sulfadiazina de prata.

As concentrações mínimas de inibição da sulfadiazina de prata são de 32,0 µg/mL para a *Escherichia coli,* 44,8 µg/mL para a *Pseudomonas aeruginosa* e 57,6 µg/mL para o *Staphylococcus aureus.* Além disso, as concentrações mínimas para efeito bactericida (CMB) são de 40,2 µg/mL e 51,2 µg/mL para *Escherichia coli* e *Pseudomonas aeruginosa*, respectivamente. Para a *Staphylococcus aureus* a CMB é igual à CMI. (SHANMUGASUNDARAM et al., 2005)

3. REVISÃO BIBLIOGRÁFICA

Recobrimento de feridas ou pele artificial é atualmente uma interessante área de pesquisa devido à sua grande importância no tratamento de traumas, queimaduras, feridas de diabete, úlceras de pele e outros danos cutâneos, tais como feridas cirúrgicas. (DENKBAS et al., 2004)

As principais funções de um recobrimento de ferida são:

- Substituir a função da pele perdida;
- Proteger a ferida da perda de fluidos e proteínas;
- Prevenir a invasão de bactérias;
- Dissipar o stress mecânico;
- Melhorar e estimular a cicatrização;
- Promover adequadas trocas gasosas e de calor com o ambiente externo;
- Ser biocompatível e não-tóxico.

Pesquisas desenvolvidas na área biomédica geraram várias opções comerciais de recobrimentos de ferida , tais como: Bioprocess®, Opsite® e Biobrane®, produzidos a partir de colágeno, ácido hialurônico e outros biomateriais. Entretanto, tais produtos apresentam alto custo, o que dificulta a aplicação e comercialização dos mesmos, principalmente nos países emergentes.

A Tabela 1 descreve outros exemplos de recobrimentos utilizados no tratamento de feridas, entretanto, estes também apresentam alto custo, além de outros inconvenientes.

Neste contexto, a busca por produtos econômica e funcionalmente viáveis ainda é uma realidade nesta área de pesquisa.

A quitosana é um biomaterial que vêm sendo extensivamente estudado devido às suas interessantes propriedades. Além de biodegradável e biocompatível, a quitosana apresenta atividades antibacteriana, fungicida,

hemostática, imuno-adjuvante e antitumoral. (KURITA, 1998)

A quitosana também apresenta vantagens econômico-ambientais, uma vez que a mesma é produzida a partir da desacetilação da quitina, extraída principalmente dos subprodutos da indústria pesqueira e cuja produção biológica anual é de cerca de 1 bilhão de toneladas. Além disso, o preço da quitosana purificada é cerca de 20 vezes menor que o do colágeno na mesma condição, o que a torna ainda mais atrativa em um país em desenvolvimento como o Brasil.

Tabela 1 – Alternativas ao tratamento de feridas.

TIPO	DESCRIÇÃO	INCONVENIENTES
Sintéticos e Semi-sintéticos	**Biobrane:** borracha de silicone/ nylon/peptídeos derivados de colágeno de porco; **Epigard:** poliuretano reticulado/ filme de poliuretano poroso; **Outros:** poli-tetra-flúor-etileno ou poliuretano/pectina ou metil celulose.	Os recobrimentos poliméricos somente são usados em feridas pequenas, capazes de cicatrizar pelo movimento das células epiteliais através da ferida, a partir das bordas; **Alto custo.**
Compostos de Colágeno	**Yannas:** camada externa de silicone e subcamada esponjosa de colágeno reticulado com glutaraldeído.	Substituto temporário; Necessita de enxerto posterior; **Alto custo do colágeno.**
Células de derme e epiderme	Consiste em uma matriz de células epidermais e fibroblastos semeados.	21 dias para o crescimento da cultura de células epidermais; **Alto custo.**

Diversos trabalhos envolvendo o uso de quitosana na preparação de recobrimento de feridas já foram publicados. Dentre eles, o estudo de MI e seus colaboradores (2001) relatou o desenvolvimento de uma membrana assimétrica de quitosana composta por duas camadas, sendo uma densa e a outra porosa, com o intuito de melhor controlar as trocas gasosas e de fluído com o meio externo.

SILVA e colaboradores (2004) também prepararam membranas de quitosana, porém utilizaram o glutaraldeído como agente reticulador. Além

da aplicabilidade na área biomédica, os autores também avaliaram as propriedades mecânicas do material obtido.

LIN-GIBSON (2003) e WELSH et al. (2002) investigaram a cinética da reação de reticulação de quitosana com hexametileno diisocianato nas fases líquida e sólida, com a finalidade de utilizar o produto obtido para recobrir feridas.

Um trabalho também interessante foi desenvolvido por OSKAYANAK e colaboradores (2005), os quais prepararam membranas para recobrimentos de feridas à base de poliuretano, obtido a partir de fontes alternativas ao petróleo.

Além destes, diversos outros trabalhos sobre blendas poliméricas, também utilizadas na preparação de membranas para recobrimento de feridas, vêm sendo publicados. Dentre os polímeros naturais, o colágeno, o alginato e o ácido hialurônico são os principais exemplos de materiais misturados com quitosana.

Além disso, polímeros sintéticos também estão sendo usados na preparação de substitutos de pele. MANGALA e colaboradores (2003) desenvolveram uma membrana para recobrimento de queimaduras à base de quitosana e poli (álcool vinílico).

Os recobrimentos de feridas também podem ser utilizados como suporte para liberação controlada de drogas, uma vez que as feridas são grandes focos para a instalação de infecção por microorganismos. *Staphylococcus aureus*, *Pseudomonas aeruginosa* e *Escherichia coli* são os potenciais patógenos encontrados em ferida de pele.

As drogas mais comuns associadas ao tratamento de feridas de pele são os antibióticos. DENKBAS e colaboradores (2004) desenvolveram esponjas de quitosana impregnadas de norfaloxina para recobrimento de ferida e, ao mesmo tempo, liberação controlada do antibiótico. LOKE e colaboradores (1999) desenvolveram um recobrimento de ferida à base de quitosana capaz de liberar clorexidina. CRUZ e colaboradores (2004) estudaram a liberação controlada de oxitetraciclina em matrizes de

quitosana e quitosana/alginato em diferentes pHs, porém o sistema de liberação não foi aplicado como recobrimento de ferida.

A gentamicina (Figura 6) é um antibiótico pertencente à família dos aminoglicosídeos, introduzido nos Estados Unidos da América em 1969 e, desde então, é mundialmente utilizada no tratamento de diversas infecções, devido ao seu baixo custo e ampla faixa de atividade antibacteriana. ZHANG e ZAHANG (2002) desenvolveram um suporte à base de quitosana e cálcio, impregnado com gentamicina, para ser usado no tratamento de osteomielite. Além deste, diversos outros trabalhos foram encontrados na literatura abordando a liberação controlada de gentamicina para o tratamento de infecções ósseas (VIRTO et al., 2002; NARAHARISETTI et al., 2005 e ZHANG et al., 1994). Entretanto, nenhum trabalho abordando a liberação controlada de gentamicina a partir de recobrimentos de feridas de pele foi encontrado, mesmo sendo o tratamento de feridas com gentamicina um procedimento muito comum.

Outro medicamento bastante utilizado como agente antimicrobiano, principalmente no tratamento de queimaduras, é a sulfadiazina de prata (Figura 7b). Devido a sua ampla faixa de atividade antibacteriana, a sulfadiazina de prata tem sido um dos fármacos mais estudados na área de recobrimentos de feridas e liberação controlada. Em 2003, MI e colaboradores apresentaram um novo tipo de recobrimento de ferida carregado com sulfadiazina, enquanto que YU et al. (2005) investigou a atividade antibacteriana de esponjas de quitosana e alginato incorporadas com sulfadiazina de prata.

Alguns trabalhos relatam o uso de antibióticos no tratamento de feridas e os efeitos deletérios que eles podem causar ao crescimento celular. CHO LEE e colaboradores (2002) mostraram o efeito citotóxico da sulfadiazina de prata, bem como o atraso no processo de epitelização, em um modelo de queimadura de segundo grau em animais.

Devido à grande complexidade que envolve o processo de cicatrização de feridas, diversos fatores devem ser considerados na elaboração de um

recobrimento para as mesmas. Os recobrimentos cientificamente propostos muitas vezes atendem somente a algumas das exigências e, portanto, a busca por um recobrimento de feridas ideal ainda é contínua.

Sendo assim, este trabalho teve como objetivo principal contribuir para o desenvolvimento de um recobrimento de ferida que fosse econômica, científica e funcionalmente viável.

4. OBJETIVOS

O objetivo geral deste trabalho foi o desenvolvimento e a caracterização de membranas à base de quitosana para recobrimento de feridas liberação controlada de fármacos à base de quitosana.

Os objetivos específicos foram:

- Aprimoramento da técnica de preparação de membranas de quitosana, através da definição de parâmetros, tais como: temperatura e tempo de secagem;
- Avaliação do efeito da neutralização na preparação e nas características finais das membranas de quitosana;
- Avaliação do efeito da adição de plastificante;
- Avaliação da citotoxicidade das membranas de quitosana;
- Avaliação da atividade antibacteriana das soluções de quitosana;
- Caracterização morfológica das membranas por Microscopia Eletrônica de Varredura e Difractometria de Raios-X;
- Caracterização das membranas por Calorimetria Diferencial Exploratória, afim de se obter os valores das temperaturas de fusão e transição vítrea e entalpia de fusão;
- Avaliação da permeabilidade ao vapor de água das membranas de quitosana, bem como parâmetros de solubilidade e umidade;

Os objetivos do estágio de doutorado no exterior, realizado na divisão de biomateriais da Universidade do Texas em San Antonio – EUA, foram:

- Estudo da aplicação de um agente reticulante alternativo para a reticulação da quitosana;
- Desenvolvimento das técnicas de reticulação da quitosana e de preparação de membranas reticuladas;

- Avaliação dos efeitos da reticulação da quitosana, bem como caracterização das membranas reticuladas;
- Avaliação do uso das membranas de quitosana reticuladas como sistema de liberação controlada de fármacos;
- Avaliação da liberação controlada dos fármacos estudados;
- Caracterização *in vitro* das membranas reticuladas de quitosana como sistemas de liberação controlada de fármacos.

5. TRABALHOS CIENTÍFICOS

4.1 Effects of Neutralization Process on Preparation and Characterization of Chitosan Membranes for Wound Dressing

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Efeitos do processo de neutralização na preparação e caracterização de membranas de quitosana para recobrimento de feridas

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Resumo: Os efeitos da neutralização na preparação e caracterização de membranas de quitosana foram avaliados por Calorimetria Diferencial Exploratória e por Microscopia Eletrônica de Varredura. A solubilidade em água e a umidade das membranas de quitosana também foram estudadas. A absorção de água em diferentes pHs foi avaliada e as propriedades mecânicas, tais como tração e deformação na ruptura foram medidas. A neutralização provocou um aumento na temperatura de transição vítrea das membranas de quitosana, além de uma diminuição na solubilidade em água, umidade e absorção de água. Um aumento nas propriedades mecânicas também foi observado após a neutralização.

Palavras-chave: biomateriais; quitosana; membranas; recobrimento de feridas; processo de neutralização.

Effects of neutralization process on preparation and characterization of chitosan membranes for wound dressing

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Summary: Neutralization process effects on preparation and characterization of chitosan membranes were evaluated by Differencial Scanning Calorimetry and Scanning Electron Microscopy. Water solubility and humidity of chitosan membranes were also studied. Swelling behavoir in different pH media was evaluated and mechanical properties such as tensile strength and elongation at break were measured. Neutralization process increased glass transition temperature of chitosan membranes and decreased their water solubility, humidity and water sorption. An improvement in mechanical properties of chitosan membranes was also observed after neutralization process.

Keywords: biomaterials; chitosan; membranes; wound dressing; neutralization process.

Introduction

Wound dressing is an area of current research interest due to its great importance in treatment of traumas, burns, diabetes wound, skin ulcers and other skin damages, such as surgical wounds[1].

An ideal wound dressing should replace the function of lost skin, protect wounds from fluid and protein losses, prevent bacterial invasion, and dissipate mechanical stress (external), and finally, improve and stimulate

wound healing[2]. Moreover, it should promote adequate gaseous and heat exchange with environment and be biocompatible and non-antigenic to avoid body rejection.[3]

Developments in the area of biomaterials offer several commercial wound dressings like Bioprocess®, Opsite® and Biobrane II®, based on collagen, hyaluronic acid and other biomaterials. However, they do not have a very accessible cost.

Chitosan is a biopolymer obtained by the deacetylation of chitin, which is the second more abundant polysaccharide found in nature. Chitin is mainly found in external skeleton of insects and in shells of crustaceans. Thus, chitosan production process is ecologically interesting and economically viable due to the use of seafood industry by-products.[4]

Moreover, chitosan exhibits various interesting biological activities, which made this polysaccharide increasingly important. Typical activities include antitumoral, immunoadjuvant, antibacterial and hemostatic activities.[5] Chitosan is metabolized by certain human enzymes, especially lysozyme, and is considered biodegradable.[6] It also can accelerate wound healing.[7] In addition, due to its positive charges at physiological pH, chitosan is bioadesive, which increases retention at the site of application.[8] For all those reasons, chitosan is one of the most important biomaterials for wound management in the recent years.

In this present study, chitosan membranes for wound dressing were prepared by a solvent evaporation technique and then neutralized, in order to evaluate the effects of neutralization process on their preparation and characterization.

Experimental

Materials

High molecular weight chitosan (Mw ~ 100,000), more than 75% deacetylated, was purchased from Aldrich Chemical Company (USA). Glacial acetic acid was purchased from Synth (Brazil). Chitosan was dissolved in an acetic acid aqueous solution (1.0 % w/w) and then was

filtered to remove any undissolved impurities.

Preparation of the membranes

Chitosan membranes were prepared by a solvent evaporation technique by using aqueous acetic acid (1.0 % w/w) as solvent. Chitosan solution was prepared by dissolving 1.5g of chitosan in 100 ml of solvent. After complete dissolution, it was filtered and cast on Petri plates. Following this, chitosan membrane (C1) was obtained by solution evaporation in an oven at 40°C. Neutralized chitosan membrane (C2) was prepared by immersion of C1 into NaOH (2% w/w)- Na_2CO_3 (0.05% w/w) aqueous solution for 1 hour. After the immersion, it was repeatedly washed with distillated water and dried at 40° C.

Modulated Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) measurements were performed on a DSC 2920 -TA Instruments. DSC curves of chitosan membranes (C1 and C2) were obtained by heating samples from –130°C up to 200° C, at heating rate of 2° C min^{-1} and modulation rate ±1° C min^{-1}, under nitrogen atmosphere, in order to estimate glass transition and melting temperatures.

Scanning Electron Microscopy

Chitosan membranes samples were cut and kept in a vacuum desiccator for 48 hours. Then, samples were coated with gold/paladium using a SC 7620 Sputter Coater – POLARON under high vacuum and 4 mA for 180 seconds. Coated samples were examined using a LEO 440i Scanning Electron Microscopy (SEM).

Water solubility and humidity

Percentage of humidity in chitosan membranes was determined by gravimetric method. Circular membranes samples (D=2 cm) were kept in a vacuum desiccator for 24 hours and weighed to obtain the initial mass (Mi). After this procedure, they were dried at 100° C for 24 hours and weighed again in order to determine the final mass (Mf).

The percentage of humidity was calculated according to the following

equation:

$$H(\%) = (Mi - Mf) * 100 / Mi \qquad \text{(Eq. 1)}$$

Chitosan membranes water solubility was determined according to Gontard et al.[9]. Circular samples (D=2 cm) were dried at 100° C for 24 hours and weighted to obtain their dry mass (Wi). After this procedure, they were immersed into 50 ml of water for 24 hours at 25° C by slowly string. Samples were then taken out and dried (100° C for 24 hours) to determine the membrane weight not soluble in water (Wf). The percentage of water solubility (W) was calculated from the following equation:

$$W(\%) = (Wi - Wf) * 100 / Wi \qquad \text{(Eq. 2)}$$

Each experiment was repeated three times and the average value was calculated.

Swelling behavior

Swelling behavior of chitosan membranes was determined by the gravimetric method. Square membranes samples (L=2 cm) were kept in a vacuum desiccator for 24 hours and their dry mass (W_D) were determined. After this procedure, they were immersed in pH 7.4 and 5.6 of phosphate buffer saline solution at 37 °C. After pre-determined periods (5, 20, 40, 60, 80, 100, 120 and 180 minutes), wet weights (W_W) of chitosan membranes were determined by first blotting the membranes with filter paper to remove adsorbed water on the surface, then immediately weighted on an electronic scale. The swelling ratio (S) of chitosan membranes in the media was calculated from the following equation:

$$S(\%) = (W_W - W_D) * 100 / W_W \qquad \text{(Eq. 3)}$$

Each experiment was repeated three times and the average value was taken as the swelling ratio.

Mechanical properties

Eletronic Digital Caliper (Fowler & NSK – Max-Cal) was used to measure

membranes thickness (δ). TA-XT2 (SMS, Surrey, UK) instrument was used to measure tensile strength (TS) and percent elongation at break (E). Tests were carried out according to ASTM D-882[10], with initial grip separation of 50 mm and cross head speed of 60 mm/min. TS was calculated by dividing the maximum load for breaking film by cross sectional area and E was determined by dividing film elongation at rupture by initial gauge length and multiplying by 100%.

Results and discussion

Differential Scanning Calorimetry

DSC results are presented in Table 1.

Table 1. Thermal properties of chitosan membranes.

Sample	Tg_1 ℃	Tg_2 ℃	Tg_3 ℃	Tm ℃	ΔH J/g
C1	-81.70	-84.10	-73.69	101.95	458.70
C2	-76.90	-75.63	-73.16	88.39	418.50

DSC analysis presented lower value of glass transition temperature for C1 and lower values of enthalpy and melting temperature for C2. It can be attributed to acetic acid molecules that act as plastcizer in C1, increasing its mobility. On the other side, neutralization process removes acetic acid molecules from C2 and promotes a molecular rearrangement of polymer chains, which is responsible for a decrease in the free volume of the system and an increase in its glass transition temperature.

Scanning Electron Microscopy

C1 and C2 scanning electron micrographs are presented in Figures 1 and 2, respectively. SEM micrographies showed that C1 surface is more regular than C2 surface, which presented some rugosity. It may be attributed to the neutralization process that removes superficial acetic acid molecules from C2. Moreover, they showed that both C1 and C2 are dense and packed membranes.

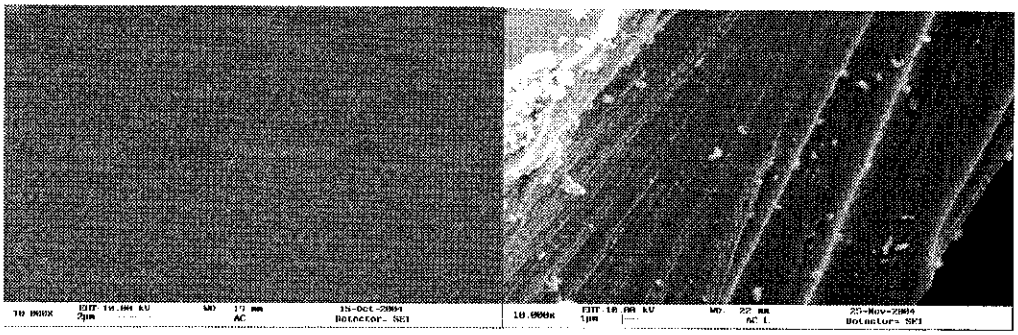

Figure 1. Superficial and Internal morphologies of C1.

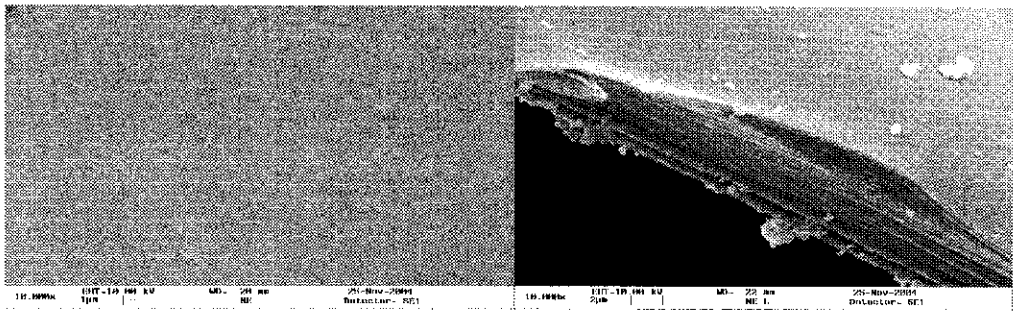

Figure 2. Superficial and Internal morphologies of C2.

Water solubility and humidity

Some parameters such as percentage of humidity (H) and of water solubility (S); thickness (δ), tensile strength (TS) and percent elongation at break (E) obtained for C1 and C2 membranes are presented in Table 2.

Table 2. Properties of chitosan membranes.

Sample	**H**	**W**	**δ**	**TS**	**E**
	%	**%**	**μm**	**MPa**	**%**
C1	17.42	20.28	40	122.31	23.45
C2	12.11	2.34	50	140.54	8.04

Chitosan is insoluble in neutral and alkaline media, but it is soluble in acid one. Residual acetic acid of C1 interacts with water and forms a weak acid solution. For this reason, C1 is much more soluble than C2. The largest humidity of C1 compared to C2 one can be attributed to the presence of acetic acid molecules in C1, which increases its hydrophilic character.

Swelling behavior

Swelling behavior of C1 and C2 at different pH's is presented in Figure 3.

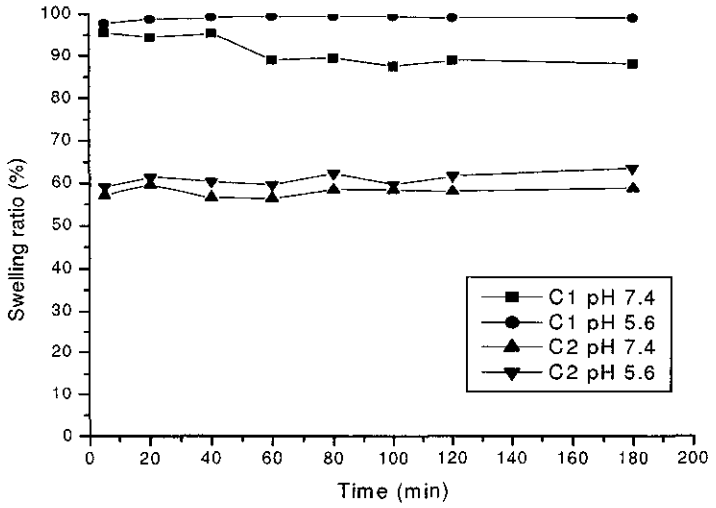

Figure 3. Swelling behavior of chitosan membranes at pH 7.4 and 5.6.

According to Figure 3, C1 presented higher water absorption in acid medium (pH 5.6) than in physiological medium (pH 7.4). It can be attributed to the protonation of chitosan amine groups in acid medium, that increases its hydrophilicity. As soon as C1 was immersed in pH 5.6 solution, it swelled and presented a gel aspect, which had lead to a more difficult removal of adsorbed water on the surface. Samples immersed in that solution for 120 and 180 minutes could not be totally taken off, since part of them were dissolved. This fact can explain the swelling ratio decreasing for those times. When immersed in pH 7.4 solution, C1 also swelled and got a gel consistence. However, it was only observed in the first three points (5, 20 and 40 minutes). After those periods, C1 probably reached the equilibrium state with the medium and its water absorption was decreased.

C2 swelling ratio was also higher in acid medium due to protonation of amine groups. Nevertheless, when compared to C1 swelling ratio, C2 one was lower in both pH solution. This can be attributed to neutralization process that removed acetic acid molecules from C2 and decreased its hydrophilicity.

Swelling behavior is an important parameter for wound dressing management. An ideal wound dressing should absorb fluids excreted by the wound to avoid accumulation of fluid between wound and dressing because of the risk of infection[13]. However, wound dressing should maintain its properties to continue replacing skin lost functions. In that way, C2 presented better results than C1, because it maintained its shape and consistence when swollen.

Mechanical properties

Skin is a viscoelastic material and is permanently subjected to slight stress. Tensile strength of skin is very dependent on the rate of loading and varies with the direction of the stress. Moreover, it is different depending on the gender, age and site. An average tensile strength of 1.8 kgf mm^{-2} (~17.6 MPa) was quoted by Wohlish[11]. However, tensile strength of wounds is weaker than skin one and varies during wound healing process.[12]

Tensile Strength (TS) and percent Elongation at break (E) of C1 and C2 are presented in Table 2. C1 presented lower tensile strength and higher elongation at break than C2, what can be explained by the presence of acetic acid molecules in C1, that act as plasticizer, lowering the tensile strength and improving the elongation at break.

According to skin tensile strength average, both chitosan membranes can replace mechanical functions of lost skin, due to their tensile strength almost 10 times higher than the skin.

Conclusions

Neutralization process promoted a molecular rearrangement on C2 due to acetic acid molecules removal and decreased free volume around the polymer chains. In addition, neutralization process reduced water solubility, humidity and water sorption of chitosan membrane by removing hydrophilic groups (acetic acid). An improvement on chitosan membrane tensile strength and a decrease on its elongation at break were observed after neutralization process and they were also attributed to the removal of acetic acid molecules that acted as a plasticizer.

Finally, neutralization process is an important step on preparation of chitosan membranes as a biomaterial for wound dressing; and also this process retards acid hidrolysis of chitosan.

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Antibacterial Activity of Chitosan Solutions for Wound Dressing

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Atividade Antibacteriana de Soluções de Quitosana para Recobrimento de Feridas

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Resumo: A quitosana tem encontrado ampla aplicação no campo biomédico devido às suas interessantes propriedades biológicas que incluem: biocompatibilidade, biodegradabilidade, atividade hemostática e efeito bacteriostático. Neste presente trabalho, a atividade antibacteriana das soluções de quitosana para recobrimento de feridas foi investigada contra *Staphylococcus aureus* (isolada de um lodo ativado) e *Escherichia coli* (ATCC 25922) que são patógenos de feridas em potencial. Além disso, os efeitos da adição de plastificante e da concentração da solução de quitosana na atividade antibacteriana foram avaliados. De acordo com o estudo da atividade antibacteriana, as soluções de quitosana, plastificadas ou não, mostraram, atividade inibitória ao crescimento de *Escherichia coli*. Entretanto, elas não inibiram o crescimento de *Staphylococcus aureus*, possivelmente pelo fato desta cepa de bactéria ter se tornado resistente devido à mutações causadas pela exposição a efluente industrial.

Palavras-chave: quitosana; recobrimento de ferida, atividade antibacteriana.

Antibacterial Activity of Chitosan Solutions for Wound Dressing

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Summary: Chitosan has found wide application in the biomedical field due to its interesting biological properties that include: biocompatibility, biodegradability, hemostatic activity and bacteriostatic effect. In this present study, antibacterial activity of chitosan solutions for wound dressing were investigated against *Staphylococcus aureus* (isolated from an activated sludge) and *Escherichia coli* (ATCC 25922) that are potential wound pathogens. Moreover, the effects of plasticizer addition and chitosan concentration on antibacterial activity of chitosan solutions were also evaluated. According to the antibacterial activity study, chitosan solutions, plasticized or not, showed inhibitory activity against *Escherichia coli.* However, they did not inhibit *Staphylococcus aureus* growth, possibly because this bacterium strain would become resistant due to mutations caused by industrial effluent exposure.

Keywords: chitosan; wound dressing; antibacterial activity

Introduction

Chitosan is a cationic copolymer obtained by alkaline deacetylation of chitin, which is a natural polymer derived from marine crustaceans' exoskeletons. Chitosan exhibits numerous interesting properties, such as antitumoral, immune-adjuvant, hemostatic and antibacterial activities[1]. Moreover, it is considered as a biocompatible and biodegradable polymer and has been shown to facilitate wound healing[2].

Due to its interesting biological properties, chitosan has been increasingly

used in several medical applications such as drug delivery systems, implants, injections and wound dressing[3, 4, 5]. Chitosan solutions have been used for treatment of skin wounds, such as skin ulcers, burns and surgical wounds.

The antimicrobial activity of chitosan has been studied extensively. It has been shown that chitosan acts by disrupting the barrier properties of the outer membrane of Gram-negative bacteria[6].

In this present study, the effects of chitosan concentration and plasticizer addition on the antibacterial activity of chitosan solutions for wound dressing were investigated. D-sorbitol, a biocompatible poly-alcohol commonly used along with chitosan, was added as a plasticizer. Besides, the antibacterial activity of chitosan solutions was evaluated against two potential Gram-negative wound pathogens: *Staphylococcus aureus* and *Escherichia coli.*

Staphylococcus aureus, the most common bacteria to cause skin wound infections[7], was isolated from an activated sludge. The activated sludge process is a wastewater treatment method in which the carbonaceous organic matter of wastewater provides an energy source for the production of new cells for a mixed population of microorganisms in an aquatic aerobic environment. Bacteria constitute the majority of microorganisms present in activated sludge. Both aerobic and anaerobic bacteria may exist in the activated sludge, but the preponderance of species is facultative, since they are able to live in either the presence of or lack of dissolved oxygen. In addition, due to the complex environment found in activated sludge, bacteria may suffer mutation, transformation and recombination.

Escherichia coli ATCC 25922, widely applied for antibiotic susceptibility assays, was used as a control Gram-negative bacterium[8].

Experimental

Materials

High molecular weight chitosan (M_w ~ 100,000), more than 75% deacetylated, and D-sorbitol were purchased from Aldrich Chemical

Company (USA). Glacial acetic acid was purchased from Synth (Brazil).

Staphylococcus aureus was isolated from an activated sludge (collected from a Waste Treatment Plant) by Selective Medium Technique and identified by Molecular Method. *Escherichia coli* American Type Culture Collection (ATCC) 25992 was gently supplied by Dr. Marcelo Brocchi from the Department of Microbiology-Institute of Biology at UNICAMP-Brazil.

Preparation of chitosan solutions

Solutions were prepared according to Table 1. Acetic acid 1.0% was used as solvent and D-sorbitol 1.0% as plasticizer.

Chitosan flakes were dissolved in acetic acid aqueous solution. Then, the solution was filtered to eliminate insoluble impurities. Plasticized chitosan solution was prepared by adding D-sorbitol to the filtered chitosan solution and the plasticizer solution was prepared by dissolving D-sorbitol in acetic acid aqueous solution.

Table 1-Solutions contents and concentrations (w/w).

Solution	Chitosan (%)	D-sorbitol (%)
Ac 1.0% (solvent)	0	0
Ch 0.5%	0.5	0
Ch 1.0%	1.0	0
Ch 1.5%	1.5	0
Ch 1.5%-So 1.0%	1.5	1.0
So 1.0%	0	1.0

Antibacterial Activity Study

Both bacteria, *Staphylococcus aureus* and *Escherichia coli,* were independently grown in nutrient broth for 24 hours at 37°C. The number of Colony-Forming Units (CFU/ml) was determined by the Serial Dilutions Method. According to this method, $3.0*10^4$ CFU/ml of *Staphylococcus aureus* and $2.0*10^3$ CFU/ml of *Escherichia coli* were counted.

The antibacterial activity study was carried out by agar diffusion technique: 30 ml of PCA (Plate Count Agar) were added to each Petri dish and then inoculated with 0.1 ml of bacterial solution (*Staphylococcus aureus* or *Escherichia coli*). Wells of 0.7 mm diameter were punched in the center of each Petri dish and 0.1 ml of test solution (Table 1) was applied to each well.

After 24 hours of incubation at 37°C, bacterial growth's inhibition zones were measured around the wells. This antibacterial activity study was carried out in triplicates.

Results and Discussion

According to antibacterial activity study results presented in Figures 1 and 2, all solutions inhibited *Escherichia coli* growth. It was expected that higher concentration of chitosan would inhibit more bacterial growth. However, Ch 0.5% showed the strongest antibacterial activity, while Ch 1.5% showed the weakest one.

This unexpected behavior can be attributed to the viscosity of chitosan solutions, which increases when chitosan concentration rises. Thus, high viscosity solutions delay chitosan diffusion in agar, while low viscosity solutions permit fast flow.

Plasticizer addition decreases solution viscosity [9] that explains stronger inhibitory activity of Ch 1.5%-So 1.0% when compared to Ch 1.5%. D-sorbitol and acetic acid also showed antibacterial activity against *Escherichia coli.*

Chitosan kills bacteria through cell membrane damage. It increases the permeability of the outer and inner membranes and ultimately disrupted bacterial cell membranes[10]. These damages are caused by electrostatic interactions between chitosan protonated amino groups and phosphoryl groups of phospholipid components of cell membranes.

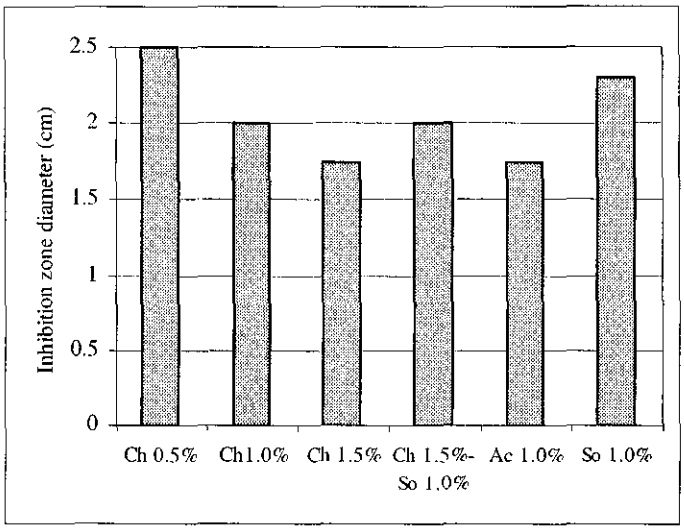

Figure 1-Inhibition zones diameter (cm) of chitosan, D-sorbitol and acetic acid solutions on *Escherichia coli* growth.

For these reasons, it was expected that chitosan solutions would inhibit *Staphylococcus aureus* growth. However, inhibition zones were not observed in Petri dishes inoculated with this bacterium. Probably, this bacterium strain, isolated from an activated sludge, would become resistant to chitosan due to mutations caused by industrial effluent exposure.

Conclusions

Chitosan solutions showed antibacterial activity against *Escherichia coli* and their inhibitory activity was inversely proportional to chitosan concentration. Moreover, D-sorbitol decreased viscosity of chitosan solution and increased its antibacterial activity.

Chitosan did not show inhibitory activity against *Staphylococcus aureus* that probably has became resistant to chitosan due to mutations caused by chemical effluent contact.

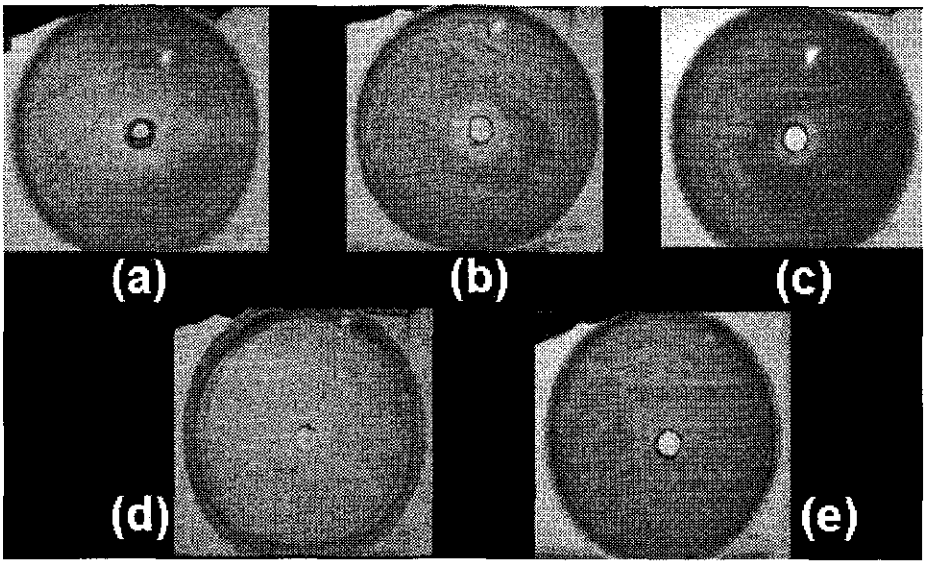

Figure 2- (a) Ch 1.0% with *Escherichia coli*; (b) Ch. 1.5%-So 1.0% with *Escherichia coli*; (c) Ch 1.5% with *Escherichia coli*; (d) So 1.0 % with *Escherichia coli*; (e) Ch 1.0% with *Staphylococcus aureus*.

Acknowledgments

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4.3 *In Vitro* Cytotoxicity Evaluation of Chitosan Membranes for Wound Dressing

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AVALIAÇÃO *IN VITRO* DA CITOTOXICIDADE DE MEMBRANAS DE QUITOSANA PARA RECOBRIMENTO DE FERIDAS

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Resumo: Três formulações de membranas de quitosana para recobrimento de feridas foram preparadas variando-se a adição de plastificante e o processo de neutralização. D-sorbitol foi usado como plastificante e NaOH-Na_2CO_3 como solução neutralizadora. O objetivo deste presente estudo foi avaliar a citotoxicidade *in vitro* das membranas de quitosana usando os testes de citotoxicidade Direta e Indireta. As células *Vero*, uma linhagem celular derivada do rim do macaco verde africano, foram escolhidas para a avaliação da citotoxicidade. As membranas de quitosana não mostraram citotoxicidade indireta, entretanto, quando em contato direto com as células *Vero,* evidências de citotoxicidade foram observadas para a membrana não neutralizada. Esta citotoxicidade direta foi atribuída ao ácido acético residual, já que o ácido acético é tóxico para as células. As membranas neutralizada e plastificada não foram diretamente tóxicas para as células e o D-sorbitol foi considerado um plastificante seguro e não-tóxico.

Palavras-chave: quitosana, membranas, recobrimento de feridas, citotoxicidade, cultivo de células, células *Vero*.

IN VITRO CYTOTOXICITY EVALUATION OF CHITOSAN MEMBRANES FOR WOUND DRESSING

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Abstract: Three formulations of chitosan membranes for wound dressing were prepared by varing the plasticizer addition and the neutralization process. D-sorbitol was used as plasticizer and NaOH-Na2CO3 as neutralizer solution. The purpose of this present study was to evaluate the in vitro cytotoxicity of these membranes by using Indirect and Direct Cytotoxixty Tests. Vero cells, a cell line derived from the kidney of the African green monkey, were chosen for cytotoxicity evaluation. Chitosan membranes did not show Indirect cytotoxicity, however, when on direct contact to Vero Cells, cytotoxicity evidences were observed for the non-neutralized membrane. This direct cytotoxicity was attributed to residual acetic acid present in this membrane, since acetic acid is toxic to cells. Neutralized and plasticized membranes were not directly toxic to cells and D-sorbitol was considered a safety and non-toxic plasticizer.

Key words: Chitosan, Wound dressing, Cell culture, D-sorbitol, Membranes.

1. INTRODUCTION

Chitosan is a partially deacetylated derivative of chitin, which is the second more abundant polysaccharide found in nature and the primary structural polymer in arthropod exoskeletons (Yamane et al., 2005). Chitosan has generated enormous interest as a biomaterial due to its various advantages such as (1) low cost, (2) easy availability, (3) biocompatibility

and (4) anti-microbial activity (Sarasam and Madihally, 2005).

The potential of chitosan as a wound dressing is based on its cationic nature and high charge density in solution. It was reported that the cationic nature of chitosan allows electrostatic interactions with anionic glycosaminoglycans and proteoglycans (Madihally et al., 1999), that may serve as a mechanism for retaining and recruiting cells, growth factors, and cytokines in the wound healing process.

Chitosan exhibits others interesting biological activities, such as anti-tumor, immuneadjuvant, and hemostatic activities (Kurita, 1998) and is considered biodegradable (Muzzarelli, 1997). Due to its positive charges at physiological pH, chitosan is bio-adhesive, which increases its retention on the site of application (Berger, 2004). It also can accelerate wound healing (Synowiecki, 2003). For all those reasons, chitosan is one of the most important biomaterials for wound dressing management in the recent years.

Chitosan is soluble in acetic acid and can easily form films and membranes. However, plasticizers can be used to improve its mechanical properties. In this present study, D-sorbitol, a bio-compatible poly-alcohol, was used as plasticizer, in order to evaluate its effects on chitosan membranes' cytotoxicity.

2. MATERIALS AND METHODS

Preparation of Chitosan Membranes.

Chitosan membranes were prepared by solvent evaporation technique by using acetic acid (1.0 % w/w) as solvent. Chitosan solution was prepared by dissolving 1.5g of chitosan in 100 ml of solvent. After total dissolution, it was filtered to remove impurities. Chitosan membrane (CA) was obtained by casting the chitosan solution in a Petri dish and drying in an oven at 40º C. Neutralized chitosan membrane (CN) was prepared by immersing CA into NaOH (2% w/w)- Na2Co3 (0.05% w/w) aqueous solution for 1 hour. After immersion, CN was repeatedly washed with distillated water and dried at 40° C. 1.0g of D-sorbitol was added to chitosan solution in order to

prepare the plasticized membrane (CP), which was obtained by casting the plasticized solution in a Petri dish and drying at 40º C. Chitosan membranes (CA, CP and CN) were sterilized by ethylene oxide exposure for 8 hours at 40°C, according to sterilization procedures.

Cytotoxicity Assay.

For cytotoxicity evaluation, Vero Cell p240, a cell line derived from the kidney of the African green monkey, Cercopithecus aeothips, was cultivated in a Ham's F10 Nutrition Media (Sigma/USA), supplemented with 10% Fetal Calf Serum (Nutricell/Brazil). Cytotoxicity Tests were carried out according to ISO 10993-5.

Indirect Cytotoxicity Test was carried out according to Mossmam's Methodology (Mossmam,1983). 10% Phenol solution was used as toxicity positive control and polypropylene (culture plate) as negative one. Viable cells number was determined by Spectrophotometric Analysis (8 = 540 nm) by using MTT solutions (Sigma/USA) for cell detection.

3. RESULTS AND DISCUSSION

Cytotoxicity testing is a rapid, standardized, sensitive and inexpensive method to determine if a material contains significant quantities of biologically harmful extractable. Besides, it can provide predictive evidence of material biocompatibility. Direct toxicity test (DT) consisted of assessing possible toxic effects of chitosan membranes on direct contact to cells, while indirect toxicity test (IT) evaluates potential deleterious effects of substances released in the culture medium by chitosan membranes.

IT results (Fig.1) indicated that CA, CP and CN did not release toxic substances to the culture media. Chitosan membranes (CA, CP and CN) presented viable cell number higher than negative control one, what can be attributed to chitosan's biological property of stimulates cell proliferation (Muzzarelli, 1988).

On the other side, DT for CA presented cell aggregation, cytoplasm reduction and cell death after 48 hours of Vero cell incubation. CA direct toxicity may be attributed to residual acetic acid, which was found to be

cytotoxic and unsuitable for wound care (Lineaweaver, 1985).

Direct cytotoxicity evidences were not observed for CP and CN and both membranes were considered satisfactory as cell growth scaffolds.

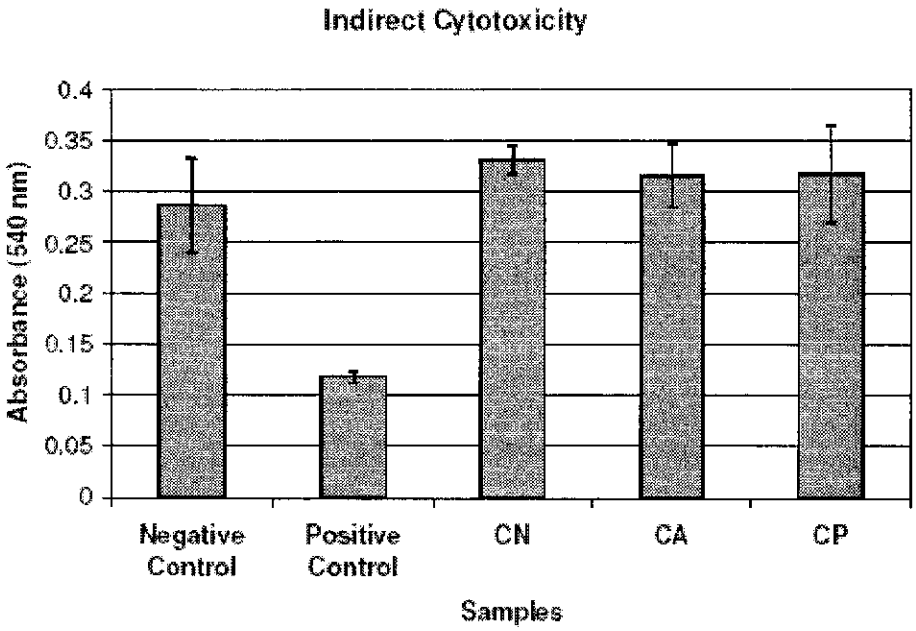

Figure 1 – Indirect cytotoxicity test for Chitosan Membranes.

4. CONCLUSIONS

Chitosan membranes' cytotoxicity was evaluated by direct and indirect tests. CA, CP and CN did not present indirect cytotoxicity. However, when on direct contact to Vero cells, CA showed cytotoxicity evidences, probably due to residual acetic acid. CP and CN did not present direct toxic effects on Vero cells and D-sorbitol was considered a non-toxic plasticizer.

Finally, neutralization is an important step in the preparation of chitosan membranes for wound dressing, since it removes residual acetic acid, which was found to be toxic to cell's growth.

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4.4 *In vitro* Cytotoxicity of Chitosan Membranes against *Vero* cells

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Citotoxicidade *in vitro* de Membranas de Quitosana contra células *Vero*

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Resumo: Três formulações de membranas de quitosana para recobrimento de feridas foram preparadas variando-se a adição de plastificante e o processo de neutralização. D-sorbitol foi usado como plastificante e NaOH-Na_2CO_3 como solução neutralizadora. O objetivo deste presente estudo foi avaliar a citotoxicidade *in vitro* das membranas de quitosana usando os testes de citotoxicidade Direta e Indireta. As células *Vero*, uma linhagem celular derivada do rim do macaco verde africano, foram escolhidas para a avaliação da citotoxicidade. As membranas de quitosana não mostraram citotoxicidade indireta, entretanto, quando em contato direto com as células *Vero,* evidências de citotoxicidade foram observadas para a membrana não neutralizada. Esta citotoxicidade direta foi atribuída ao ácido acético residual, já que o ácido acético é tóxico para as células. As membranas neutralizada e plastificada não foram diretamente tóxicas para as células e o D-sorbitol foi considerado um plastificante seguro e não-tóxico.

Palavras-chave: quitosana, membranas, recobrimento de feridas, citotoxicidade, cultivo de células, células *Vero*.

In vitro Cytotoxicity of Chitosan Membranes against *Vero* Cells

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ABSTRACT: Three formulations of chitosan membranes for wound dressing were prepared by varying plasticizer addition and neutralization process. D-sorbitol was used as plasticizer and NaOH-Na_2CO_3 as neutralizer solution. The aim of this present study was to evaluate *in vitro* cytotoxicity of chitosan membranes by using Indirect and Direct Cytotoxicity Tests. Vero cells, a cell line derived from the kidney of the African green monkey, were chosen for cytotoxicity evaluation. Chitosan membranes did not show indirect cytotoxicity, however, when on direct contact to Vero Cells, cytotoxicity evidences were observed for non-neutralized membrane. This direct cytotoxicity was attributed to residual acetic acid present in this membrane, since acetic acid is toxic to cells. Neutralized and plasticized membranes were not directly toxic to cells and D-sorbitol was considered a safety and non-toxic plasticizer.

Keywords: Chitosan, membranes, wound dressing, cytotoxicity, cell culture, Vero cells.

1 INTRODUCTION

The loss or failure of an organ or tissue is one of the most frequent, devastating, and costly problems in human health care. Tissue engineering, is a new research filed that applies the principles of biology and engineering to the development of functional substitutes for damaged tissue.

Central to successful tissue engineering is the ability of cells to adhere to an extra cellular material and proliferate. This ability mainly depends on the interactions between the cells and the material surface. Thus, an ideal scaffold for tissue engineering should demonstrate considerable mechanical properties, suitable biodegradability and, most importantly, good biocompatibility.

Chitosan is a partially deacetylated derivative of chitin, which is the second more abundant polysaccharide found in nature and the primary structural polymer in arthropod exoskeletons [Yamane et al., 2005]. Chitosan has generated enormous interest as a biomaterial due to its various advantages such as (1) low cost, (2) easy availability, (3) biocompatibility and (4) anti-microbial activity [Sarasam and Madihally, 2005]. The potential of chitosan as a tissue engineering scaffold is based on its cationic nature and high charge density in solution. Due to its cationic nature, chitosan is allowed for electrostatic interactions with anionic glycosaminoglycans and proteoglycans [Madihally et al., 1999]. These ionic interactions may serve as a mechanism for retaining and recruiting cells, growth factors, and cytokines within the tissue scaffold.

Chitosan exhibits others interesting biological activities, such as anti-tumor, immune-adjuvant, and hemostatic activities [Kurita, 1998,]. Moreover, it is metabolized by certain human enzymes, especially lysozyme, and is considered biodegradable [Muzzarelli, 1997]. Due to its positive charges at physiological pH, chitosan is bio-adhesive, which increases its retention on the site of application [Berger, 2004]. It can also accelerate wound healing [Synowiecki, 2003].

Due to all those properties, chitosan is one of the most important biomaterials for wound dressing management in the recent years. Chitosan can easily form films and membranes. However, plasticizers can be used to improve its mechanical properties. In this study, D-sorbitol, a biocompatible poly-alcohol, was used as plasticizer, in order to evaluate its effects on chitosan membranes' cytotoxicity.

2 MATERIALS AND METHODS

2.1 Chitosan membranes production

Chitosan membranes were prepared by solvent evaporation. 1.0% (w/w) Acetic Acid (Synth/Brazil) solution was used as solvent. 1.5g of High Molecular Weight Chitosan (Aldrich/USA) was dissolved in 100 ml of solvent. Then, chitosan membranes (CA) were obtained by casting chitosan solution in Petri dishes and drying at 40°C.

1.0 g of D-sorbitol (Aldrich/USA) was added to chitosan solution in order to prepare plasticized membranes (CP). Neutralized membranes (CN) were prepared by immersing CA into NaOH (2% w/w)-Na2Co3 (0.05% w/w) aqueous solution for 1 hour. Then, CN were repeatedly washed with distillated water and dried at 40° C.

Chitosan membranes (CA, CP and CN) were sterilized by ethylene oxide exposure for 8 hours at 40°C, according to sterilization procedures.

2.2 Cell culture

Vero cells are recommended for studies of cytotoxicity and for cell-substratum interactions with biomaterials. This cell line was established from the kidney of the African green monkey (Cercopithecus aethiops) and obtained from Adolfo Lutz Institute, São Paulo, Brazil.

Cells were cultured in Ham-F10 medium (Sigma) supplemented with 10% fetal calf serum (FCS, Nutricell Nutrientes Celulares, SP, Brazil) at 37°C and kept in culture flask of 25mL or 50mL before inoculation (Corning, Cambridge, MA, USA). Nutritional medium was replaced periodically.

2.3 Cytotoxicity assay

Vero cells were cultured on different chitosan membranes (CA, CP, and CN) for 24 hours. Six repetitions were made of all experiments. Test was carried out according to Mossmann's Methodology [Mossmann, 1983]. Briefly, substrates were incubated in 96 well plates (Corning) with cultured medium without FCS for 24h at 37° C. After this, 100L of cell suspension

(2.5 x 105 cells/mL) in Ham F-10 with 10% of FCS was added to the wells containing different chitosan samples.

Wells were washed twice with 0.1M phosphate buffered saline (PBS) pH 7.4, at 37°C and incubated with 100μL of Ham F-10 medium. The assay mixture (10μL per well) containing 5mg/mL of 3-(4,5-dimetiltiazol-2-il)-2,5 diphenyl tetrazolium bromide (MTT, Sigma) was added to each well and incubated for 4h at 37°C.

After 4h, 100μL of isopropanol acid (Isofar Ind, RJ, Brazil) was added to each well and 3 hours later, cells were quantified spectrophotometrically, based on absorbance at 540nm by using a Biorad Model 550 micro plate reader Spectrophotometer (Hercules, CA, USA).

According to the ISO 10993-5, cells on poly-propylene culture plate by themselves were used as positive control, while culture medium with phenol (2%) was used as negative toxicity control. Absorbance of all experimental conditions (chitosan samples, negative or positive controls) without cells was also read for MTT reaction control.

Comparison of continuous variables for all groups was done with ANOVA. When a significant difference was found, Turkey HSD multiple comparison test was performed ($p < 0.05$).

2.4 Cell morphology by Light microscopy and Scanning Electron microscopy

For cell morphologic analysis by light microscopy (LM), 2.5 x 105 cells/mL were inoculated in 24 wells culture plate (Corning) containing a chitosan sample (CA, CP or CN) or one of the controls (positive or negative). Cells were feed with Ham F-10 medium with 10% FCS. Alive cells were observed and photographed in inverted microscope IX-50 (Olympus, Hatagaya, Tokyo, Japan) after 48h, 120h and 240h of cultured. For all experiments, n=5.

For cell morphologic analysis by scanning electron microscopy (SEM), 2.5 x 105 cells/mL were inoculated on different chitosan membranes (CA, CP and CN) in 24 well cultured plate (Corning) feed with Ham F-10 medium

with 10% FCS. Cells cultured on a glass cover slip, at the same culture conditions, were used as control. After 48h, samples were fixed in 3% glutaraldehyde (Sigma) in 0.1M phosphate buffer at pH 7.2 for 45 minutes at 4℃, and post-fixed with 1% OSO4 (Sigma) for 2h at 4℃. Specimens were then dehydrated in graded series of ethanol, critical point dried (Balzers CDT 030) and coated with gold in a sputter coater (Balzers CDT 050). The coated specimens were observed and photographed with a JEOL JSM-5800 scanning electron microscope.

3 RESULTS

3.1 Cytotoxicity assay

Cytotoxicity testing is a rapid, standardized, sensitive and inexpensive method to determine whether a material contains significant quantities of biologically harmful extractable. Besides, it can provide predictive evidence of material biocompatibility. Direct cytotoxicity test consisted of assessing possible toxic effects of chitosan membranes on direct contact to cells, while Indirect Cytotoxicity test evaluates potential deleterious effects of substances released in the culture medium by chitosan membranes.

Indirect Cytotoxicity results (Figure 1) indicated that CA, CP and CN did not release toxic substances to the culture media. Chitosan membranes (CA, CP and CN) showed viable cell number higher than negative control that can be attributed to chitosan's biological property of stimulates cell proliferation.

On the other hand, for direct toxicity evaluation, a possible toxic effect of CA was observed (Figure 2).

3.2 Cell morphology by light microscopy

For negative control (culture plate), spreading cells growing were found at all incubation times studied. After 48hs and 120hs, we could observe a confluent cells monolayer on the culture plates. Round cells that indicate cell division could also be seen at these incubation times. Past 240hs, rounded cells on the substrate were found, indicating lost of viability.

Besides, numerous cells were observed flouting on the culture medium (Figures 3A).

For positive control of toxicity (phenol), round and no viable cells on the substrate were observed for all incubation times. The cell number at all periods was, apparently, the same, indicating no cell proliferation on that samples (Figures 3B).

For CA sample, round and aggregated cells on the substrate were found at the first 48hs. However, cells aggregation was observed in small number. After 120hs of incubation, more spreading cells could be observed in the cellular aggregates. These cells were apparently trying to migrate for other regions of CA. Past 240hs, a no confluent cell layer on CA was observed (Figures 3C).

Similar cell behavior was observed for CP and CN. However, at the first 48hs, we observed a no confluent spreading cell monolayer on CP and CN samples. After 120hs, a confluent cell layer could be observed. Moreover, past 240hs of incubation, multi-layers of cells on CP and CN samples were found (Figures 3D and 3E, respectively).

3.3 Cell morphology by Scanning Electron Microscopy

By SEM, retracted cell around cell fragments we found on CA. In some regions we could observe spreading cell near to cell fragments (Figures A1 and A2). We also could observe spreading cell on substrate on CP. In some regions, we found cell connected by thin processes (Figures B1 and B2). For CN, we observed spreading cells with large processes linking them. Many vesicles and/or microvillus were found on cell surface (Figures C1 and C2).

4 DISCUSSION

The indirect cytotoxicity indicated that there was no liberation of toxic substances from the different chitosan samples to the culture medium. On the other hand, CA showed some direct toxic activity in the MTT assay. This result was confirmed by the morphological pattern of Vero cells cultured on CA. Rounded and aggregated cells in small areas of CA were

observed. Morphological pattern was compatible with cells submitted to some toxic effect. After initial contact, some cells were capable to proliferate and migrate from cellular aggregates to CA surface. However, when compared to the other chitosan samples studied (CP and CN), CA showed small number of cells. CA direct toxicity can be attributed to residual acetic acid, which was found to be cytotoxicity and unsuitable for wound care [Lineaweaver, 1985].

It was not observed direct cytotoxicity evidences for CP and CN and both membranes were considered satisfactory as cell growth scaffolds. CP and CN samples showed viable cell number higher than negative control. Mori et al. examined the effects of chitin and its derivatives on fibroblasts proliferation. Their results indicated that chitosan has no proliferation effect on in vitro fibroblasts growth [Mori et al. 1997]. However, other reports showed that chitosan can indirectly accelerate fibroblast proliferation in vivo [Ueno et al., 1999]. Ding et al., when studied chitosan immobilization on PLLA surface, did not observe prominent effects of chitosan on cell proliferation (Ding et al., 2004). Our results are in accordance to Mori et al. and Ding et al. since a decrease in the number of cells was observed for all chitosan substrates (CA, CP and CN) when compared to the negative control (culture plate).

Thus, our in vitro study results suggest that CP and CN can support the initial attachment and spreading of Vero cells. These findings are in accordance to previous reports that showed similar morphological pattern of cells cultured on chitosan surfaces.

Microscopic observations at 1h revealed that osteoblasts were attached and beginning to spread. In contrast, fibroblasts showed rounded morphologies with markedly reduced attachment levels [Fakhryet al., 2004]. Previous reports have shown conflicting results about the effects of chitosan on fibroblasts. While chitosan has been shown to have stimulatory effect on fibroblast activity [Muzzarelli et al., 1988; Lahiji et al., 2000], other results have shown some inhibitory effects of chitosan on fibroblasts growth [Mori et al., 1997; Berschtet al., 1994].

Mei et al. showed the cell morphology on polycaprolactone (PCL) and chitosan-modified scaffolds. It was reported that chitosan modification dramatically enhances cellular affinity and compatibility for PCL scaffolds. Cells were more firmly adhered on chitosan-modified PCL and more elongated and stretched on this substrate. Moreover, cell adhesion and proliferation on PCL scaffolds were considerably improved after chitosan modification [Mei et al., 2005].

Conflicting results among published data on chitosan-mediate fibroblast cells attachment may be explained by differences in cell population analyzed and by variation in properties of tested chitosan [Fakhry et al., 2004].

After 240hs of incubation on chitosan samples, morphological analysis showed cells growing such as many layers. That growth pattern indicates a satisfactory proliferation of cells on those chitosan substrates. It also indicates that CP and CN can support cellular growth after normal decrease of viability, as well observed in negative control for the same period of incubation. Our morphologic observations showed reduction of cell spreading in chitosan substrates when compared to culture plate (negative control). Similar trend was previously cited by Sarasam and Madihally who observed drastic decrease in the cell spreading area from control to chitosan [Sarasam and Madihally, 2005].

Cell spreading and morphology on chitosan substrates still are an unclear event. Structurally, chitosan is a linear polysaccharide consisting of □(1□4) linked D-glucosamine residues with variable number of randomly located N-acetyl-glucosamine groups. The average molecular weight ranges from 50 to 1000 kDa [Yamane et al., 2005]. Chitosan does not have one specific binding domain for cell adhesion mediated by integrins receptors. Also, any intracellular signaling pathway activated by chitosan contact is known [Hynes and Zhao, 2000]. Cells generally attach to an artificial culture substrate by binding to specific anchoring proteins that become adsorbed to substrate surface. Chitosan acts as a positively charged center in solution, and the outside of the cell membrane carries negative charges.

For Ding et al., the electronic interaction between cell and substrate surface could modulate the cytoskeleton and cell morphology (Ding et al., 2004). On the other hand, Huang et al. suggest that cell spreading and morphology could be modulated by the structural characteristics of chitosan scaffolds. In that work, authors seeded cells on bi-dimensional (2D) and three-dimensional (3D) chitosan substrates. On 2-D condition, cells were restricted to spread on a flat plane and the important factor affecting cellular activity was whether the substrate contains or no cell adhesion binding domains. In contrast, 3-D matrices provided spatial advantages for cell–cell and cell-matrix adhesion as well as support for cell traction [Huang et al., 2005]. Reduction in cell size was thought to be the result of strong electrostatic interactions associated with the deacetylation degree [Mao et al., 2004]. Changes on cellular activity induced by different deacetylation degrees could not be significantly relative to other adhesive forces [Huang et al., 2005]. Nevertheless, cells proliferation inhibition found on chitosan scaffolds could be due to reduced adhesion and not to strong adhesion [Mao et al., 2004].

5 CONCLUSIONS

Chitosan membranes' cytotoxicity was evaluated by Direct and Indirect Tests. CA, CP and CN did not show Indirect Cytotoxicity. However, when on direct contact to Vero Cells, CA showed cytotoxicity evidences that were attributed to residual acetic acid. CP and CN did not show direct toxic effects on Vero Cells and D-sorbitol was considered a non-toxic plasticizer. Finally, neutralization was an important step in preparation of chitosan membranes for wound dressing because it removed residual acetic acid, which was found to be toxic for cells growth.

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7 FIGURE LEGENDS

Figure 1. Indirect Cytotoxicity Test for Chitosan Membranes.

Figure 2. Direct Cytotoxicity Test for Chitosan Membranes.

Figure 3. Morphology of Vero cells cultured on different chitosan membranes, obtained by light microscopy.

A) Negative control for cytotoxicity (CT-);

B) Positive control for cytotoxicity (CT+);

C) Chitosan membrane (CA);

D) chitosan plasticized membrane (CP);

E) chitosan neutralized membrane (CN). All assays were performed for 48, 120 and 240hs of cultured.

Figure 4. Morphology of Vero cells cultured on different chitosan membranes, obtained by scanning electron microscopy. A) Chitosan membrane (CA); B) chitosan plasticized membrane (CP); C) chitosan neutralized membrane (CN).

Figure 1

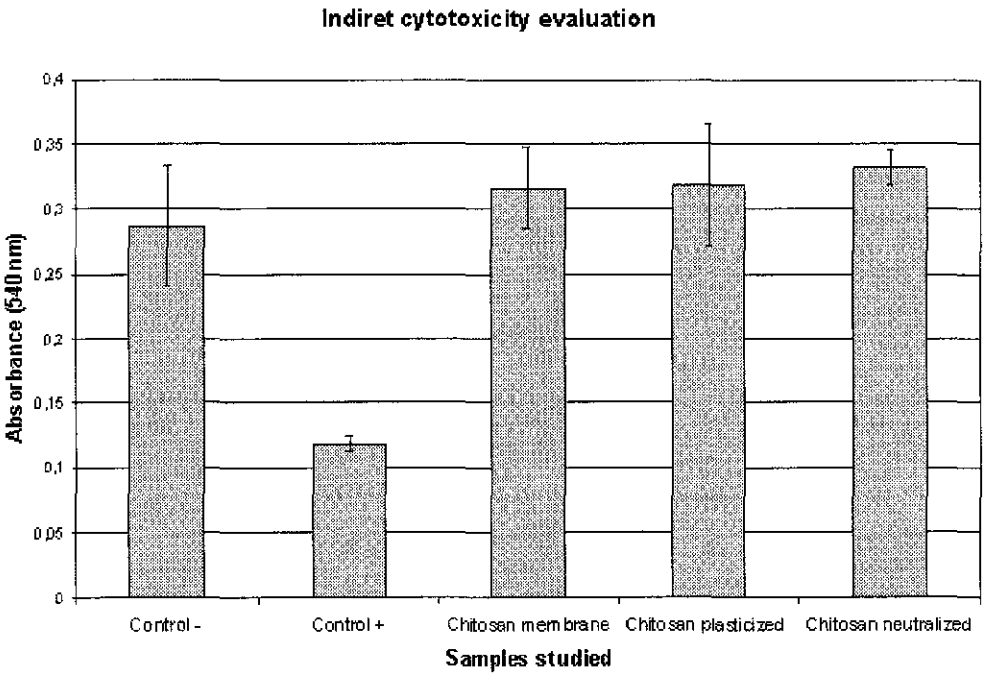


Figure 2

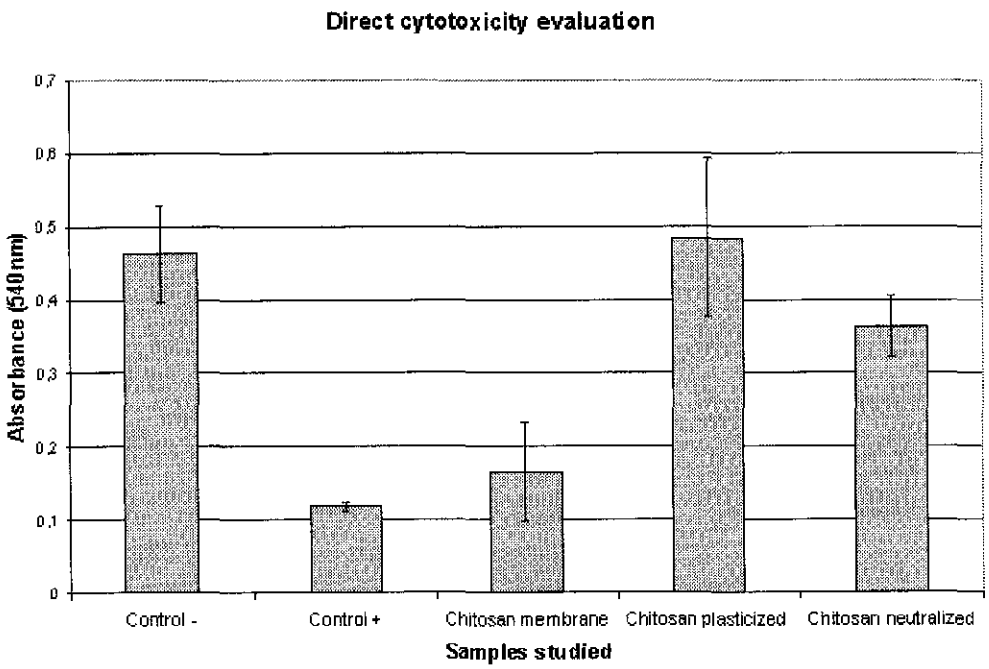
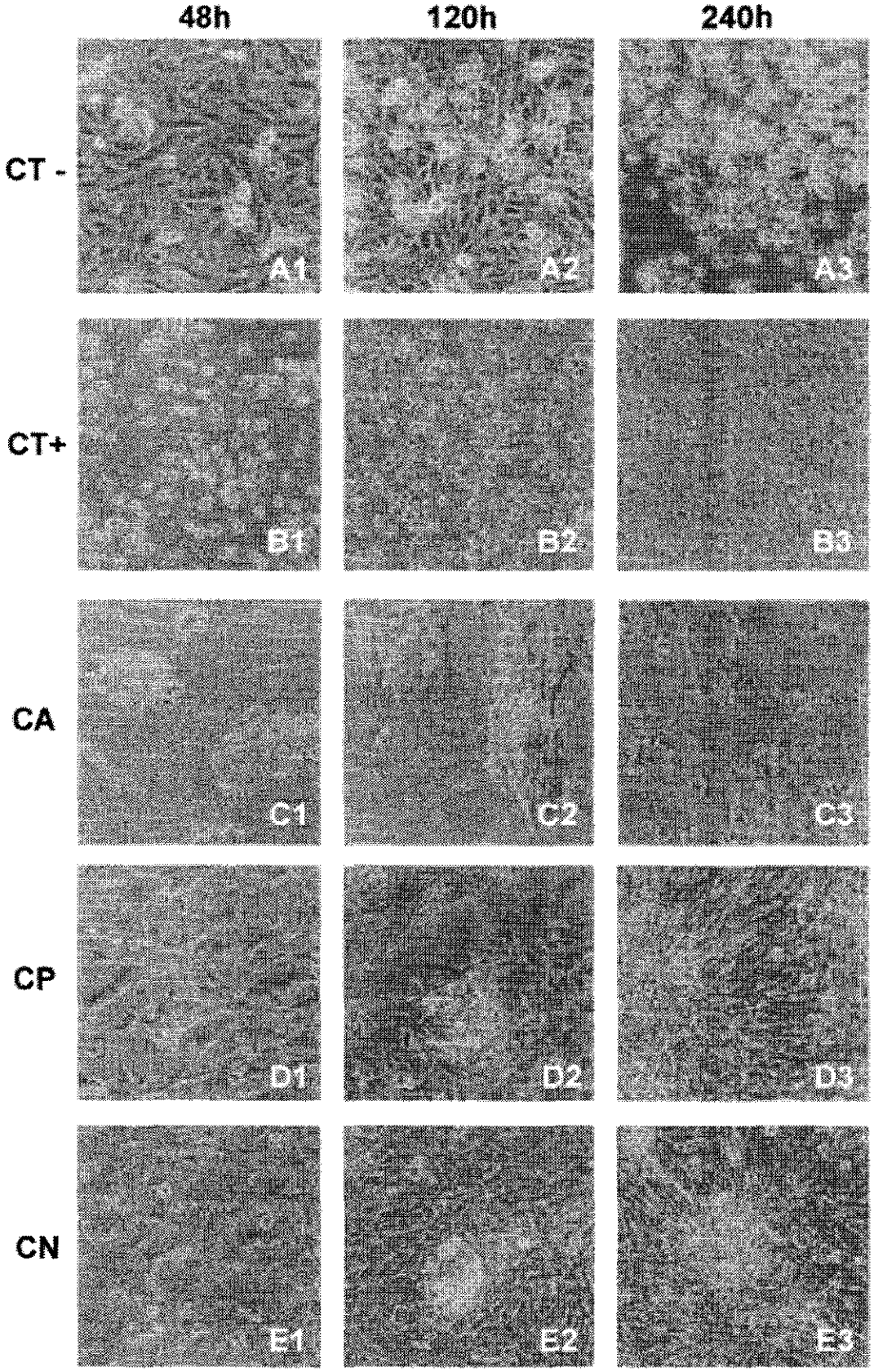


Figure 3


48h
120h
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CT -
CT+
CA
CP
CN
A1
A2
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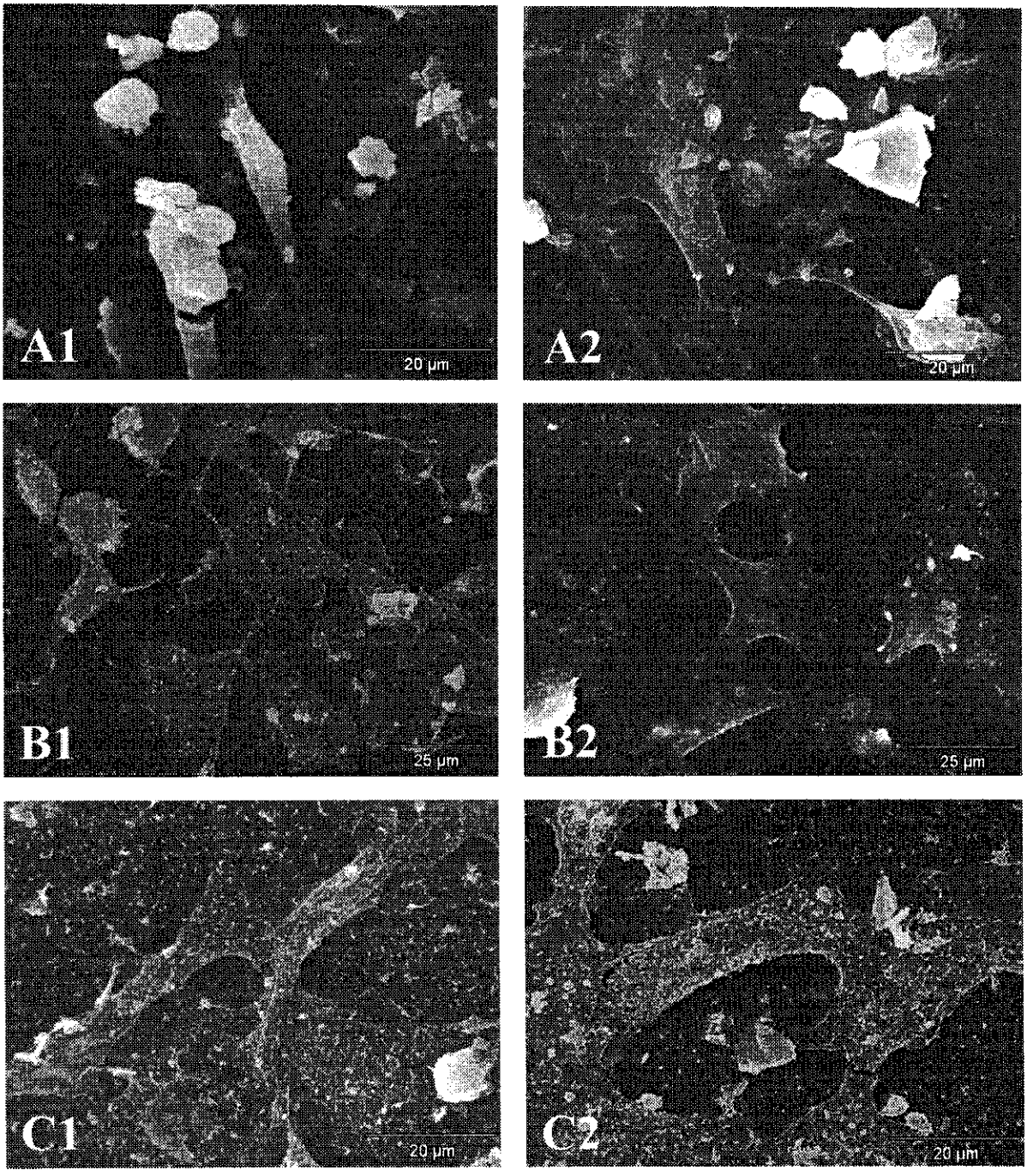
E2
E3

Figure 4



4.5 Crystallinity *versus* Water Vapor Permeability of Chitosan Membranes for Wound Dressing

Publicado nos anais da Advances in Materials and Processing Technology (AMPT) Conference, 2006.

Cristalinidade *versus* Permeabilidade ao Vapor D'água de Membranas de Quitosana para recobrimento de feridas

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RESUMO

Os efeitos da cristalinidade na permeabilidade ao vapor de água das membranas de quitosana foram avaliados neste presente estudo. D-sorbitol, um poli-álcool biocompatível, foi usado como plastificante para avaliar seu efeito nas propriedades das membranas de quitosana. As membranas de quitosana foram morfologicamente caracterizadas por microscopia eletrônica de varredura e difractometria de raios-X. As propriedades térmicas foram avaliadas por calorimetria diferencial exploratória e as propriedades mecânicas, tais como tração e deformação na ruptura também foram medidas. As membranas plastificadas apresentaram maior permeabilidade ao vapor d'água e deformação ao romper, porém menor tração e cristalinidade. Este comportamento pode ser atribuído à maior mobilidade das cadeias poliméricas promovida pela adição de plastificante.

PALAVRAS-CHAVE: quitosana, D-sorbitol, barreira ao vapor d'água, difractometria de raios-X.

Crystallinity *versus* Water Vapor Permeability of Chitosan Membranes for Wound Dressing

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ABSTRACT

Effects of crystallinity on water vapor permeability of chitosan membranes were evaluated in this present study. D-sorbitol, a biocompatible poly-alcohol, was used as plasticizer to evaluate its effects on chitosan membranes' properties. Chitosan membranes were morphologically characterized by Scanning Electron Microscopy and X-Ray Diffraction analyses. Thermal properties were evaluated by Differential Scanning Calorimetry and mechanical properties, such as tensile strength and elongation at break, were also measured. Plasticized membranes showed higher water vapor permeability and elongation at break, but lower tensile strength and crystallinity. This behavior can be attributed to the higher mobility of polymer chains promoted by plasticizer addition.

KEYWORDS: Chitosan, D-sorbitol, water vapor barrier, X-ray diffractometry.

INTRODUTION

Chitosan is a polysaccharide derived from chitin that is the second most abundant polysaccharide found in nature. Chitin is largely found in external skeleton of insets and in shell of crustaceans. Chitosan is mainly obtained

by deacetylation of chitin but it can also be found in the cell wall of some fungi, such as Mucor rouxii [1]. Both chitin and chitosan have been widely studied because of their interesting biological properties.

Chitosan interesting properties include biocompatibility, biodegradability, bacteriostatic effect, hemostatic and antitumoral activities, besides promote wound healing [2,3]. Due to its unique properties, chitosan has found wide application in biomedical area, such as: implants, drug delivery matrix, scaffolds for tissue engineering, skin substitutes and wound dressings [4,5 and 6].

Chitosan is soluble in acetic acid solution and can easily form films and membranes by solvent evaporation technique. However, depending on desired properties, plasticizer can be added to improve flexibility and plasticity of membranes [7]. Polyols, such as sorbitol and glycerol, plasticize effectively due to their ability to reduce internal hydrogen bonds while increasing intermolecular space [8].

D-sorbitol is naturally found in berries and fruits and is synthetically prepared by high-pressure catalytic hydrogenation of glucose. Besides, sorbitol is also produced by the human body. Due to its low toxicity and consequently high biocompatibility, D-sorbitol was used as plasticizer in this study.

Crystallinity probably is the major physical characteristic that determines the functional properties of polymers. It plays an important role in adsorption efficiency and swelling behavior [9]. Besides, mechanical, thermal and barrier properties are also affected by crystallinity. Therefore, the goals of this present study were to evaluate the effectiveness of D-sorbitol as plasticizer, as well as to investigate the effects of plasticizer addition on chitosan membranes' crystallinity, and hence, on their thermal, mechanical and barrier properties.

EXPERIMENTAL

Materials

High molecular weight chitosan (Mw ~ 100,000), more than 75% deacetylated, and D-sorbitol were purchased from Aldrich Chemical Company (USA). Glacial acetic acid was purchased from Synth (Brazil).

Preparation of Membranes

Chitosan membranes were prepared by solvent evaporation technique by using acetic acid (1.0 % w/w) as solvent. Chitosan solution was prepared by dissolving 1.5g of chitosan in 100 ml of solvent. After total dissolution, it was filtered to remove impurities.

Chitosan membrane (CA) was obtained by casting the chitosan solution in a Petri dish and drying in an oven at 40º C. Neutralized chitosan membrane (CN) was prepared by immersing CA into NaOH (2% w/w)-Na_2Co_3 (0.05% w/w) aqueous solution for 1 hour. After immersion, CN was repeatedly washed with distillated water and dried at 40 ° C.

1.0g of D-sorbitol was added to chitosan solution in order to prepare the plasticized membrane (CP), which was obtained by casting the plasticized solution in a Petri dish and drying at 40º C.

Differential Scanning Calorimetry (DSC)

DSC analyses were performed on a DSC 2920 -TA Instruments. DSC curves of chitosan membranes were obtained by heating samples from –130 ºC up to 200 ° C, at heating rate 2 ° C min^{-1} and modulation rate ±1 ° C min^{-1}, under nitrogen atmosphere, in order to estimate glass transition and melting temperatures.

X-Ray Diffractometry

X-ray diffraction of chitosan membranes was performed in a Powder Diffractometer HZG/4A (Freiberger Preazisionsmechanik, Freiberg, Germany). The relative intensity was recorded in the scattering range (2θ) of 4 to 40º.

Scanning Electron Microscopy (SEM)

Chitosan membranes samples were cut and kept in vacuum desiccators for 48 hours. Then, samples were coated with gold/palladium using a SC 7620 Sputter Coater – POLARON under high vacuum and 4 mA for 180 seconds. Coated samples were examined using a LEO 440i Scanning Electron Microscopic.

Water Vapor Permeability (WVP)

WVP of chitosan membranes was measured according to ASTM 96-00 [10] by the Desiccant method. Chitosan membranes were sealed to the open mouth of a test dish containing anhydrous calcium chloride, and the assembly was placed in a desiccators with controlled atmosphere (75 ± 2% RH) at room temperature. The dish assembly was daily weighted and the water vapor transmission rate was calculated. Tests were carried out in triplicates.

Mechanical Properties

Electronic Digital Caliper (Fowler & NSK – Max-Cal) was used to measure membranes thickness and TA-XT2 (SMS, Surrey, UK) Instrument was used to measure tensile strength (TS) and elongation at break (E). Tests were carried out according to ASTM D-882 [11], with initial grip separation of 30 mm for CA and CN and 50 mm for CP. Cross head speed was fixed in 1 mm/s in all tests.

RESULTS AND DISCUSSION

Differential Scanning Calorimetry and X-ray Diffractometry

According to Table 1, CA showed higher melting fusion enthalpy that indicates higher crystallinity. These results were confirmed by X-ray Diffraction analysis (Figure 1), in which CA showed strong reflection at 2θ around 10-12º and 18-20º, besides a small peak around 16º that has been attributed to the anhydrous crystal lattice [12].

Moreover, CA showed lower glass transition temperature when compared to CN, which can be attributed to the residual acetic acid present in CA that acts as plasticizer [13]. Thus, neutralization process removed residual

acetic acid from CN and promoted a molecular rearrangement that was responsible for decreasing the free volume around the polymer chains. Therefore, CN showed higher glass transition temperature and lower melting one.

Besides, according to Figure 1, CN showed small peak around 10-12º and reflection at 2θ around 20-22º, as observed by Srinivasa *et al* [14], which indicates some crystallinity.

Table 1. Thermal Properties of chitosan membranes.

Sample	Tg_1 (°C)	Tg_2 (°C)	Tg_3 (°C)	Tm(°C)	ΔH(J/g)
CA	-81.70	-84.10	-73.69	101.95	458.70
CN	-76.90	-75.63	-73.16	88.39	418.50
CP	-87.51	-85.65	-74.02	102.70	76.82

As expected, CP showed the most amorphous behavior that can be attributed to the plasticizer addition.

Arvanitoyannis *et al* [15] observed low values of fusion enthalpy and glass transition temperatures in chitosan and gelatin edible films when different amounts of sorbitol were added.

CP showed lower fusion enthalpy and glass transition temperature and this amorphous behavior was confirmed by X-ray diffractometry, since CP did not show any strong reflection around 2θ.

Scanning Electron Microscopy

Although thermal and x-ray analyses showed considerable differences among CA, CN and CP, no significant structural changes among samples was observed by SEM analysis (Figure 2). All samples were dense and package membranes. Nevertheless, CN showed some superficial rugosity, which can be attributed to neutralization treatment.

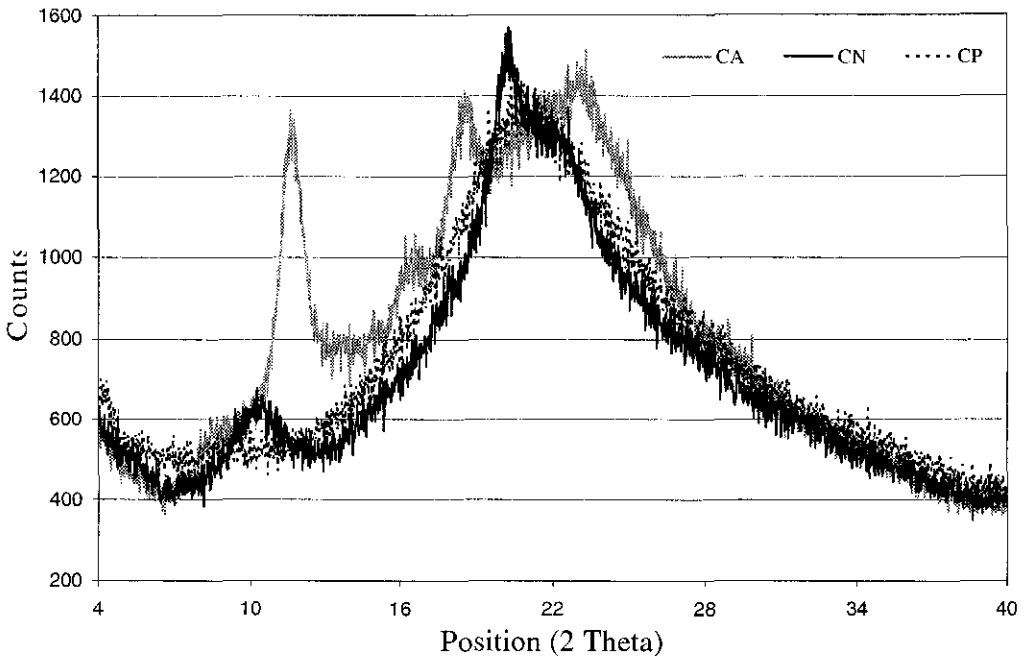

Figure 1. X-ray Diffractometry of chitosan membranes.

Water Vapor Permeability and Mechanical Properties

Plasticizers are theorized to decrease the intermolecular forces along polymer chains, increasing flexibility while decreasing the barrier properties of films. Thus, as expected, D-sorbitol addition increased WVP and elongation at break of CP by decreasing its crystallinity and hence increasing the mobility of the polymer chains. On the other hand, CP showed lower tensile strength when compared to CA and CN that can be explained by its lower degree of crystallinity and glass transition temperature.

Table 2. Barrier and Mechanical Properties of chitosan membranes.

Sample	Thickness(mm)	WVP(g*mm/m^2*dKPa)	TS (MPa)	E (%)
CA	0.0436 ± 0.0028	5.07 ± 0.32	58.62 ± 1.87	18.85 ± 2.43
CN	0.0704 ± 0.0043	5.74 ± 0.05	79.83 ± 4.49	8.30 ± 2.14
CP	0.0718 ± 0.0089	6.67 ± 0.60	14.70 ± 0.15	83.12 ± 1.66

According to Table 2, CA showed lower WVP due to its higher density of crystals that obstructs the penetrant's (water vapor) diffusion. In addition, CN showed the highest tensile strength and the lowest elongation at break.

This mechanical behavior can be attributed to the neutralization process that promoted a molecular rearrangement in CN, increasing its glass transition temperature by decreasing the free volume around the polymer chains.

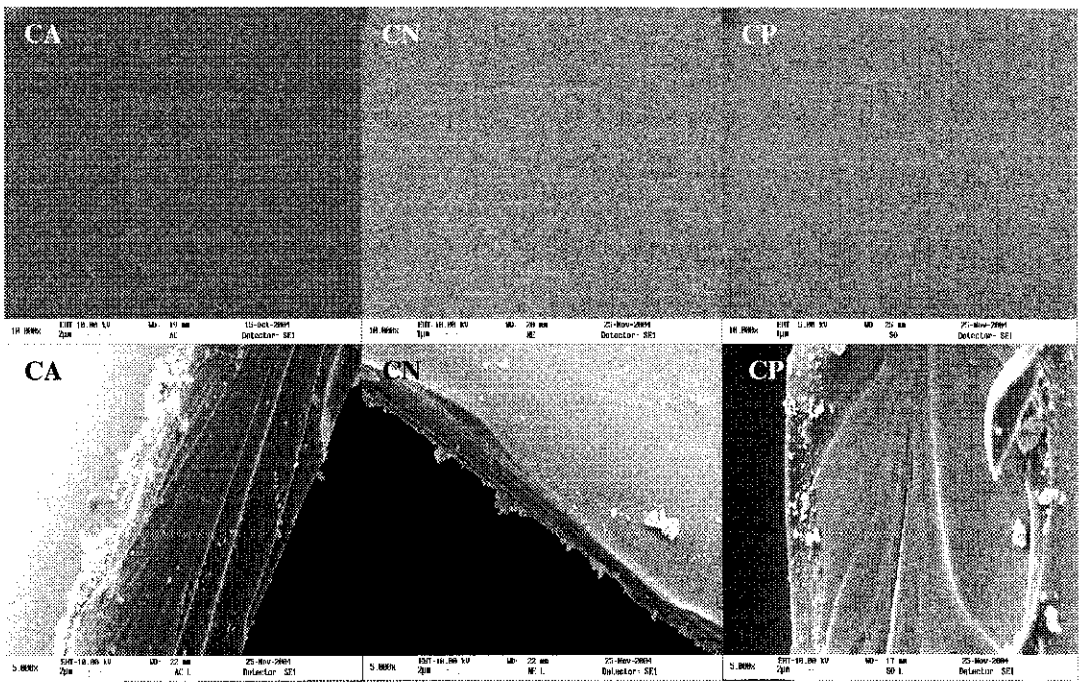

Figure 2. Micrographs of chitosan membranes.

CONCLUSIONS

Plasticizer addition to CP decreased its crystallinity by reducing internal hydrogen bonds (intermolecular forces) while increasing the free volume around the polymer chains. Moreover, D-sorbitol addition improved WVP and flexibility of chitosan membranes that are very important skills for wound dressing.

Although tensile strength had been decreased by plasticizer addition, it still can be considerate for wound dressing, since an average tensile strength of 17.6 MPA for normal skin was quoted by Wohlish [16]. Moreover, the tensile strength of wounds is lower than the skin one and it also varies during the wound healing process [17].

Finally, D-sorbitol was effective as plasticizer but future studies must be done to evaluate its stability in chitosan membranes.

ACKNOWLEDGENTS

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4.6 *In Vitro* Gentamicin Sustained and Controlled Release from Chitosan Cross Linked Films

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Liberação sustentada e controlada in vitro de gentamicina a partir de filmes reticulados de quitosana

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RESUMO: A gentamicina é um antibiótico pertencente à família dos amino-glicosídeos usada no tratamento de infecções causadas por vários tipos diferentes de bactéria, particularmente as infecções por bactérias Gram-negativas. As preparações tópicas de gentamicina são usadas no tratamento de infecções de pele; entretanto, elas devem ser aplicadas à área infectada pelo menos 3 vezes ao dia. Neste trabalho, um novo recobrimento de ferida foi estudado para liberar gentamicina enquanto cobre e protege a ferida, reduzindo a dosagem e o número de doses do antibiótico. A quitosana, um biopolímero biocompatível e biodegradável, com atrativas características biológicas, foi utilizada na preparação dos filmes carregados com antibiótico. O hexametileno 1,6-Di(aminocarboxisulfonato), um diisocianato bloqueado solúvel em água, foi usado como agente reticulante e o ácido acético diluído como solvente. O ácido acético residual foi neutralizado em uma solução NaOH-Na_2CO_3 e os efeitos do tempo de neutralização foram investigados. Além disso, a liberação controlada de gentamicina *in vitro* foi observada por duas semanas e os efeitos da concentração inicial deste antibiótico, do grau de reticulação da quitosana e do tempo de neutralização das membranas na cinética de liberação da gentamicina foram avaliados.

PALAVRAS-CHAVE: gentamicina, quitosana, recobrimento de feridas, reticulação, liberação sustentada e controlada.

In vitro Gentamicin Sustained and Controlled Release From Chitosan cross linked Films

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Abstract

Gentamicin, an amino-glycoside antibiotic used to treat infections caused by many types of bacteria, is used topically to treat skin infection; however, these formulations should be applied to affected area at least three times a day with mandatory cleaning at each application. In this work, a novel wound dressing film was investigated for controlled and sustained delivery of gentamicin while covering and protecting the wound. Chitosan, a biocompatible and biodegradable biopolymer with attractive wound healing properties, was crosslinked with Hexamethylene 1,6-Di(aminocarboxysulfonate) to control the retention and release of encapsulated gentamicin from the formulated film. In vitro gentamicin release from the above antibiotics loaded films in phosphate buffered saline at physiological pH and temperature was studied for 2 weeks. The effects of gentamicin initial concentration, and cross-linking ratio on the kinetics of gentamicin release were evaluated.

Keywords: gentamicin, chitosan, wound dressing film, cross-linking, controlled and sustained drug release.

1. Introduction

Gentamicin is one of the most consumed antibiotics because of its low cost, broad antibacterial spectrum of action, low rate of primarily resistant pathogens, low allergy rate, good stability and water solubility (Virto, Frutos, Torrado, & Frutos, 2003). Gentamicin binds to components in the

bacterial cell and causes the production of abnormal proteins; the production of these abnormal proteins is ultimately fatal to the bacteria. Gentamicin can treat many types of bacterial infections, particularly Gram-negative infection; it eliminates bacteria that cause infections in lung, skin, bone, joint, stomach, blood, and the urinary tract infections. However, it is not absorbed from the gut and is consequently only given by injection or infusion.

Gentamicin topical preparations are used to treat the infections of skin. Topical preparations are available in two dosage forms: cream and ointment. Skin wound treatment with topical gentamicin includes three basic steps: 1- washing the affected area with water and soap; 2- application of a small amount of the cream or ointment to the affected area and, finally, 3- covering the treated area with a gauze dressing. This procedure should be repeated at least three times a day, or in accordance with a physician's prescription. Thus, traditional gentamicin topical treatment is painstaking, besides which it can lead to failure in infection control or delay in wound healing, since the dosage is imprecise and delivered discontinuously. For these reasons, a novel antibiotic-releasing wound dressing was investigated in the present work.

As most of the common polymers are synthetic, their biocompatibility and biodegradability are quite limited compared to the natural polymers such as cellulose, chitin, chitosan and their derivatives. However, many naturally abundant polymeric materials also exhibit a limitation in their reactivity, biodegradability and processability (Mass, Mass, & Tighe, 1998; Illum, 1998), but chitosan is recommended as suitable functional material, because of its excellent biocompatibility, biodegradability, non-toxicity, and adsorption properties, etc. In addition, chitosan can accelerate wound healing and has shown hemostatic and bacteriostatic activities (Mass, Mass, & Tighe, 1998; Berger, 2004). The source of chitosan is chitin, the natural waste derived from the discarded shells of crab and shrimp etc. and the second most abundant polysaccharide. Chitosan finds wide applications such as implants, drug delivery matrix, scaffolds for tissue

engineering, skin substitutes and wound dressings (Hoemann, 2005; Denkbas, 2004; Ueno, Mori, & Fujinaga, 2001). In bio-delivery applications, chitosan has been used as a vehicle for drug, protein and gene delivery (Chen, Wu, Mi, Lin, YU, & Sung, 2004; Yuan, Chesnutt, Utturkar, Haggard, Yang, Ong, & Bumgardner, 2007; Sinha Singla, Wadhawan, Kaushik, Kumria, & Bansal, 2004).

Chitosan is soluble in acid solutions and can easily form films and membranes by solvent evaporation. However, due to its positive charge in acid medium, caused by amino group protonation, it becomes extremely hygroscopic hydrogel; the retention and the release of a biomolecule depends on the swelling/deswelling of hydrogel, which may vary by several factors including the amount of water in hydrogel. To increase the time frame and consistency of kinetics of drug delivery, hydrophilic polymers need to be cross-linked (Jameela, Kumary, Lal, & Jayakrishnan, 1998). Several methods are available for the cross-linking of chitosan, the most common being the one using glutaraldehyde as the cross-linker (Hirano, Yamaguchi, Matsuda, Miura, & Kondo, 1977). However, there are concerns over the toxicity of the cross-linking agents used, especially the glutaraldehyde, the residual retention of which may compromise the biocompatibility of chitosan delivery system.

Recently, a novel water-soluble, bisulfite blocked diisocyanate has been prepared and used as a cross-linking agent for the network formation of chitosan gel (Welsh, Schauer, Qadri, & Price, 2002; Lin-Gibson, Walls, Kennedy, & Welsh, 2003). In fact, the bisulfite blocked diisocyanate is the analogous Di-aminocarboxysulfonate, which must be devoid of any toxic effects of the relative diisocyanate. Also, the bisulfite derivatization of a diisocyanate group makes the blocked crosslinker soluble in water to allow for easy processability of crosslinked chitosan gel formations. Thus, 1,6-Hexamethylene dilsocyante (HMDI), a well studied diisocyanate was selected as cross-linking agent and reacted with sodium bisulfite to prepare the analogous blocked diisocyanate: Hexamethylene 1,6-Di-(aminocarboxysulfonate) (HDACS; Fig. 1).

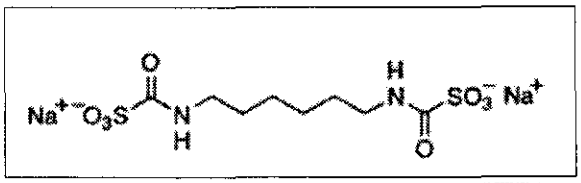

Fig. 1: Structure of **Hexamethylene 1,6-Di(aminocarboxysulfonate)**

Therefore, this research designs a chitosan formulation, crosslinked through the aforesaid water soluble blocked diisocyante crosslinker, and loaded with gentamicin in a mild acidic solution and eventually casted into a film, for sustained and consistent delivery of the antibiotic. The residual acid was neutralized with NaOH-Na_2CO_3 aqueous solution for varied time lengths. The resultant biomaterial was characterized for crosslinkage by FTIR, for hydrophilicity and swelling behavior and for the in vitro gentamicin controlled release over an extended period of 2 weeks; the effects of gentamicin initial concentration, cross-linking ratio and neutralization time on the kinetics of gentamicin release were also evaluated.

2. Experimental

2.1 Materials and Instrumentation

High molecular weight chitosan (Mw ~100,000, >75% deacetylated), Acetone, acetic acid, sodium bisulfite, ninhydrin, sodium hydroxide, sodium carbonate and hexamethylene diisocyanate (HMDI) were purchased from Aldrich Chemical Co., Milwauki, Wisconsin. Gentamicin sulfate and phosphate buffered saline (PBS) were purchased from Sigma Chemical Co. St. Louis, MO.

FTIR analyses were performed on a MIDAC - M series FTIR instrument from MIDAC Corporation (Costa Mesa, California); Contact angle instrument (Model: VCA-2000) was obtained from AST Products, Billerica, MA.

2.2 Synthesis of Hexamethylene 1,6-Di(aminocarboxysulfonate)

In a 100 mL round-bottom flask containing a magnetic stir bar, 6.73 g (40 mmol) of HMDI was added to 8.36 g $Na_2S_2O_5$ (44 mmol) dissolved in 15.53

mL H_2O and stirred for 20h. The product was precipitated with acetone and dried in vacuum. Insoluble polymeric byproducts were removed by dissolving the product in water (30 mL) followed by filtration. Product was isolated from the filtrate by precipitation in acetone and dried in vacuum, resulting in a white powder, as described earlier (Lin-Gibson, Walls, Kennedy, & Welsh, 2003).

2.3 Experimental Design for in-vitro gentamicin release from crosslinked chitosan

The effects of cross-linking ratio (0, 30 and 50%), initial antibiotic concentration (0, 1 and 10%) and neutralization time (0, 30 and 60 minutes) on the kinetics of in vitro release of gentamicin were investigated in a 3x3 factorial design (Table-1). The drug release was followed for 2 weeks and a colorimetric procedure, based on the ninhydrin reaction with amines present in gentamicin, was employed for released gentamicin quantification.

Table 1 – Experimental Design.

Sample #	**Concentration of HDACS, %***	**Concentration of Gentamicin Sulfate, %****	**Neutralization time, (min.)**
1	0	0	0
2	0	1	30
3	0	10	60
4	30	0	30
5	30	1	60
6	30	10	0
7	50	0	60
8	50	1	0
9	50	10	30

*based on NH_2 availability on chitosan; **based on chitosan concentration.

This outline uses the Taguchi "robust" statistical design in which the main effects are identified.

2.4 Cross-linking reaction

Crosslinking was performed by a modification in the procedure as described earlier (Lin-Gibson, Walls, Kennedy, & Welsh, 2003). Briefly, in a 250 mL flask, 1.5g of chitosan was dissolved in 100 mL of 1.0% aq. acetic acid with vigorous stirring. After total dissolution, the desired amount of Hexamethylene 1,6-Di(aminocarboxysulfonate) was added and the solution was heated to 60℃ under stirring for 24 h.

2.5 Preparation of gentamicin loaded films

After cross linking reaction, the desired amount of gentamicin sulfate was added to the solution and stirred for 24 h. One mL of the resultant solution was placed in each of the circular plastic molds of 16 mm diameter. The contents in the molds were allowed to dry at room temperature to form the circular films. The dried samples were then treated with aqueous NaOH (2.0% w/w)-Na_2CO_3 (0.05% w/w) solution to remove residual acetic acid. The time for alkaline treatment was chosen for different samples according to schedule shown in Table-1 above.

2.6 FTIR analyses

FTIR analyses on above thin film samples were performed on a MIDAC - M series FTIR instrument. The samples were scanned in the range of 500 to 2000 cm^{-1}.

2.7 Contact Angle

The experiments for contact angle measurements were performed in order to evaluate water affinity (hydrophilicity), an important parameter for wound dressings. The water contact angle with chitosan films was obtained by carefully placing a drop of water on the film surface and then, after 30 seconds, both right and left angles of contact were measured by the contact angle instrument, using a reported method (Zhang, Radomyselskiy, Datta, Zhao, & van Ooij, 2003).

2.8 Swelling Behavior

Swelling behavior of the above films was assessed by the gravimetric method. Samples films were kept in a vacuum desiccator for 24 h before determining dry mass (mD) by weighing to +/-0.0001g places on an electronic balance. Then, the samples were immersed for 24 h in 0.1M PBS maintained at pH 7.4 at 37℃. The soaked samples were then blotted with filter paper to remove non-absorbed surface water and then weighed again to determine wet mass (mW). Swelling ratio (S) was calculated, using the following equation:

$$S\ (\%) = [(m_W - m_D)/m_W] \times 100$$

Reported values are averages of three samples.

2.9 In vitro release

Experiments on In vitro release of gentamicin from the above chitosan films were performed at 37℃ in 0.1M PBS, pH 7.4. The release medium was collected after each 24 h and replaced with fresh buffer each time. This procedure was repeated for 14 days and the experiment was carried out in triplicate. Gentamicin concentration was measured indirectly by reaction with ninhydrin for 15 minutes at 95℃, followed by determination of ultraviolet absorbance at 400 nm by an established method (Frutos, Torrado, Perez-Lorenzo, & Frutos, 2000).

3. Results and discussion

Topical formulations of chitosan for wound healing and related applications and their effects on wound related components are being intensely investigated (Ueno, Mori, & Fujinaga, 2001). The use of chitosan for the controlled delivery of biomolecules is on rise recently (Yuan, Chesnutt, Utturkar, Haggard, Yang, Ong, & Bumgardner, 2007; Sinha, Singla, Wadhawan, Kaushik, Kumria, & Bansal, 2004). But, it forms extremely hygroscopic hydrogels and the controlled retention and release of a biomolecule from chitosan could be achieved by an appropriate

biocompatible cross-linker that has physicochemical compatiobility with chitosan. This work evaluates such a crosslinked chitosan based controlled release device to be later used for wound dressing film for sustained gentamicin release.

3.1 Cross-linker and cross-linking

To attain the crosslinking, first HDACS was synthesized as a crosslinker by a known method; it was then incorporated in required amounts (Table-1) in acidic solution of chitosan and the crosslinking, by the formation of urea linkage between HDACS and preferably the amino group of chitosan, was realized by mild heating for 24 h. Apparently, crosslinking must affect the hydrophilic behaviors of the crosslinked chitosan film, therefore after neutralization of residual acid, the water-contact angles and swelling behavior of the resultant films were measured. However, confirmation of chitosan crosslinking with HDACS was done by FTIR. The examination of the Hexamethylene 1,6-Diurea crosslinked chitosan films through various parameters used in this work is described as follows:

3.1.1 FTIR

The development of cross-linking was shown by a well resolved peak between 1565 cm-1 and 1475 cm-1, characteristic of NH deformation in secondary amides (Amide II band). In addition, a peak at about 1600 cm-1, representing C=0 stretch in secondary amides (Amide I band), was only observed for cross linked films, and its resolution was greater for the 50% cross linked samples (Welsh, Schauer, Qadri, & Price, 2002).

3.1.2 Contact angle measurement

The results obtained for the non-cross linked chitosan film were Right: 85.7° and Left: 85.2°. However, for both cross linked films (30% and 50% cross linked), the contact angles were 0°, since the water drop spread on the films. Thus, cross-linking increased the hydrophilic character of chitosan films.

3.1.3 Swelling behavior

The swelling behavior of chitosan films is presented in Figure 2. As expected, cross-linking suppressed swelling. However, this effect was inversely proportional to the degree of cross-linking, which can be attributed to the hydrophilic character of the cross linking agent.

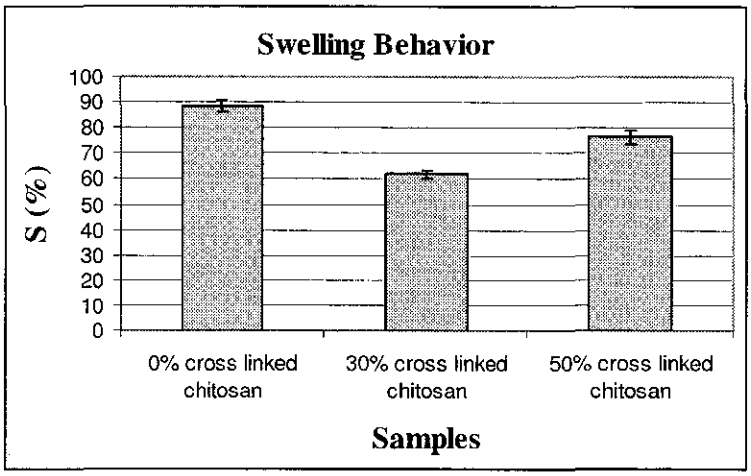

Figure 2 – Swelling behavior of Chitosan and HDACS cross-linked samples.

3.2 In vitro gentamicin release study

Results of in vitro release of gentamicin are presented in Figure 3. Samples 1, 4 and 7 were not plotted because their gentamicin initial concentration was 0 (Table 1).

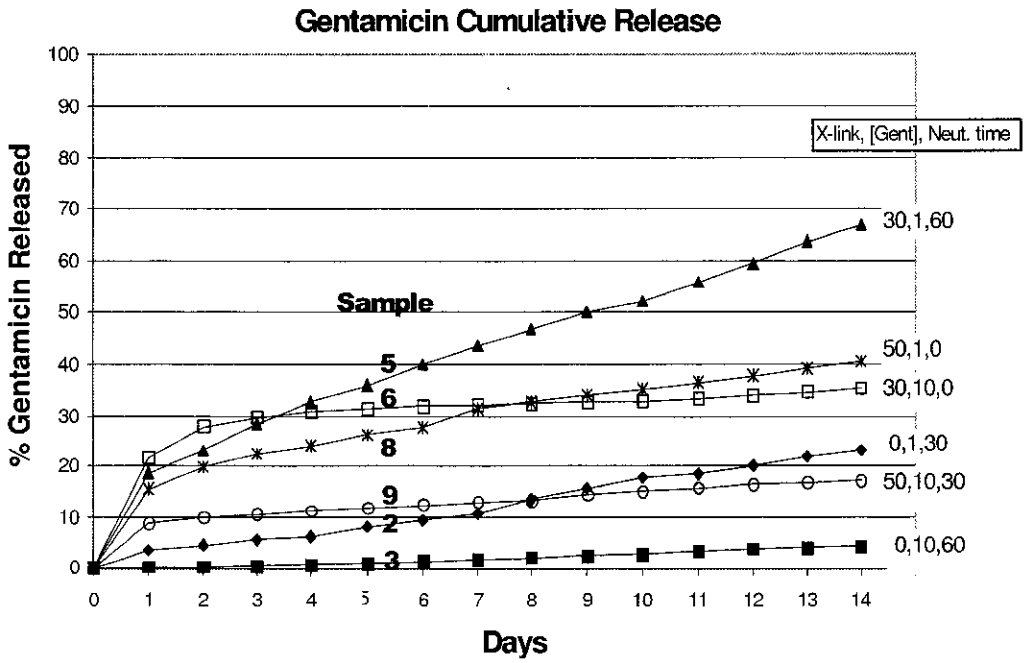

Figure 3 – Gentamicin cumulative release.

Since samples 2 and 3 were not cross linked, a considerable high amount of initial release of gentamicin was expected form these samples compared to those with crosslinking. However, the contrary effect was observed. Because of the good solubility of gentamicin in aqueous medium, probably most of gentamicin was released to the neutralization media when these samples were neutralized. Thus, after neutralization, the initial concentration of gentamicin for in vitro release experiment was lower than expected. However, this effect was not observed for cross-linked samples, because cross-linking created a polymeric network that might have acted as a barrier to fast diffusion of gentamicin during neutralization process.

Comparing sample pairs with the same degree of cross linking and different amounts of gentamicin (5 and 6; 8 and 9), it was observed that higher gentamicin initial concentration promoted slower percent release and the lower gentamicin initial concentration showed higher percent release. Therefore, the mechanism of gentamicin diffusion was mainly governed by its initial concentration. Indeed, the diffusion rate was inversely proportional to gentamicin initial concentration.

Comparing couples of sample pairs with the same gentamicin initial concentration, but different degrees of cross linking (5 and 8; 6 and 9), higher cross linking produced slower release. This was of course expected as the higher cross-link density increases the barrier for gentamicin diffusion. Thus, the diffusion rate was inversely proportional to the degree of cross-linking.

Pseudomonas aeruginosa, Escherichia coli and Staphylococcus aureus are the most common pathogens found in skin infected wounds. Minimal inhibition concentration of gentamicin for *Pseudomonas aeruginosa* and *Escherichia coli* varies between 0.06 through 8 μg/mL and for *Staphylococcus aureus*, it is between 0.12-1.0 μg/mL (Skundrik, Medovic, & Kostic, 2002). Therefore, the initial concentration of gentamicin should be selected according to pathogen in target and also to the treatment period. Prolonged treatments require slow diffusion and hence higher gentamicin initial concentration; for short treatments, the minimal inhibition concentration is reached even with lower gentamicin initial concentrations.

4. Conclusions

Chitosan – HDACS cross-linking reaction was efficient and confirmed by FTIR and hydrophilic behavior analyses. Cross-linked films swell less than chitosan film itself; however, this effect was inversely proportional to cross linker concentration, due to the hydrophilic character of the cross linking agent. Moreover, according to contact angle analysis, cross-linked films became more hydrophilic than the chitosan film itself.

In vitro gentamicin controlled release was carried out at 37° C and in pH 7.4 in order to simulate the body conditions. Results showed that the diffusion rate is governed by initial concentration of gentamicin and degree of cross-linking. Higher gentamicin initial concentration and degree of cross linking promoted the slower release: after 14 days of release, less than 20% of gentamicin initial concentration had been delivered (sample 9). On the other hand, lower gentamicin initial concentration and degree of cross-

linking promoted the faster release, with almost 70% of the gentamicin initial concentration released after 14 days (sample 5).

The process of neutralization seems to be limiting for non-crosslinked gentamicin loaded chitosan films. In this case, gentamicin initial concentration was altered by neutralization, since gentamicin was rapidly removed by dissolving into the neutralization media.

Additional experiments on wound healing were carried out with selected samples and will be published in communication later.

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4.7 *In vitro* Silver sulfadiazine Controlled Release from Chitosan cross linked Films

Liberação Controlada de Sulfadiazina de Prata *In vitro* a partir de Filmes Reticulados de Quitosana

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RESUMO: A sulfadiazina de prata é um agente antibacteriano eficiente e amplamente utilizado no tratamento de queimaduras, devido ao seu amplo espectro de atividade contra bactérias Gram positivas e Gram negativas. A pomada de sulfadiazina de prata tem sido usada para tratar feridas de queimadura infeccionadas; entretanto, este tratamento é doloroso, podendo levar à falha ou ao atraso da cicatrização, além de ter que ser repetido pelo menos duas vezes ao dia. Neste trabalho, desenvolveu-se um recobrimento de ferida carregado com sulfadiazina de prata , com a função de cobrir e proteger a ferida, além de liberar o princípio ativo aol longo do tempo, reduzindo a dosagem e o número de doses do do mesmo. A quitosana, um biopolímero biocompatível e biodegradável com atrativas características biológicas, foi utilizada na preparação dos filmes carregados de sulfadiazina de prata. Como agente reticulante, utilizou-se o hexametileno 1,6-Di(aminocarboxisulfonato), que é um diisocianato bloqueado e solúvel em água. A liberação de sulfadiazina de prata *in vitro* foi acompanhada por 2 semanas. Os efeitos da concentração inicial da sulfadiazina de prata, do grau de reticulação da matriz polimérica e do tempo de neutralização na cinética de liberação da sulfadiazina de prata foram avaliados.

PALAVRAS-CHAVE: sulfadiazina de prata, quitosana, recobrimento de feridas, reticulação.

In vitro Silver sulfadiazine Controlled Release from Chitosan cross linked Films

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ABSTRACT: Silver sulfadiazine is an effective and widely used antibacterial agent for treatment of burn injuries, due to its broad spectrum of activity against Gram-negative and Gram-positive bacteria. Silver sulfadiazine cream has been used to treat burn wound infections; however, this treatment is painstaking, can lead to failure in infection treatment or delay in wound healing and should be repeated at least twice a day. In this work, a novel wound dressing was developed to controlled delivery silver sulfadiazine while covering and protecting the wound, as well, reducing the drug dosage and dosing times. Chitosan, a biocompatible and biodegradable biopolymer with attractive biological characteristics, was used to prepare the antibiotic loaded films. Hexamethylene 1,6-Di(aminocarboxysulfonate), a water soluble and blocked diisocyante, was used as cross-linker agent. *In vitro* sulfadiazine release was observed for 2 weeks. The effects of silver sulfadiazine initial concentration, cross-linking ratio and neutralization time on the kinetics of sulfadiazine release were evaluated.

KEYWORDS: silver sulfadiazine, chitosan, wound dressing, cross-linking.

INTRODUCTION

Sulfadiazine (Figure 1) is a sulfonamide antibiotic which eliminates bacteria that cause infections by stopping the production of folic acid inside the bacterial cell. It is commonly used in combination with silver to treat skin

wounds. Silver sulfadiazine has been a standard treatment for burns, ulcers and infected wounds due to its broad spectrum of activity against both Gram positive and negative bacteria, besides fungus, even at low concentration.[1] Silver sulfadiazine cream has been traditionally applied to wounds; however, it can not allow long-term protection of the wound from infection. For this reason, topical silver sulfadiazine should be applied to the wound at least twice a day, according to doctor prescription. Thus, traditional treatment can lead to failure in infection treatment or delay in wound healing, since the dosage is not precisely and continuous. Moreover, absorption of silver ions has been a major concerned during usage of silver sulfadiazine cream in deep burns that may be cytotoxic for treated tissues. [2]

Due to all these reasons, a novel silver sulfadiazine loaded wound dressing was developed, in order to protect the wound, while releases antibacterial agent to treat or avoid bacterial infection.

Chitosan is a biopolymer derived from chitin, the second most abundant polysaccharide found in nature. Chitosan has been extensively studied in biomaterials field. It is biocompatible and biodegradable. In addition, chitosan can accelerate wound healing and has shown hemostatic and bacteriostatic activities.[3,4] Due to its unique properties, chitosan has found wide application in biomedical area, such as: implants, drug delivery matrix, scaffolds for tissue engineering, skin substitutes and wound dressings.[5,6 and 7]

Chitosan is soluble in acid solutions and can easily form films and membranes by solvent evaporation technique. However, due to its positive charges in acid medium, caused by its amino group protonation, chitosan is extremely hygroscopic. In this way, it is necessary to crosslink its chains, in order to prepare an efficient drug delivery matrix.

Diisocyanates are usually used as cross-linker agent in Chemical Industries. However, because of reactivity with and insolubility of most commercially available diisocyanates in water, it should be reacted with a

bisulfite to protect, or block, reactivity and to increase water solubility before be reacted with chitosan. Thus, the product of block reaction, stable in acidic aqueous solutions, with increased pH or temperature, readily reacts with amines, forming a urea linkage, at a rate much greater than that characteristic of competing reactions with alcohols or water. Furthermore, stability in the presence of amines and consequent long-term storage may be increased by the choice of an aliphatic diisocyanate, rather than a more reactive aromatic one. For this reason, HMDI (1,6-HexaMethylene Dilsocyante), a well studied diisocyanate frequently used in the Polyurethane Industry, was selected as cross-linker agent and hence reacted with sodium bisulfite, in order to prepare a blocked diisocyanate: **Hexamethylene 1,6-Di(aminocarboxysulfonate)**.

The effects of cross-linking ratio (0, 30 and 50%), silver sulfadiazine initial concentration (0, 1 and 10%) and neutralization time (0, 30 and 60 minutes) on the *in vitro* release of silver sulfadiazine were evaluated. The release was followed for 2 weeks and sulfadiazine concentration was determined by UV Spectrophotometry.

Figure 1- Sulfadiazine and silver sulfadiazine structures.

MATERIALS AND METHODS

Materials

High molecular weight chitosan (>75% deacetylated), silver sulfadiazine and hexamethylene diisocyanate were purchased from Aldrich. Sodium bisulfite Reagent Plus® was purchased from Sigma-Aldrich.

Synthesis of Hexamethylene 1,6-Di(aminocarboxysulfonate)

In a 100 mL round-bottom flask containing a magnetic stir bar, 6.73 g (40 mmol) of HMDI was added to 8.36 g $Na_2S_2O_5$ (44 mmol) dissolved in 15.53 mL H_2O and was stirred for 20h at room temperature. The product was

precipitated in acetone and dried in vacuum. Insoluble polymeric byproducts were removed by dissolving the product in water (30 mL) followed by filtration. Product was isolated from the filtrate by precipitation in acetone and dried in vacuum, resulting in a white powder.[8]

Experimental Design for in vitro controlled release

Matrix experiments using orthogonal arrays were applied for experiment design. Samples were prepared according to Table 1.

Cross linking reaction

In a 250 mL flask, 1.5g of chitosan was dissolved in 1.0% acetic acid solution. After total dissolution, the desired amount of Hexamethylene 1,6-Di(aminocarboxysulfonate) was added and the solution was heated up to 40°C under stirring for 24 h.

Preparation of silver sulfadiazine loaded films

After cross linking reaction, the desired amount of silver sulfadiazine was added to the solution and stirred for 24 h. Then, the resulted solution was cast in the molds and dried at room temperature. Dried samples were then neutralized in Ethanol 70% solution according to Table 1.

Table 1 – Experimental Design.

Sample	**Concentration of Hexamethylene 1,6-Di (aminocarboxysulfonate), %***	**Initial Concentration of Silver Sulfadiazine, %****	**Neutralization time, min.**
1	0	0	0
2	0	5	30
3	0	10	60
4	30	0	30
5	30	5	60
6	30	10	0
7	50	0	60
8	50	5	0
9	50	10	30

*based on NH_2 availability on chitosan; **based on chitosan concentration.

FTIR

FTIR analyses were performed in a MIDAC – M series FTIR instrument in the range of 500 up to 2000 cm^{-1}.

Contact Angle

Contact angle measured was performed in an AST model VCA 2000 instrument, in order to evaluate samples' water affinity (hydrophilicity) that is an important parameter for wound dressings.

Swelling Behavior

Swelling behavior of chitosan films was determined by the gravimetric method. Samples (D= 1 cm) were kept in a vacuum desiccators for 24 h and then, their dry mass (m_D) were determined by weighting them on an electronic scale. After this procedure, samples were immersed in 0.1M PBS pH 7.4 at 37°C. Past 24 h, samples were blotted with filter paper to remove the adsorbed water on the surface, and weighted again in order to determine their wet mass (m_W). The swelling ratio (S) of chitosan films was calculated according to the following Equation:

$$S\ (\%) = [(m_W - m_D)/m_W\ ] * 100$$

In vitro release

In vitro release of sulfadiazine from chitosan films was performed at 37°C in 0.1M PBS, pH 7.4. The release medium was collected at predetermined time intervals (24 h) and replaced with fresh buffer. This procedure was repeated for 14 days and carried out in triplicates. Samples were analyzed by UV Spectrophotometer at 236 nm to determine the concentration of released sulfadiazine.

RESULTS

FTIR

According to the infra red spectra, cross linking reaction could be supported by the presence of a peak better resolved in between 1565 cm^{-1} and 1475 cm^{-1}, that represents NH deformation in secondary amides (Amide II band). In addition, a peak around 1600 cm^{-1}, that represents C=0 stretch in secondary amides (Amide I band), was only observed for cross linked films, and its resolution was greater for the 50% cross linked sample.[9]

Contact Angle

The water contact angle with chitosan films was obtained by dropping 1 drop of water on the film surface and then, after 30 seconds, both right and left angles of contact were measured. The results obtained for the not cross linked chitosan film were **R: 85.7°** and **L: 85.2°**. However, for both cross linked films (30% and 50% cross linked), the contact angles were **0°**, since the water drop spread on the films. It means that cross linking reaction increased the hydrophilic character of chitosan films.

Swelling behavior

Swelling behavior of chitosan films is presented in Figure 2. As expected, cross linking reaction decreased the swollen of films. However, this effect was inversely proportional to the degree of cross linking. It can be attributed to the cross linking agent hydrophilic character.

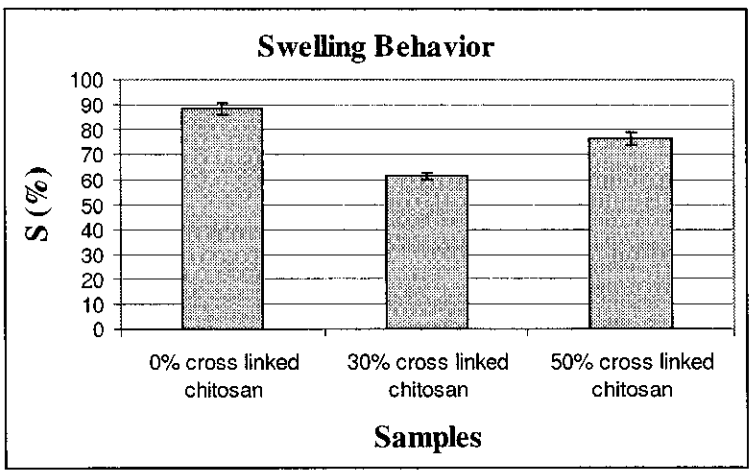

Figure 2 – Swelling behavior of samples.

In vitro release

Silver sulfadiazine is poorly water soluble (0.34 mg/100 ml) and after dissociation, releases silver as well as sulfadiazine ions. Thus, silver sulfadiazine release can be divided in two steps: sulfadiazine release and silver release. Because there is no interaction between sulfadiazine and chitosan, sulfadiazine release depends on the solubility of silver sulfadiazine in the dissolution medium. However, chitosan contains amino groups which are capable of binding silver and hence, silver release

depends also on the chemical interactions between silver ions and chitosan. Moreover, both releases depend on the mass transport resistance caused by chitosan films.

According to Figure 3, all samples displayed a burst release in the first days. This initial release is very important to fast control bacterial infection in wounds treatment. After this initial period, slow release of sulfadiazine was observed.

Figure 4 well illustrated sulfadiazine cumulative release. Samples 2, 5 and 8, containing 5% silver sulfadiazine initial concentration, showed similar behavior and have released around 30% of theirs initial concentration after 14 days, while samples 3, 6 and 9 (10% silver sulfadiazine initial concentration) have released more than 15%.

According to these results and due to silver sulfadiazine low molecular weight, chitosan films mass transport resistance did not control sulfadiazine release, but silver sulfadiazine solubility did. Samples 3, 6 and 9 that contained twice the silver sulfadiazine initial concentration of samples 2, 5 and 8 showed approximately half of cumulative silver sulfadiazine release observed for samples 2, 5 and 8.

Staphylococcus aureus, Pseudomonas aeruginosa and *Escherichia coli* are the most common pathogens found in burns and skin wounds. Silver sulfadiazine minimal inhibition concentration (MIC) was found to be 32 µg/mL for *Escherichia coli,* 44.8 µg/mL for *Pseudomonas aeruginosa* and 57.6 µg/mL for *Staphylococcus aureus.* [1] Besides, minimal bactericidal concentration (MBC) was 40.2 µg/mL and 51.2 µg/mL for *Escherichia coli* and *Pseudomonas aeruginosa*, respectively. For *Staphylococcus aureus* MBC was equal to MIC.

Thus, samples 2, 5 and 8 released enough amount of silver sulfadiazine to inhibit these three pathogens just in the first day, while samples 3, 5 and 9 only did not release enough silver sulfadiazine to inhibit *Staphylococcus aureus*. From the second day on, all samples have kept minimum inhibitory concentration for all three pathogens. Therefore, the presence of infection

as well as the identification of pathogen should be considered before sample selection since minimal silver sulfadiazine should be released to avoid silver excess and body absorption.

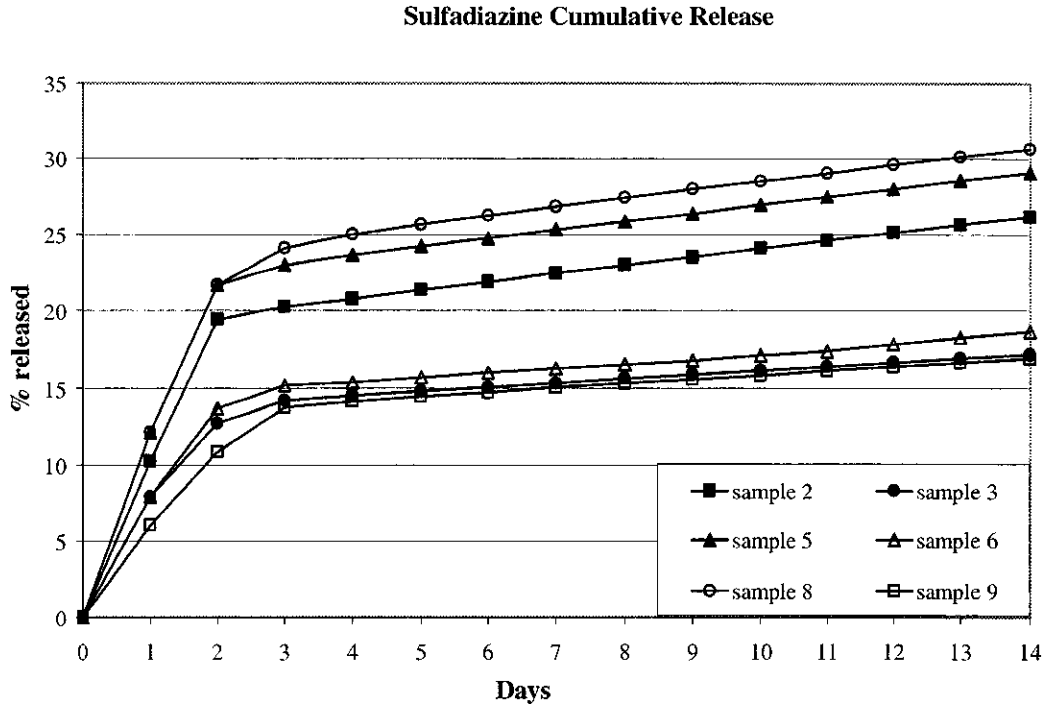

Figure 3 – Sulfadiazine cumulative release.

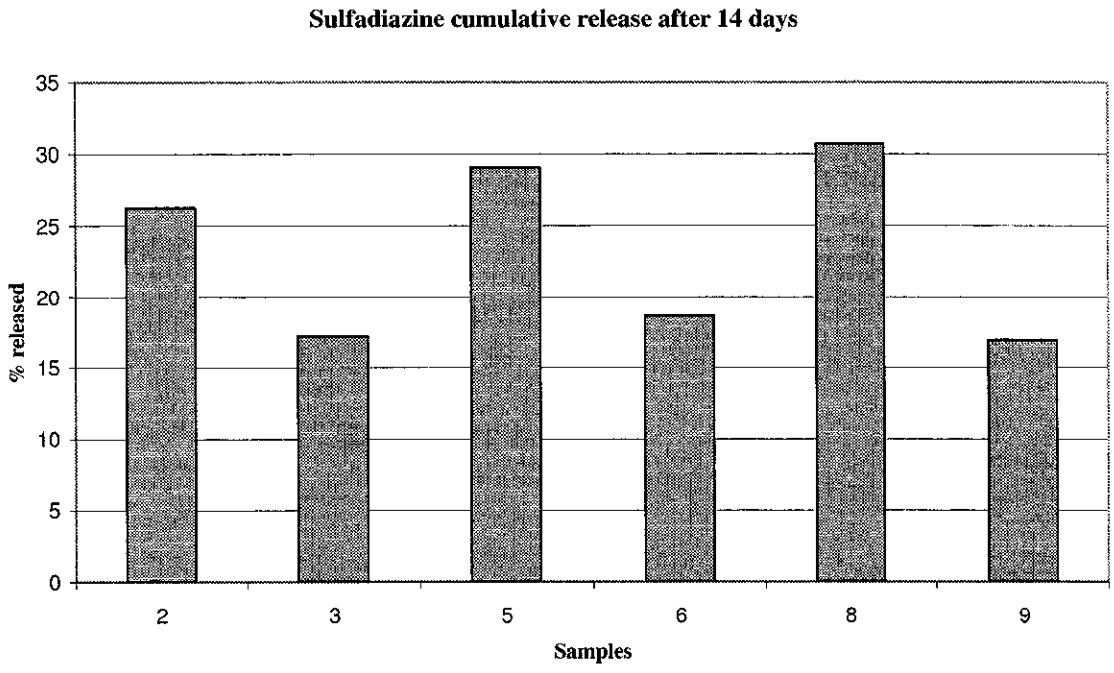

Figure 4 – Sulfadiazine cumulative release after 14 days.

CONCLUSION

Chitosan - Hexamethylene 1,6-Di (aminocarboxysulfonate) cross linking reaction was efficient and confirmed by FTIR and swelling analyses. Cross linked films swollen less than chitosan one and this effect was inversely proportional to cross linker concentration, due to the hydrophilic character of the cross linking agent. Moreover, according to contact angle analysis, cross linked films became more hydrophilic than chitosan film. Besides, the effect of cross linker concentration could not be evaluated because the water drop spread on both cross linked films.

Silver sulfadiazine release was controlled by its solubility in the dissolution medium. Because of silver sulfadiazine low molecular weight, mass transport resistance did not govern the diffusion process.

Samples with higher silver sulfadiazine initial concentration should be used to treat infected wounds, while the samples with lower one could be used to avoid wound infections.

Additional experiments such as *in vitro* and *in vivo* wound healing were carried out with selected samples and will be published in a further paper.

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4.8 *In Vitro* Wound Healing Responses to Drug Loaded Cross Linked Chitosan Films

Avaliação *in vitro* da cicatrização de feridas tratadas com filmes reticulados de quitosana carregados com fármacos

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RESUMO: Atualmente, o uso de cobaias em experimentos de pesquisa tem sido amplamente questionado pelas Instituições e Organizações protetoras de animais. Por outro lado, testes preliminares e validação científica da hipótese de pesquisa são requeridos antes de um teste *in vivo* ser aprovado. Devido às numerosas etapas a serem completadas até se conseguir a aprovação de um estudo *in vivo*, este procedimento pode levar muito tempo. Por todas estas razões, o desenvolvimento de um teste *in vitro* capaz de substituir a pesquisa em animais seria muito interessante. Com esse objetivo, este trabalho dedicou-se ao desenvolvimento de um novo modelo de ferida *in vitro* para a avaliação do potencial cicatrizante de filmes de quitosana carregados de antibióticos. MG-63, uma linhagem de células derivada de um osteosarcoma, foi usada no modelo *in vitro*, enquanto que os filmes de quitosana foram carregados com gentamicina e sulfadiazina de prata, um antibiótico e um agente antibacteriano, respectivamente.

PALAVRAS-CHAVE: quitosana, antibiótico, cicatrização de ferida, cultivo celular, *in vitro.*

In vitro wound healing responses to drug loaded cross linked chitosan films

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ABSTRACT: Currently, animals use in research experiments has been widely questioned by Animal Protect Institutions and Organizations. Moreover, preliminary tests and scientific validation of research hypothesis are required before *in vivo* tests be approved. Due to the numerous steps to be accomplished before *in vivo* study approval, this procedure could take a long time. For all these reasons, the development of an *in vitro* test that could replace animal research would be very interesting. Therefore, the aim of this work was the development of a novel *in vitro* wound model for evaluation of the healing potential of drug loaded chitosan films. MG-63, a cell line derived from an osteosarcoma, was used in the *in vitro* model, while chitosan films were loaded with gentamicin and silver sulfadiazine, an antibiotic and an antibacterial agent, respectively.

KEYWORDS: chitosan, antibiotic, wound healing, cell culture, *in vitro.*

INTRODUCTION

Animal testing or animal research refers to the use of animals in research experiments. Worldwide, about 100 million animals [1] are used annually and either killed during the experiments or subsequently euthanized. Animal research is mainly carried out inside universities, medical schools and pharmaceutical companies, although some commercial facilities provide animal-testing services to industry. [2]

Groups that supports animal research affirm that it has played a vital role in every major medical advance and that many major developments, such as penicillin (mice), organ transplant (dogs), and poliomyelitis vaccine (mice, monkeys) involved animal research. [3] However, the topic is controversial. Opponent groups argue that animal testing is unnecessary, poor scientific practice, poorly regulated, that the costs outweigh the benefits, or that animals have an intrinsic right not to be used for experimentation.

Nevertheless, most scientists and governments agree that animal testing should cause as little suffering to animals as possible, and that animal tests should only be performed where necessary, respecting the three **R**'s principle: **R**educing, **R**eplacement and **R**efinement. In this context, the development of *in vitro* tests that could replace preliminary animals testing is an interesting, current and crucial research field.

Chitosan is a biopolymer derived from chitin, the second most abundant polysaccharide found in nature. Due to its interesting biological properties such as: biocompatibility, biodegradability, antibacterial activity and bio-adhesion, it has been widely studied and applied as a biomaterial in the biomedical area.[4 and 5] Biomedical applications of chitosan involve implants, drug delivery matrix, scaffolds for tissue engineering, skin substitutes and wound dressings [6, 7 and 8]. In this present work, drug loaded chitosan films for wound dressing were prepared and their wound healing potential was *in vitro* evaluated.

Gentamicin, an amino-glycoside antibiotic, and silver sulfadiazine, a standard antibacterial agent for burns treatment, were used to prepare the drug loaded chitosan films. They are topically used for treatment of skin infections and burn wounds for a long time. Gentamicin is one of the most consumed antibiotics in the world. Its high consumption is due to its low cost, wide antibacterial spectrum of action, low rate of primarily resistant pathogens, low allergy rate, as well as good thermo-stability and water solubility. [9]

Silver sulfadiazine possesses a wide spectrum of activity against Gram positive and negative bacteria and fungus. [10] Besides, it is effective even at low concentrations due to the presence of silver ions that kill bacteria and do not cause pathogen resistance.

For the in vitro model, human MG-63 cells, a cell line derived from an osteosarcoma, was cultured. Wounds were created and the healing potential of drug loaded chitosan films were qualitatively evaluated by the wound filling rate.

MATERIALS AND METHODS

Materials

High molecular weight chitosan (>75% deacetylated), hexamethylene diisocyanate and silver sulfadiazine were purchased from Aldrich. Gentamicin sulfate salt was purchased from Sigma and sodium bisulfite Reagent Plus®, from Sigma-Aldrich. MG-63 cell line was gently supplied by Dr. Cohen.

Synthesis of cross linking agent

In a 100 mL round-bottom flask containing a magnetic stir bar, 6.73 g (40 mmol) of HMDI was added to 8.36 g $Na_2S_2O_5$ (44 mmol) dissolved in 15.53 mL H_2O and was stirred for 20h at room temperature. The product was precipitated in acetone and dried in vacuum. Insoluble polymeric byproducts were removed by dissolving the product in water (30 mL) followed by filtration. Product was isolated from the filtrate by precipitation in acetone and dried in vacuum, resulting in a white powder. [11]

Preparation of chitosan film

1.5g of chitosan was dissolved in 100 ml of 1.0% acetic acid solution. After total dissolution, solution was cast in the molds and dried at room temperature.

Cross linking reaction

In a 250 mL flask, 0.8g of hexamethylene 1,6-di(aminocarboxysulfonate) was added to 100 ml of 1.5% chitosan solution and heated up to 40℃ under stirring for 24 h.

Preparation of chitosan cross linked film

The solution above described was cast in the molds and dried at room temperature in order to prepare the chitosan cross linked film.

Preparation of gentamicin loaded film

After cross linking reaction, 1% gentamicin sulfate was added and homogenized for 24 h. Then, the resulted solution was cast in the molds and dried at room temperature. Dried samples were then neutralized in Ethanol 70% for 30 minutes and sterilized in UV light.

Preparation of silver sulfadiazine loaded film

The same procedure was used to prepare silver sulfadiazine loaded films. However, 5% silver sulfadiazine was used instead of 1% gentamicin sulfate.

Cell culture

MG-63 cells were seeded in six-well tissue culture plates (Corning Co, Houston, TX). The seeded cells were incubated in α-MEM (Minimum Essential Media Alpha Medium) with 5% fetal bovine serum (FBS) and humidity atmosphere of 5% CO_2 and 95% air at 37° C. After cells reached confluence, wounds were created across the surface of each well (Figure 1) and the media were changed. The wounds were then examined microscopically to ensure that cellular and extra-cellular materials were removed from the wound sites.

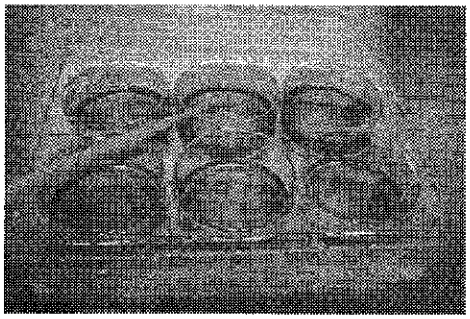

Figure 1 – *In vitro* wounds.

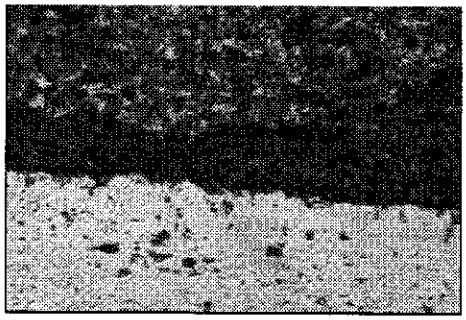

Figure 2 – In vitro wound healing at 0 day.

At this moment, chitosan films were placed in the wells and in each plate, the positive control was a well with no film. In order to evaluate chitosan effect on wound filling rate, a chitosan film was incubated. Besides, a cross linked chitosan film was also incubated to evaluate cross linking agent effect on *in vitro* wound healing. Gentamicin and silver sulfadiazine effects were evaluated by incubation of drug loaded cross linked chitosan films. Samples were identified according to Table 1 and the experiment was carried out on duplicates. At every two days, culture media were replaced in order to provide essentials nutrients for cellular growth.

Table 1 – Samples identification.

Sample	Content
A	No film (positive control)
B	Chitosan film
C	Chitosan cross linked film
D	Gentamicin loaded chitosan cross linked film
E	Silver sulfadiazine loaded chitosan cross linked film

Determination of wound filling rate

Wound filling rate was qualitatively measured after 0, 7, 14 and 21 days of incubation by using a microscope with photography apparatus.

After each desired period, films were removed from the wells and kept in formol for 24 hours for further investigation. Then, the cells were stained with 20 ml of T-blue for 2 minutes at 37° C. After staining, all media were removed and cells were washed with 1 ml of 0.1M phosphate buffer solution (PBS) pH 7.4. Then, PBS was removed and 1 ml of formol was

added. Cells were kept at 4℃ for 24 hours and, after this procedure, formol was replaced for ethanol 70%. At this moment, cells were ready to be photographed and the wound filling rate was qualitatively determined by the amount of cells that grown in each wound site.

RESULTS

Figure 2 illustrates the wound at 0 day. As expected, cells were stained, while the wound site was not. I*n vitro* wound healing results for 7, 14 and 21 days are presented in Figure 3. According to Figure 3, samples D and E showed inferior wound filling rate when compared to positive control (sample A) in the first week. However, this effect was not observed for sample D in the next week. At 21 days, excepted for sample E, wound sites were almost totally filled by cells.

Lee *et al* [12] reported the cytotoxic effect of silver sulfadiazine on HaCaT cells and delayed epithelialization in animal model. Besides, a marked inhibition of 3T3 fibroblasts growth, caused by 1% silver sulfadiazine cream, was observed by Mi *et al* [13]. However, according to authors, this inhibition was significantly reduced by the use of a silver sulfadiazine incorporated asymmetric chitosan membrane.

Gentamicin ototoxicity and neuphrotoxicity have been extensively studied. [14 and 15] Nevertheless, these deleterious effects seem to be dosing dependent and have not been well established yet. Lin *et al* [16] reported that application of 2% gentamicin eye drops in porcine cornea disturbed the corneal epithelial healing rate significantly. Bertolaso *et al* [17] reported the biochemical mechanism underlying gentamicin cytotoxicity in OC-k3 cells. However, cytotoxic effect of gentamicin on fibroblast growth is not well discussed. According to figure 3, gentamicin deleterious effect only is significant in the first week of *in vitro* wound healing. Probably, this is an adaptation period to the cells and after that, wound filling rate is recovered. Past 21 days, sample D wound filling is almost equivalent to sample A (positive control).

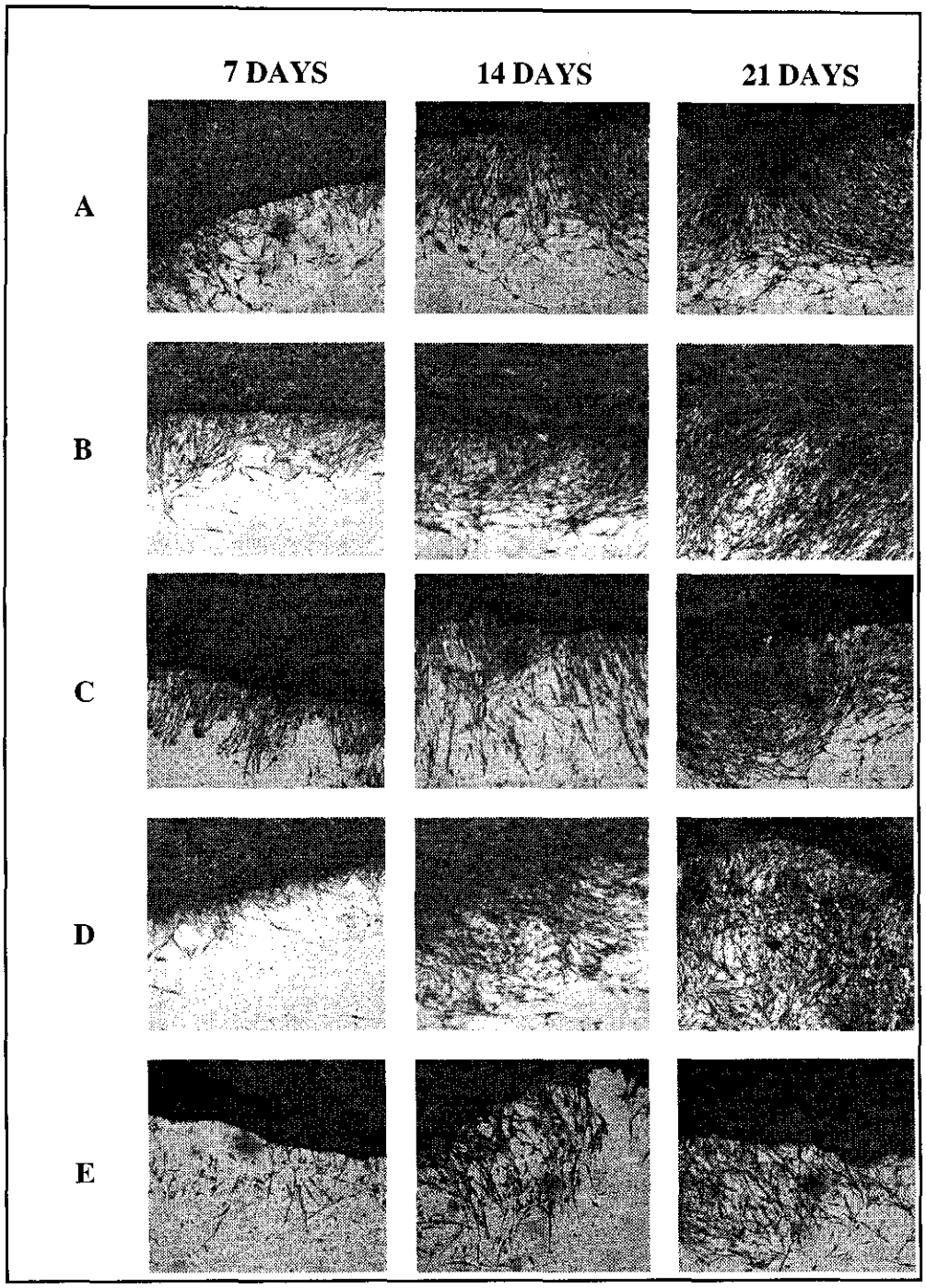

Figure 3 – In vitro wound healing results.

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Chitosan and cross linked chitosan films (samples B and C, respectively) did not show cytotoxicity, but stimulated cells growth. Ueno *et al* [18] reported that chitosan stimulated migration and proliferation of fibroblast and also collagen production in dogs' wound healing. Moreover, wound healing experiments using mouse model have shown that the application of chitosan hydrogel onto an open wound induced significant wound contraction and accelerated wound healing. [19]

CONCLUSION

In vitro wound healing model results have shown to be comparable to those obtained in previous animal studies. Therefore, the presented *in vitro* wound healing model could replace preliminary animal testing, collaborating to the three R principles: reduction, replacement and refinement.

As expected, chitosan films enhanced cells growth. In addition, cross linked chitosan films was not cytotoxic for MG-63 cells and thus, hexamethylene 1,6-di(aminocarboxysulfonate) can be considered a safety cross linking agent.

Wound healing deleterious effects of drug loaded chitosan films on *in vitro* wound healing were more pronounced for silver sulfadiazine that disturbed wound filling during all 21 days. Cells growth was initially delayed by gentamicin, however, after this adaptation period, cells started to grow fast. In this context, the use of drugs in wound treatments should be carefully evaluated since it could delay wound healing, instead of accelerate it.

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6. CONCLUSÕES

- As soluções de quitosana apresentaram atividade antibacteriana contra a bactéria *Escherichia coli*, e a inibição do crescimento foi inversamente proporcional à concentração de quitosana;
- A solução de plastificante (d-sorbitol), bem como a solução solvente (ácido acético 1.0%) também apresentaram atividade antibacteriana contra a bactéria *E. coli;*
- A membrana de quitosana (CA) apresentou a menor permeabilidade ao vapor d'água devido à sua alta cristalinidade;
- Apesar de apresentar propriedades mecânicas aceitáveis, CA apresentou citotoxicidade direta às células *Vero* devido à presença de ácido acético residual;
- A neutralização foi uma etapa muito importante na preparação das membranas de quitosana, pois:

1- Neutralizou o ácido acético residual que é diretamente citotóxico;

2- Diminuiu o grau de hidratação das membranas, evitando a formação de gel após contato com a água;

3- Aumentou a permeabilidade ao vapor de água;

- A membrana neutralizada (CN) apresentou o menor grau de hidratação e permeabilidade ao vapor d'água intermediária quando comparada às membranas plastificada (CP) e não-neutralizada (CA);
- O D-sorbitol é um plastificante seguro para as células, porém, devido ao seu caráter altamente higroscópico, o grau de hidratação das membranas de quitosana aumentou muito com a adição deste plastificante;
- A adição de plastificante também aumentou a permeabilidade ao vapor de água e a flexibilidade das membranas de quitosana;

- As membranas de quitosana CN e CP mostraram-se satisfatórias para uso como recobrimentos de feridas; entretanto, as mesmas devem ser reticuladas a fim de serem utilizadas como sistemas de liberação controlada de fármacos;
- A reação de reticulação da quitosana com o hexametileno 1,6-di-(aminocarboxisulfonato) foi bem sucedida. Os graus de reticulação foram 30 e 50%;
- O hexametileno 1,6-di-(aminocarboxisulfonato) mostrou-se um excelente agente reticulante. Como o material reticulado não ficou quebradiço, a adição de plastificante não foi necessária;
- O grau de hidratação das membranas de quitosana reticuladas foi inversamente proporcional à concentração de agente reticulante, devido à elevada hidrofilicidade do mesmo;
- A reticulação aumentou o caráter higroscópico das membranas de quitosana, e consequentemente, a biocompatilidade do material;
- De acordo com os testes , as membranas reticuladas de quitosana mostraram-se eficientes na liberação controlada de gentamicina e sulfadiazina de prata;
- O modelo de cicatrização de feridas *in vitro* utilizando a linhagem celular MG - 63, mostrou-se um excelente método para se estudar os efeitos dos agentes antimicrobianos no processo de cicatrização;
- As membranas de quitosana, reticuladas ou não, favoreceram o crescimento *in vitro* das células MG -63 quando adicionadas ao meio de cultivo celular;
- A membrana de quitosana 30% reticulada, carregada com 1% de gentamicina, inibiu inicialmente o crescimento *in vitro* das células MG - 63;

- A membrana de quitosana 30% reticulada, carregada com 5% de sulfadiazina de prata, inibiu parcialmente o crescimento *in vitro* das células MG - 63;
- A utilização ou não de antibióticos ou agentes antimicrobianos deve ser seriamente considerada durante o tratamento de feridas, uma vez que a utilização dos mesmos pode comprometer o processo de cicatrização.

7. SUGESTÕES PARA TRABALHOS FUTUROS

- Estudar a liberação controlada de agentes antiinflamatórios e outros tipos de medicamentos, utilizando os mesmos sistemas de liberação à base de quitosana reticulada;
- Avaliar as propriedades mecânicas, térmicas e de permeabilidade dos sistemas de liberação obtidos;
- Desenvolver sistemas de liberação controlada de fármacos para recuperação de cartilagem de eqüinos.

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9. APÊNDICES

9.1 TESTE *IN VIVO*

Como parte complementar ao presente estudo, foi feito um teste preliminar qualitativo *in vivo*. O animal indicado e utilizado para tal teste foi o rato, devido ao seu tamanho/porte adequado e ao curto período de cicatrização destes animais. O teste in vivo foi conduzido da seguinte forma:

1- Foram selecionados 10 animais adultos, sadios, de sexo aleatório, pesando cerca de 500 g cada;

2- Os animais foram anestesiados e depilados na parte dorsal;

3- Oito feridas foram feitas no dorso de cada animal com o auxílio de um aplicador de cola quente, com a finalidade de simular uma queimadura;

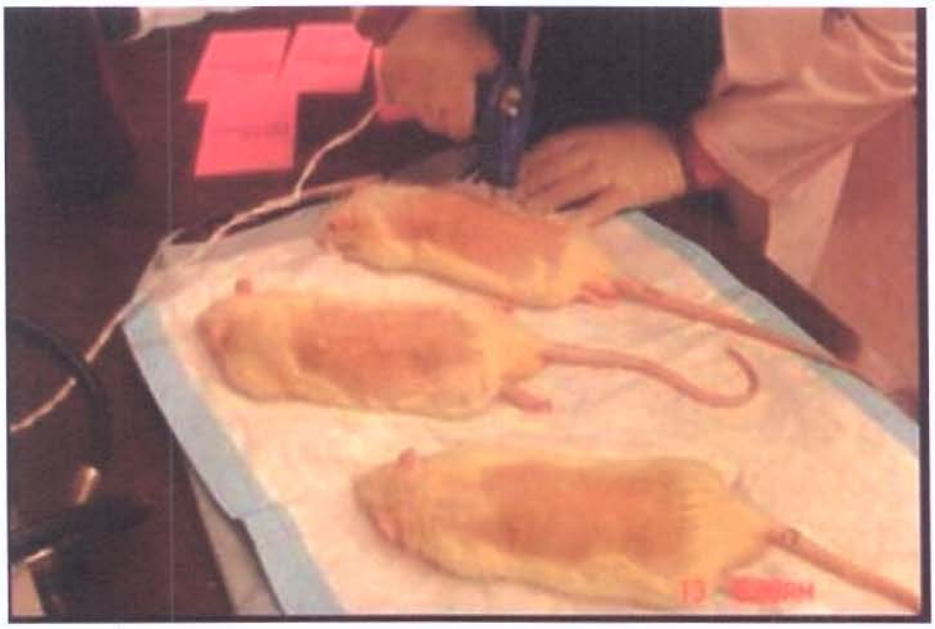

Figura 8 – Aplicação da cola quente.

4- Após 24 horas da aplicação da cola quente, os animais foram novamente anestesiados para a remoção do tecido morto e aplicação dos seguintes tratamentos: **A – sem tratamento (controle); B – membrana de quitosana; C – membrana de quitosana 30% reticulada carregada com gentamicina a 1% e D – membrana de quitosana 30% reticulada e carregada com 1% de sulfadiazina de prata.**

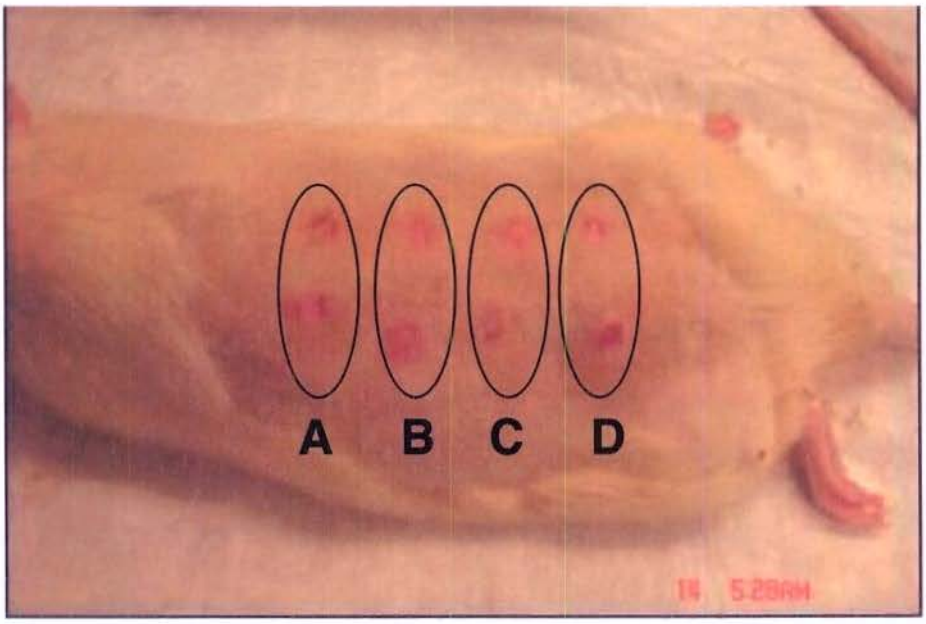

Figura 9 – Aplicação dos tratamentos nas feridas.

5- Depois de aplicados os tratamentos, foi passada bandagem para garantir que os tratamentos não fossem removidos pelos animais. Os animais foram então acondicionados em gaiolas individuais, com regime de 12 horas de luz/dia e alimentados normalmente;

6- Após 7 dias, as bandagens foram retiradas e a cicatrização das feridas foi avaliada de acordo com os tratamentos aplicados.

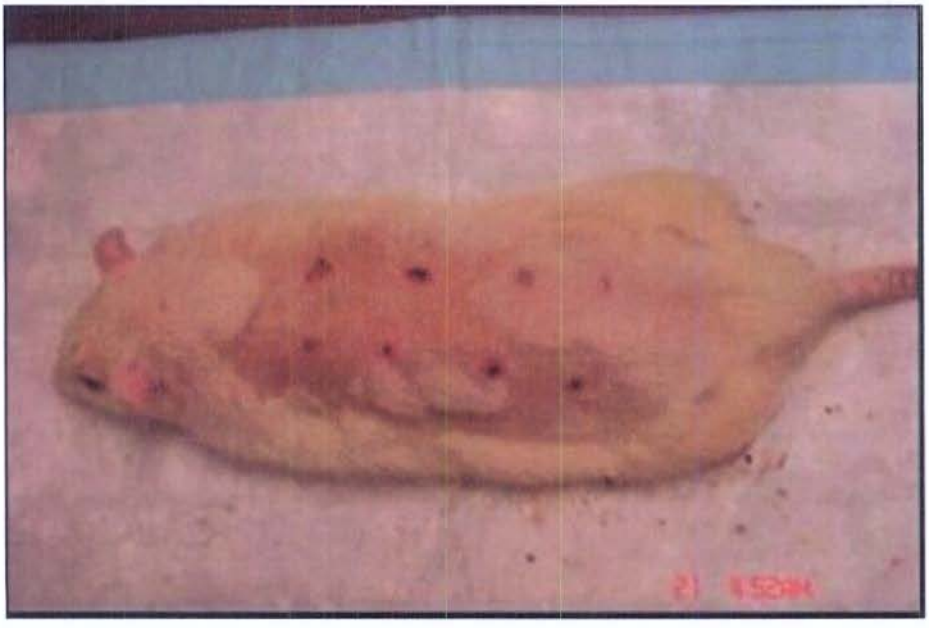

Figura 10 – Cicatrização das feridas após 7 dias.

Os sinais de cicatrização observados para os tratamentos à base de quitosana foram: diminuição do raio da ferida e presença de pêlos. Em todas as situações, as feridas tratadas evoluíram melhor que a ferida controle. Entretanto, não foi possível observar diferença significativa entre os tratamentos B, C e D.

O ensaio *in vivo* foi feito na University of Texas Health Science Center, respeitando os protocolos internacionais e as normas para a realização de testes com animais.

2.6 CURVAS DE DSC

Estão apresentadas a seguir, as curvas de DSC das membranas de quitosana (CA), quitosana neutralizada (CN) e quitosana-D-sorbitol (CP).

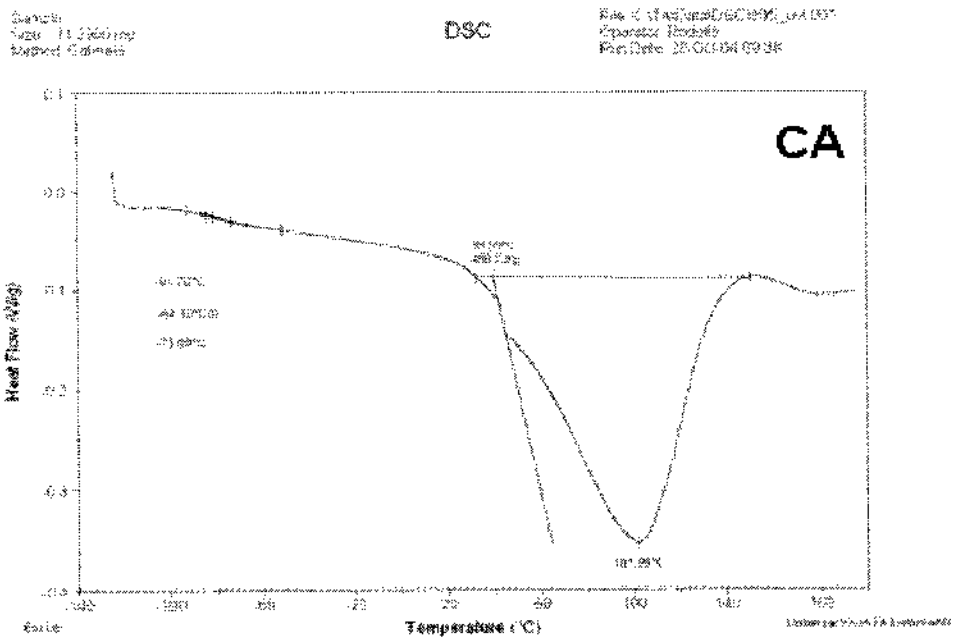

Figura 11 – Curva de DSC para a membrana de quitosana.

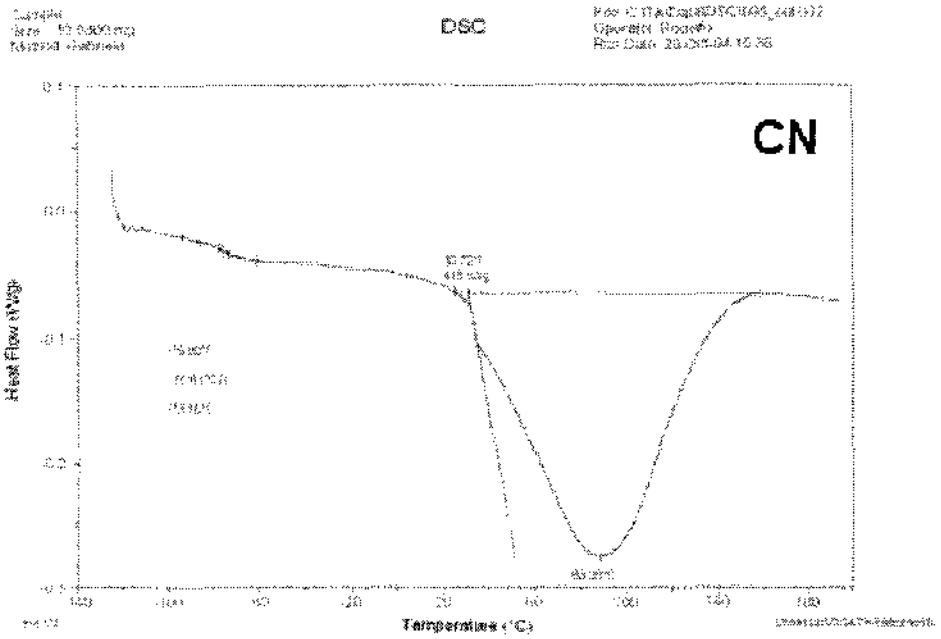

Figura 12 – Curva de DSC para a membrana de quitosana neutralizada.

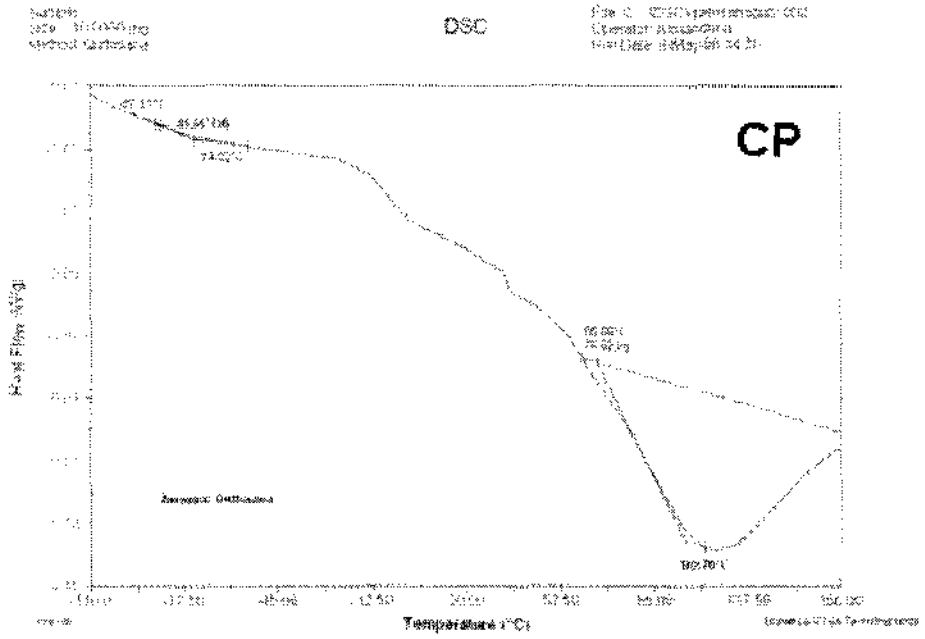

Figura 13 – Curva de DSC para a membrana de quitosana plastificada.