UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE
CURSO DE QUÍMICA MEDICINAL

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PREPARAÇÃO, AVALIAÇÃO *IN VITRO* E ANÁLISE MORFOLÓGICA DE MATRIZES QUITOSANA-GELATINA EM DIFERENTES PROPORÇÕES COMO UM SISTEMA DE ENTREGA DE PEPTÍDEO PARA CICATRIZAÇÃO DE FERIDAS

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Trabalho de Conclusão de Curso de Graduação apresentado ao curso de Química Medicinal da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito parcial para obtenção do grau de Bacharel em Química Medicinal.

Orientadora: Prof.ª Drª. Maria Ismenia Zulian Lionzo

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RESUMO

O uso de matrizes de quitosana e gelatina como sistemas de liberação de fármacos é estudado há anos. A quitosana e a gelatina possuem propriedades para aplicação biomédica devido às suas características químicas, biocompatibilidade e degradabilidade. Neste trabalho, lipossomas associados ao peptídeo modelo (insulina), inseridos em filmes de quitosana:gelatina (40:60, 50:50 e 60:40) foram preparados a fim de avaliar a liberação *in vitro* do peptídeo associado, bem como o inchamento, degradação e propriedades morfológicas. Além disso, a integridade da molécula de insulina, antes e depois do ensaio de liberação foi avaliada. Os filmes mostraram liberação superior na proporção 40:60 em relação às outras proporções (40:60 > 50:50 > 60:40) e foram observados altos valores de inchamento e degradação, na mesma ordem. Além disso, foram observadas microfases e rugosidade na superfície dos filmes, por microscopia óptica, com a adição dos lipossomas carregados de insulina. Os resultados demonstraram que a modulação da proporção de gelatina na matriz de quitosana leva a um aumento na liberação da molécula associada, provavelmente devido a mecanismos de inchamento e degradação da matriz.

Palavras-chave: quitosana; gelatina; lipossomas, peptídeo; matrizes; drug delivery.

ABSTRACT

The use of chitosan and gelatin matrices as drug delivery systems has been studied for years. Chitosan and gelatin have properties for biomedical application due to its chemical characteristics, biocompatibility and degradability. In this work, liposomes associated with the model-peptide (insulin), inserted into chitosan:gelatin films (40:60, 50:50 and 60:40) were prepared in order to evaluate the *in vitro* release of the associated peptide, as well as swelling, degradation and morphological properties. In addition, the integrity of the insulin molecule, before and after release assay, was evaluated. The films showed superior release in the 40:60 ratio compared to the others proportions (40:60 > 50:50 > 60:40) and higher values of swelling and degradation were observed, in the same order. Furthermore, microphases and rugosity was observed in the films surface, by optical microscopy, with the addition of the insulin-loaded liposomes. The results demonstrated that the modulation of gelatin ratio in the chitosan matrix lead to an increase in the release of the associated molecule, probably because of mechanisms of matrix swelling and degradation.

Keywords: chitosan; gelatin; liposomes, peptide; matrices; drug delivery.

SUMÁRIO

INTRODUÇÃO **10**
FUNDAMENTAÇÃO TEÓRICA **12**
OBJETIVOS **14**
Objetivo geral 14
Objetivos específicos 14
PREÂMBULO **15**
1 INTRODUCTION **16**
2 MATERIALS AND METHODS **17**
2.1 Liposome preparation 17
2.2 Polymer matrices obtainment 17
2.3 Dynamic Light Scattering (DLS) and Zeta-potential (ζ) 18
2.4 Microscopic Analysis 18
2.5 Swelling degree and weight loss 18
2.6 Release profile of insulin-loaded liposomes 18
2.7 Electrospray Ionization Quadrupole Time-of-flight Mass Spectrometry (ESI-QTOF-MS) 19
3 RESULTS AND DISCUSSION **19**
3.1 Dynamic Light Scattering (DLS) and Zeta-potential (ζ) 19
3.2 Microscopic Analysis 19
3.3 Swelling degree and weight loss 20
3.4 Release profile of insulin-loaded liposomes 21
3.5 Electrospray Ionization Quadrupole Time-of-flight Mass Spectrometry (ESI-QTOF-MS) 21
4 CONCLUSIONS **22**
Declaration of competing interest **23**
Acknowledgements **23**
REFERENCES **23**
REFERÊNCIAS **25**

INTRODUÇÃO

O Brasil enfrenta, atualmente, uma série de mudanças no setor saúde: com o aumento da expectativa de vida e redução da natalidade, a população está setornando cada vez mais idosa — processo denominado de transição demográfica. Trata-se de faixas etárias que, por sua vez, possuem maior prevalência das doençascrônicas não transmissíveis e, associado ao sucesso no combate a algumas doenças transmissíveis causadoras de morbimortalidade, verifica-se o fenômeno da transição epidemiológica, ou seja, mudanças nos padrões e frequências na distribuição de condições de saúde em uma população (Conselho Nacional de Secretários de Saúde (CONASS), 2014).

Além disso, a transição tecnológica traz novas oportunidades e ferramentas para o diagnóstico e tratamento das situações de saúde que visam à recuperação dasaúde e o bem-estar e a qualidade de vida dos pacientes. O desenvolvimento e a incorporação de tecnologias médicas, entretanto, não ocorrem rapidamente no dia a dia dos serviços de saúde, pois necessitam de estudos robustos que atestem sua eficiência e segurança (CONASS, 2014).

Em 2021, o Ministério da Ciência e Tecnologia instituiu a Política de Ciência, Tecnologia e Inovação em Materiais Avançados, por meio do Decreto nº 10.746, entendendo que há necessidade de inovação na área da saúde e fomentando o incentivo às pesquisas de novos materiais que facilitem e agilizem a recuperação da saúde dos pacientes, desenvolvam novos materiais e auxiliem na formação de recursos humanos para o fortalecimento do setor no país (BRASIL, 2021).

Na área da saúde, o Plano de Ação de Ciência, Tecnologia e Inovação para Tecnologias Inovadoras e Convergentes, de 2019, traz em seu primeiro volume, Nanotecnologia, diversos temas estratégicos, dentre os quais se destacam os “materiais carreadores para sistemas de liberação de fármacos ou suplementos e (nano)(bio)materiais para engenharia tecidual” (BRASIL, 2019, p. 11). Trata-se de uma área que tem trazido propostas promissoras para o tratamento das afecções cutâneas, como o uso de pele de tilápia (colágeno) associadas com nano-hidroxiapatita e quitosana (QIANQIAN *et al.*, 2021), membranas de celulose associadas à quitosana e óxido de cério (KALAYCIOĞLU *et al.*, 2020) e células-tronco associadas (ou não) à bioimpressão (KUCHARZEWSKI *et al.*, 2019; ROSHANGAR *et al.*, 2021).

Lesões na pele, associadas a feridas crônicas, queimaduras e úlceras de pressão são problemas de saúde que necessitam de recursos terapêuticos adicionais para o seu manejo. Neste contexto, matrizes de polímeros biodegradáveis possuem aplicabilidade na reabilitação da pele lesada. Essas matrizes podem proteger o ferimento e, conforme sua composição, facilitar a regeneração do tecido (DEĞIM *et al.*, 2011).

Os sistemas matriciais são sistemas de liberação modificada de substâncias ativas. No caso de matrizes, o sistema de liberação consiste em um polímero que reveste a substância ativa e promove sua liberação com velocidade controlada. Matrizes poliméricas podem ser aplicadas em diversas áreas, incluindo sob a forma de filmes de revestimento cutâneo, que podem, por exemplo, serem utilizados no tratamento de queimaduras (STOICA; CHIRCOV; GRUMEZESCU, 2020).

No Brasil, esse tipo de sistema terapêutico é adquirido por importação, principalmente dos Estados Unidos, com elevado custo. Por isso, desenvolver um sistema com matéria-prima nacional pode facilitar a oferta pelo Sistema Único de Saúde (SUS) de forma universal e menos onerosa, facilitando o acesso pelos pacientes (RODRIGUES; COSTA; KISS, 2018).

Muitos casos de queimaduras são registrados no país e as úlceras de pressão são um sério problema para pacientes acamados. Também chamadas de escaras, essas feridas são oriundas do contato entre a pele e a superfície, mais especificamente da pressão ocasionada pelo próprio corpo nos pequenos vasos sanguíneos superficiais. Com o tempo, esses vasos se rompem, ocasionando feridas difíceis de tratar, de forma que a principal forma de intervenção é a prevenção.

Neste cenário, o uso dos biomateriais poliméricos e a nanotecnologia representam uma alternativa para tratamento de agressões na pele, pois podem proteger o tecido e, ao mesmo tempo, promover a liberação controlada de ativos associados.

Considerando o estado da arte da utilização de sistemas de liberação utilizando matrizes, o uso da quitosana e o colágeno como constituintes é promissor, pois suas características intrínsecas contribuem harmonicamente para a formação estrutural dos filmes (ambos os géis podem ser secos e moldados conforme a necessidade, e a dependência da quitosana pelo pH do meio permite um intercâmbio entre suas formas solúveis e insolúveis, além do colágeno ser uma proteína hidrodispersível, que pode modular a desintegração do material), bem como a biocompatibilidade com o organismo (VEDOVATTO *et al.*, 2020). A inovação na área farmacêutica tem impacto importante na saúde da população e tem sido guiada por pesquisa e desenvolvimento (LI; LI, 2021), portanto, estudos que visem novas opções terapêuticas são de excepcional importância.

FUNDAMENTAÇÃO TEÓRICA

A quitosana, de estrutura β-(1→4)-*N*-acetil-d-glucosamina, é um copolímero natural proveniente da reação de desacetilação da quitina, que é o segundo biopolímero mais abundante encontrado na natureza, atrás apenas da celulose. A quitina pode ser encontrada no exoesqueleto de artrópodes, como o camarão, e na parede celular de fungos e leveduras (RINAUDO, 2006). Tanto a quitosana quanto a quitina são utilizadas para a produção de biofilmes, curativos e sistemas de liberação de ativos, por serem ambas biodegradáveis — com degradação mais rápida do que a convencional — e biocompatíveis — com os riscos biológicos avaliados e aprovados –, podendo levar à elaboração de biomateriais com boas propriedades farmacêuticas e biológicas (YOUNES; RINAUDO, 2015).

A quitosana possui um processamento mais simples que o da quitina, mas sua estabilidade é menor devido ao seu caráter mais hidrofílico e maior sensibilidade ao pH, mas por ser um polieletrólito, forma complexos eletrostáticos com macromoléculas de carga oposta. Além disso, a associação da quitosana a uma matriz compósita com colágeno pode melhorar a adesão do filme à pele (YOUNES; RINAUDO, 2015). Sabe-se que as células da epiderme e, consequentemente, a proliferação celular, apresentam cargas majoritariamente aniônicas na superfície; logo, a quitosana (que possui cargas positivas em sua estrutura), associa-se à pele eletrostaticamente; e a adição de colágeno à matriz confere uma maior biocompatibilidade ao material, pois propicia a afinidade celular, por ser um constituinte da matriz extracelular (MATURAVONGSADIT *et al.*, 2021).

As matrizes podem ter suas propriedades moduladas por outros aditivos. Por exemplo, a inserção de lipossomas nas matrizes ajuda a manter sua estabilidade e aprimora sua biocompatibilidade (YOUNES; RINAUDO, 2015). Os lipossomas são nanovesículas formadas por fosfolipídios dispostos em uma bicamada lipídica, com núcleo aquoso, e essas nanoestruturas permitem o encapsulamento de drogas hidrofílicas e hidrofóbicas, pois são estruturas anfipáticas. Dessa forma, são adequadas para encapsulação de peptídeos e macromoléculas (ELOY *et al.*, 2014).

Já é descrito na literatura a utilização de hidrogel de quitosana com lipossomas e fator de crescimento epidermal (EGF), que é um peptídeo, para a cicatrização de feridas (DEĞIM *et al.*, 2011). Outra pesquisa, utilizando EGF, produziu matrizes compósitas de quitosana e colágeno associadas a lipossomas, mostrando ser possível a produção de filmes finos que sejam biocompatíveis com sistemas celulares (VEDOVATTO *et al.*, 2020). Os lipossomas, apesar de oriundos de fosfolipideos anfotéricos, apresentam carga superficial, majoritariamente, negativa e formam complexos polieletrolíticos com a quitosana.

Os sistemas de liberação de ativos compostos por lipossomas podem comportar tanto fármacos, quanto peptídeos e macromoléculas. A insulina é um peptídeo largamente utilizado para o controle da diabetes mellitus, principalmente do tipo 1, pois é a proteína facilitadora da entrada de glicose nas células (BARBOSA *et al.*, 2020). Peptídeos, como o EGF e a insulina, nas suas formas livres, sofrem degradação no meio, o que pode ser minimizado ao serem encapsulados em uma nanoestrutura (BATISTA; CARVALHO; MAGALHÃES, 2007). Dessa forma, esses sistemas promovem a liberação do ativo encapsulado, de forma lenta e gradual, afim de otimizar a quantidade do fármaco em contato com o tecido.

Estima-se que ocorrem no Brasil mais de 1 milhão de acidentes com queimaduras por ano, de primeiro, segundo e terceiro graus, e destes, 10% necessitam de atendimento hospitalar, sendo que 0,25% poderão ir a óbito devido as lesões (CRUZ; CORDOVIL; BATISTA, 2012). Além disso, as úlceras de pressão são feridas que acometem severamente pacientes internados por longos períodos e os tratamentos convencionais, que são de uso tópico e, principalmente, a prevenção através de monitorização e movimentação constante do paciente, não são sempre resolutivos, além dos pacientes necessitarem de cuidado constante, com um envolvimento de profissionais de saúde (MERVIS; PHILLIPS, 2019).

Dessa forma, o desenvolvimento de novos tratamentos para afecções cutâneas bem como, a aplicação de tecnologias que utilizem matérias-primas mais baratas, tornando os produtos mais acessíveis às pessoas são essenciais. Neste sentido, a universidade é um espaço profícuo para a realização de trabalhos que possam ir ao encontro das necessidades dos indivíduos e sociedade trazendo avanços e inovação.

OBJETIVOS

OBJETIVO GERAL

Preparar e caracterizar matrizes poliméricas, através da variação da razão quitosana:gelatina, a fim de atestar o aumento da liberação do peptídeo modelo incorporado a lipossomas.

OBJETIVOS ESPECÍFICOS

a) conhecer os mecanismos envolvidos na interação entre os componentes da matriz, caracterizando quanto à sua morfologia, utilizando microscopia óptica, degradação e inchamento em meio aquoso;
b) determinar o perfil de liberação do peptídeo através de quantificação por *high performance liquid chromatography* (HPLC);
c) analisar a integridade e estabilidade da insulina liberada por espectroscopia de massas.

PREÂMBULO

PREPARATION, *IN VITRO* EVALUATION AND MORPHOLOGICAL ANALYSIS OF CHITOSAN-GELATIN MATRICES IN DIFFERENT RATIOS AS PEPTIDE DELIVERY SYSTEM FOR WOUND HEALING

(A ser submetido no periódico **International Journal of Pharmaceutics**)

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Preparation, *in vitro* evaluation and morphological analysis of chitosan-gelatin matrices in different ratios as peptide delivery system for wound healing

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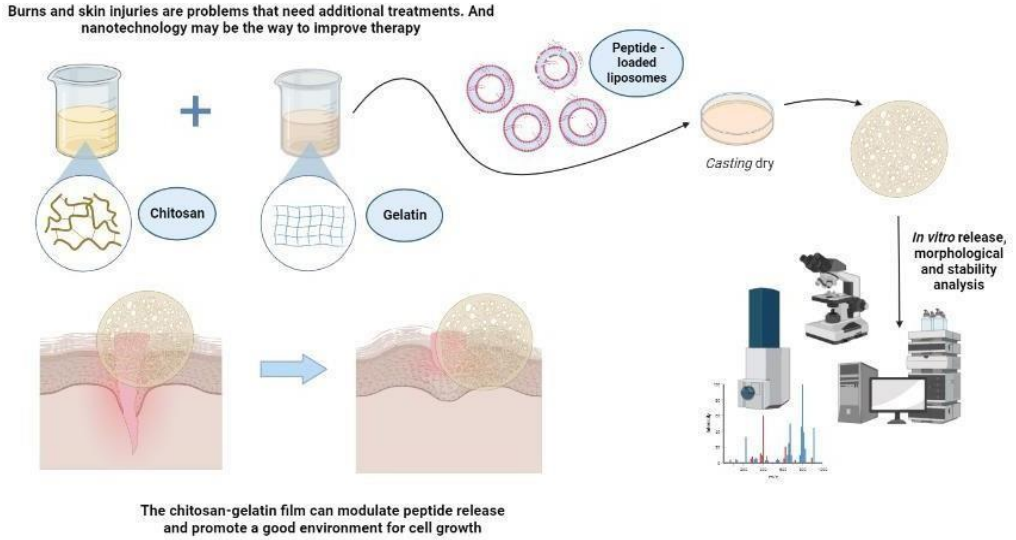
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ABSTRACT

The use of chitosan and gelatin matrices as drug delivery systems has been studied for years. Chitosan and gelatin have properties for biomedical application due to its chemical characteristics, biocompatibility and degradability. In this work, liposomes associated with the model-peptide (insulin), inserted into chitosan:gelatin films (40:60, 50:50 and 60:40) were prepared in order to evaluate the *in vitro* release of the associated peptide, as well as swelling, degradation and morphological properties. In addition, the integrity of the insulin molecule, before and after release assay, was evaluated. The films showed superior release in the 40:60 ratio compared to the others proportions (40:60 > 50:50 > 60:40) and higher values of swelling and degradation were observed, in the same order. Furthermore, microphases and rugosity was observed in the films surface, by optical microscopy, with the addition of the insulin-loaded liposomes. The results demonstrated that the modulation of gelatin ratio in the chitosan matrix lead to an increase in the release of the associated molecule, probably because of mechanisms of matrix swelling and degradation.

GRAPHICAL ABSTRACT



1. Introduction

Tissue engineering and regenerative medicine have been developing over the years and its principles are the restructuring and function improvement of injured tissues, which includes cell implants, delivery of epidermal growth factor (EGF) and the use of matrices, such as scaffolds (Ahmed et al., 2018). The construction of synthetic, natural, biological, degradable or non-degradable scaffolds represents an important advance in the area of tissue regeneration, because this technology provides the ideal environment for cell growth, such as those formed by highly porous biomaterials that supply a regular amount of nutrients and oxygen to the epithelial cells (Kulkarni et al., 2021; Zou et al., 2018).

The use of hydrogels, especially those made of naturally derived macromolecules - chitosan or synthetic polymers, for example -, has a great potential for biocompatibility and biodegradability (Dragan and Dinu, 2019), as they can easily adhere to skin (Chaudhari et al., 2016; Liu et al., 2018). Skin injuries associated with chronic wounds, burns and pressure ulcers are health problems that require additional therapeutic resources for their management. In this context, biodegradable polymer matrices have applicability in the rehabilitation of injured skin. These matrices can protect the wound and, depending on their composition, facilitate tissue regeneration (Değim et al., 2011; Güneş and Tıhmınlıoğlu, 2017).

Matrix systems can act as modified release systems for active substances. The release system consists of a polymer that coats the active substance and promotes its release in a controlled speed. Polymeric matrices can be applied in a variety of areas, including in the form of skin coating films, which can, for example, be used in the treatment of burns (Fini and Orienti, 2003; Stoica et al., 2020).

Many cases of burns accidents are reported in Brazil: it is estimated the occurrence of more than 1 million first, second and third-degree burn accidents per year, of which 10% require hospital care, and 0.25% may die due to their injuries (Cruz et al., 2012). In addition, pressure

ulcers are wounds that severely affect patients hospitalized for long periods of time, and conventional treatments - mainly topical and based on monitoring and constant patient movement - are often ineffective, besides the fact that patients require constant care, with an involvement of health professionals (Mervis and Phillips, 2019).

In this context, the use of polymeric biomaterials and nanotechnology can represent an alternative for the treatment of such skin injuries, as they can protect the tissue and, at the same time, promote the controlled release of associated actives. Furthermore, this type of therapeutic system is imported, mainly from the United States, at high cost. Therefore, developing a system with national raw material can facilitate the offer by the Sistema Único de Saúde (SUS) in a universal and less expensive way, facilitating the access by patients (Rodrigues et al., 2018).

Chitosan or β-(1→4)-N-acetyl-d-glucosamine is a natural copolymer derived from the deacetylation reaction of chitin, which is the second most abundant biopolymer found in nature, after cellulose. Chitin can be found in the exoskeleton of arthropods, such as shrimp, and in the cell wall of fungi and yeast (Rinaudo, 2006). Both chitosan and chitin are used on the production of dressings and active release systems, because they are both biodegradable - with faster degradation than conventional - and biocompatible - with the biological risks evaluated and approved - and can lead to the elaboration of biomaterials with good pharmaceutical and biological properties (Bano et al., 2017; Younes and Rinaudo, 2015).

Chitosan is less stable than chitin because it is more hydrophilic and more sensitive to pH, but since it is a polyelectrolyte, it forms electrostatic complexes with oppositely charged macromolecules. In addition, the association of chitosan with a composite matrix containing collagen can improve the adhesion of the film to the skin (Younes and Rinaudo, 2015). The chitosan have the property of provoke an immunologic response, stimulating the inflammatory and immunologic cells, to promote tissue regeneration faster than the natural process, without a stimulus (Vigani et al., 2019). Furthermore, the structural characteristics of the chitosan stimulate the recognition of the epithelial cells, because it is similar to the componentes of extracellular matrix, for which is indicated to use in tissue engineering (Matica et al., 2019).

Matrices can have their properties modulated by other additives. For example, inserting liposomes into the matrices helps maintain their stability and improves their biocompatibility (Kumar et al.,2020; Younes and Rinaudo, 2015). Liposomes are nanovesicles formed by phospholipids arranged in a lipid bilayer, with an aqueous core, and allow the encapsulation of both hydrophilic and hydrophobic drugs. Thus,they are suitable for encapsulation of peptides and macromolecules (Eloy et al., 2014).

The use of chitosan hydrogels with liposomes and a peptide, for wound healing is already described in the literature (Değim et al., 2011). Other research using EGF produced composite matrices of chitosan and gelatin associated with liposomes, showing that it is possible to produce thin films that are biocompatible with cellular systems (Vedovatto et al., 2020). Despite coming from amphoteric phospholipids, liposomes have mostly negative surface charge and form polyelectrolyte complexes with chitosan (Lionzo et al., 2013).

The release systems composed by liposomes can hold drugs, peptides and macromolecules. Insulin is a peptide widely used to control diabetes mellitus, especially type 1, since it is the facilitator protein of glucose entry into cells (Barbosa et al., 2020). Peptides, such as EGF and insulin, in their free forms, suffer degradation in the environment, which can be minimized when encapsulated in a nanostructure (Batista et al., 2007). Thus, these systems promote the release of the encapsulated active, slowly and gradually, in order to optimize the quantity of drug in contact with the tissue.

Innovation in the pharmaceutical field has an important impact on population health and has been driven by research and development (Li and Li, 2021), so studies that target new therapeutic options are of great importance. Previous research show that the use of chitosan and collagen as constituents of matrices is promising: their intrinsic characteristics contribute harmonically to the structural formation of films, as both gels can be dried and molded as needed, and the pH-dependence of chitosan allows an exchange between its soluble and insoluble forms. Furthermore, collagen is a hydrodispersible protein, which can modulate the disintegration of the material (Vedovatto et al., 2020).

In the present study, the increase of gelatin amount in a chitosan:gelatin film was proposed with the aim of improve the release of a liposome-encapsulated peptide (insulin) dispersed on the matrice. For this, three different chitosan:gelatin formulations were proposed and the morphology of the films were evaluated by optical microscopy. The integrity and stability of the films, in phosphate buffer, by swelling and weight loss were accessed. In addition, the insulin release profile of the matrices was plotted by the quantification of insulin by high performance liquid chromatography. To estimate the integrity and stability of insulin molecule after the release assay, the molecule was evaluated by time-of-flight mass spectrometry.

2. Material and methods

2.1 Liposome preparation

The liposomes were prepared by reverse-phase evaporation method adapted from (Vedovatto et al., 2020). Initially, 0.6 mg of 1,2-Dipalmitoyl-*sn*-glycero-3-phosphate, monosodium salt (Lipoid PA16/0, Germany) and 5.4 mg of Hydrogenated phosphatidylcholine from Soybean (Lipoid S PC-3, Germany), were solubilized in 5 mL of chloroform and 400 µL of phosphate buffer (PBS). After 5 minutes in the ultrasonic bath, the organic solvent was removed using a rotary evaporator (R-300, Buchi, Switzerland). After total evaporation, 3 mL of insulin [3,5 mg/mL] (Humulin®R, Lilly, Indianapolis, USA) and 2 mL of PBS were added in the solution. The solution was put in an ultrasonic bath for an additional 10 minutes, allowing liposome formation. Samples without insulin were also prepared using 5 mL of PBS.

2.2 Polymer Matrices obtainment

Aqueous acetic acid (0.7%, AlphaTec, São Paulo, Brazil) and medium molecular weight chitosan (2.6%, Sigma-Aldrich, St. Louis, USA) dispersions were prepared and preserved with methylparaben (0.05 wt%, Cosmetrade, Porto Alegre, Brazil). After a 24-hour hydration period, the small insoluble fibers were removed by centrifugation (15 minutes at 3500 rpm, Novatécnica NT-815, Piracicaba, São Paulo, Brazil). At the same time, an aqueous gelatin solution (2.0 wt%, CRQ, São Paulo, Brazil), preserved with methylparaben (0.7 wt%) was prepared and stored at room temperature for 24 hours. Solution of medium molecular weight chitosan was mixed with the gelatin solution in the ratios 40:60, 50:50, 60:40, v/v in a magnetic stirrer (20 minutes at 500 rpm). Those mixtures [3 mL of chitosan + 4.5 mL of gelatin (40:60), 3.75 mL of chitosan + 3.75 mL of gelatin (50:50) and 4.5 mL of chitosan + 3.0 mL of gelatin (60:40)] were used to prepare the matrices without insulin by solvent *casting* method, adapted from (Lionzo e tal., 2012) at plastic Petri dishes (6 cm of diameter), dried at incubator (40ºC) for 24 hours and left on a desiccator containing silica-gel for additional 24 hours. To complete the final volume (10 mL) in the dishes, 2.5 mL of distilled water was added in the dispersions.

For the liposomes and drug-containing matrices, 2.5 mL of liposomes with insulin or without insulin solution were stirred with 7.5 mL of chitosan-gelatin dispersions, for 15 minutes at 1,200 rpm. Then, mixtures were poured into plastic Petri dishes for the *casting* step. After that, samples were dried at desiccator for 24 hours and then left in a drying incubator at 40 ºC for additional 24 hours. Following that, all the matrices were

neutralized with an NaOH 1 M solution for 30 seconds and then washed in purified water for 30 seconds. After that, they were dried again at the dissecator. The samples were packed between PVC films and protected from light exposure during the subsequent months when the physical-chemical analysis was conducted.

2.3 Dynamic Light Scattering (DLS) and Zeta-potential (ζ)

The size (nm) and Zeta potential (mV) of liposome samples, with and without insulin, diluted in 10 mM NaCl solution, were analyzed by Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK), in triplicates, in collaboration with Faculdade de Farmácia at Universidade Federal do Rio Grande do Sul (UFRGS).

2.4 Microscopic Analysis

The samples were analyzed by optical microscopy (Optical Microscope CX22LED, Olympus, Tokyo, Japan). Aiming to obtain images from the surface of the films, before and after the peptide release, the matrices with and without liposomes/insulin or both. Sample pieces were cut in 1 cm² of area. The magnifications used was 40x.

2.5 Swelling degree and weight Loss

In order to evaluate the swelling properties of the films, the percentage of hydration of the samples was determined. Portions of 1 cm^2 of each film were weighed and immersed individually in phosphate buffer solution (PBS, pH 7.35) at 37 ± 1.0 ºC. The immersion times were 15 minutes, 30 minutes, 1 h, 1h 30min, 24 h and 48 h. After immersion, the excess of surface humidity was removed with a filter paper and the films were weighed on analytical balance (Shimadzu AY-220, Tokyo, Japan). The percentage of hydration was calculated using the following equation:

$$\%\, hydration = \frac{W_t - W_0}{W_0}.100 \qquad (1)$$

Where W_0 is the dry weight of the portions before hydration and Wt is the weight after immersion in the buffer.

For the weight loss, the portions were placed in a desiccator and weighed after being completely dried to determine the degradation caused during the study. The equation below was used to calculate the percentage:

$$\%\, degradation = \frac{W_0 - W}{W}.100 \qquad (2)$$

Where W_0 is the dry weight before the test and W is the weight of the portions dried in the desiccator after immersion in the buffer. The essays were made in triplicate for each sample.

2.6 Release profile of insulin-loaded liposomes

To design the peptide release profile of liposomes, an *in vitro* assay was performed simulating physiological temperature and pH conditions. Initially, sodium phosphate dibasic/monobasic buffer (NEON, São Paulo, Brazil) was prepared (GEHAKA PG 1800, sens: 99.3%, São Paulo, Brazil). After that, 3 samples of each matrix (40:60, 50:50 and 60:40, in triplicate) were cut in 1cm², weighed and a water bath was prepared on a hot plate with magnetic stirring at temperature 37±1.0 ºC.

The 9 film samples and 4 mL of phosphate buffer were transferred to 9 glass flasks with lids. Under magnetic stirring and temperature control, the samples were kept for 72 hours in analysis, and a total of 10 samples of 400 µL each were collected (0.25, 16, 20, 24, 40, 44, 48, 64, 68 and 72 hours). To maintain the same buffer condition, at each collection, the same volume was added. At each collection, the release samples were packed in eppendorfs and frozen for later analysis in high performance liquid chromatography (HPLC) (Fig. 1).

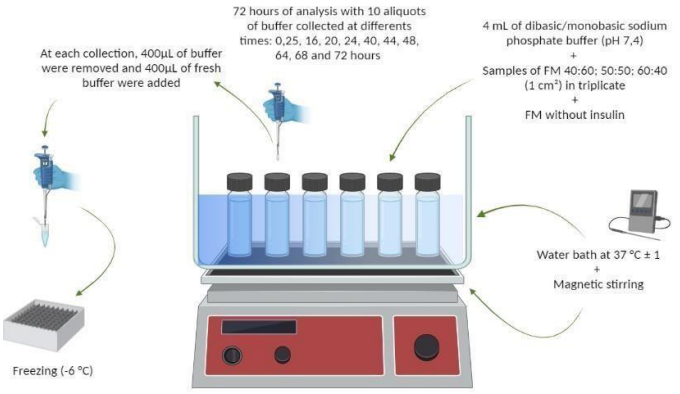


Fig.1. The process of the release profile assay

Furthermore, in order to evaluate the films composed of chitosan/gelatin and liposomes, pieces (1.0 cm x 1.0 cm) of the matrices were cut and placed in three glass flasks (40:60, 50:50 and 60:40) under the same conditions (pH 7.35, phosphate buffer) and put in a water bath at 37±1 ºC for 24 hours. After this time, the extract was collected. Additionally, insulin recovery was evaluated, where a spiked peptide solution was added to the matrix extract, which was prepared in the same way as the loaded matrix evaluation assay, at a concentration of 30 µg/mL.

The quantification of insulin released by the films was performed in HPLC Shimadzu Prominence model with a spectrophotometric Diode Array Detector SPDM20A model (Kyoto, Japan). We used a previously validated method for insulin quantification, tested for the conditions used in this work. The mobile phases used were prepared and filtered: distilled water acidified with 0.05% trifluoroacetic acid (TFA suitable for HPLC > 99.0%, Sigma-Aldrich, St. Louis, USA) and acetonitrile (suitable for UV/HPLC 99.92%, Sal-R, São Paulo, Brazil) acidified with 0.05% trifluoroacetic acid. At the same time, 6 solutions of Humilin®R were prepared in phosphate buffer pH 7.35 with increased concentrations of insulin (5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, 25 µg/mL and 30 µg/mL) in 5 mL volumetric flasks from a "mother solution" of insulin, in order to determine the calibration curve of insulin. The quantification of the analyte was evaluated according to the buffer used and the retention time for insulin in the phosphate buffer was defined at 4.7 minutes.

The samples collected from release were thawed to room temperature and 300 µL of sample were added to vials with 700 µL of mobile phase acidified water with 0.05% TFA. Overall, 90 samples were collected among 10 collection points.

To obtain the samples for HPLC analysis, 300 µL of the extract of each solution were collected, filtered in a sterile 0.45 µm PTFE filter and added to vials with 700 µL of mobile phase acidified water with 0.05% TFA. The samples were analyzed at 40°C temperature and 1 mL/min flow rate, as shown in Fig. 2.

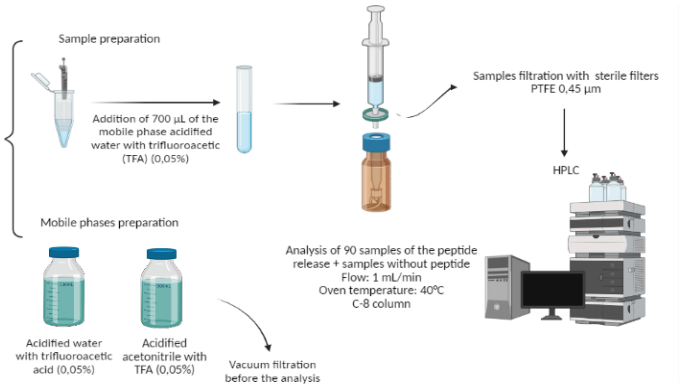


Fig. 2. Scheme of preparation of the samples to the HPLC analysis

2.7 Electrospray Ionization Quadrupole Time-of-flight Mass Spectrometry (ESI-QTOF-MS)

The insulin degradation in the release samples (24 hours point) and the extract with known concentration of insulin were analyzed by electrospray ionization quadrupole time-of-flight mass spectrometry using a Brunker Datonics (Billerica, MA, USA) micrOTOF-QIII with ionization electrospray Apollo II model. For this analysis, the samples of the release assay (24 hours point), the spiked insulin solutions (30 µg/mL), the insulin in distilled water standard (1 mg/mL) and the insulin in PBS (15 µg/mL) were injected directly, and the analysis conditions was: End plate offset voltage = 500 V, capillary voltage = 4,5 kV, nebulizer pressure = 3.0 bar, dry gas temperature = 250 °C and dry gas flow rate = 4.0 L/min. The MS method was conducted according to the 50-2500 m/z and 3 Hz spectra range was applied. The method was adapted from (Hamidli et al., 2022).

3. Results and discussion

3.1 Dynamic Light Scattering (DLS) and Zeta-potential (ζ)

The mean size obtained for the liposomes without insulin, in triplicate, was 432±38.1 nm, PDI = 0.955 and the zeta potential was -29.7±6.22 mV. The preparation by reverse-phase evaporation method produces a heterogeneous liposome population with different sizes and form aggregates with multilamellar nanovesicles (Ahmed et al., 2019). Joanitti et al. produced liposomes with phosphatidylcholine and cholesterol by three different methods: reverse-phase evaporation, freeze-thaw and by the hydration and dehydration method. The reverse phase evaporation method demonstrated larger particle diameter compared to the others (528 nm) (Joanitti et al., 2018).

Thus, the average size of the obtained liposomes without insulin may be related to the aggregation between the nanovesicles and the influence of the preparation method itself. The liposomes with insulin mean size, in triplicate, was 126±69.1 nm, PDI = 0.261 and the zeta potential was -29.73±2.33 mV. The difference in size between the formulation without insulin and with insulin, may be related to the addition of the peptide itself. According to Hofmann et al. the addition of saline solution in the preparation of liposomes can reduce aggregation, even though this characteristic is normal in liposomes over time. They prepared liposomes using cationic/anionic DPPCs and derivatives, associated with cholesterol and noticed a decrease in the diameter of the liposomes with NaCl solution and highlighted that cholesterol may be able to ensure colloidal stability (Hofmann et al., 2020).

In another study, Von Zuben et al. produced insulin-associated liposomes with and without functionalization, and identified a larger average diameter value of liposomes without insulin compared to those associated with the peptide, and highlight that PDI values greater than 0.5 suggests a high polydispersity with the formation of aggregates following electrostatic interactions between the peptide and the liposomes (Von Zuben et al., 2021).

That is, the insertion of insulin may be reducing the formation of aggregates and decreasing the average size of liposomes, as highlighted by the PDI values obtained, considerably higher for liposomes without insulin, indicating a greater formation of aggregates and consequent increase in the average size, which is not observed for insulin-associated liposomes. The close zeta potential values between liposomes with and without insulin indicate a similar surface for both formulations. The peptide may be alocated in the internal or external aqueous phase or liposome formulation.

3.2 Microscopic Analysis

The images of the films composed by 40:60, 50:50 and 60:40 chitosan:gelatin ratios, with and without liposomes (containing or not insulin) and the films after the release assay, are shown in the Fig. 3. The films 40:60 chitosan:gelatin (1a) presented homogeneity between chitosan and gelatin. And the addition of liposomes (1b) caused an increase of rugosity, with the formation of microphases, evidenced by the interactions with the nanovesicles. The addition of liposome-insulin conjugate (1c) caused an attenuation of the chitosan-gelatin microphases and the rugosity is observed in the surface. The films after the release (1d) showed preservation of the microphases and also showed a maintanance of opacity.

The films 50:50 chitosan:gelatin without liposomes (2a) also showed a homogeneity between chitosan and gelatin, and microphase formation is also visible, differently from (1a), where this texture is absent. The presence of liposomes (2b) also evidences the presence of microphases and the increase of the rugosity. The liposome-insulin presence (2c) showed microphase conservation and rugosity, also observed in the film after the release (2d). The films 60:40 chitosan:gelatin without liposomes (3a) showed fewer microphases and higher homogeneity between chitosan and gelatin than those presented in the 2a image.

The addition of the liposomes (3b) caused an evident increase of the rugosity, compared with 1b and 2b, and the maintanance of the microphases. Similar results were showed in another study, but observed from electron microscopy analisys (Lionzo et al., 2012). The presence of the insulin (3c) also caused an attenuation (the microphases strucutre is less visible) in the structure, compared with 3b, as well as, the maintenance of the microphases presence and rugosity. In 3d, after the release assay, the films preserved the microphases, rugosity but seemed to be less opaque.

This way, the films made only with chitosan-gelatin (1a, 2a and 3a) showed homogeneity and with the most presence of microphases in the 50:50 film (2a). All the matrices with liposomes showed increased rugosity (the higher in the 60:40 (3b)) and microphases (1b, 2b and 3b). The presence of the liposomes and also the major concentration of chitosan, can be related to this characteristics.

The addition of insulin (1c, 2c and 3c) maintains the microphases structures and rugosity, but causes a attenuation of the microphases, decreasing the expressivity shown in (1b, 2b and 3b). In the matrices which stayed 72 hours submerged in buffer (1d, 2d and 3d) it is possible to see the maintenance of the microphases and the rugosity. In a study published, Vedovatto et al. made optical microscopy of chitosan-gelatin films with liposomes, where also saw the increase of the rugosity with the liposomes, as well the attenuation of the microphases structure caused by the peptide, in this case the epidermal growth factor (EGF) (Vedovatto et al.,2020).

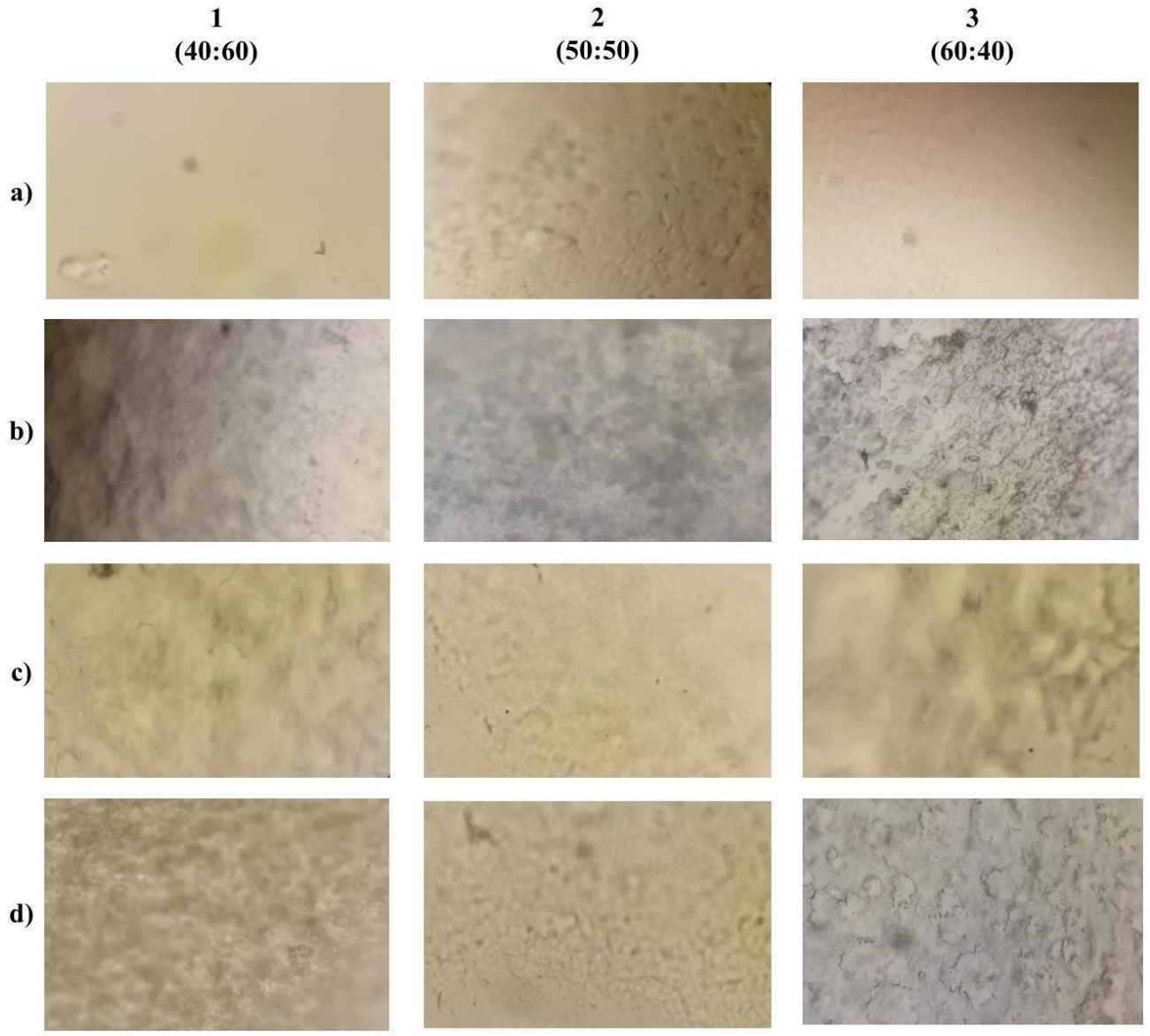


Fig. 3. Optical microscopy compiled images (40x). 1a: 40:60 chitosan:gelatin, 1b: 40:60 chitosan:gelatin with liposomes, 1c: 40:60 chitosan:gelatin with liposomes and insulin, 1d: 40:60 chitosan:gelatin whit liposomes and insulin after the release assay. 2a: 50:50 chitosan:gelatin, 2b: 50:50 chitosan:gelatin with liposomes, 2c: 50:50 chitosan:gelatin with liposomes and insulin, 2d: 50:50 chitosan:gelatin whit liposomes and insulin after the release assay. 3a: 60:40 chitosan:gelatin, 3b: 60:40 chitosan:gelatin with liposomes, 3c: 60:40 chitosan:gelatin with liposomes and insulin, 3d: 60:40 chitosan:gelatin whit liposomes and insulin after the release assay.

3.3 Swelling degree and weight loss

The results showed high absorption of the buffer, above 150% (Fig. 4) for all films. At 30 minutes of the assay, the 40:60 and 50:50 matrices showed swelling close to 250%, unlike the 60:40 matrices, which showed swelling around 200%. After this time, the films started to absorb less buffer. The 40:60 matrices continue to maintain the highest percentages of absorption until 48 hours, not showing values below 200% at any point. Initially, the 50:50 matrices absorb the buffer very quickly, but the absorption speed and amount gradually decrease.

Matrices with a higher presence of chitosan (60:40) absorb less buffer from the beginning, maintaining the percentage of hydration below 200%. Moreover, the chitosan is hydrodispersible and is capable to contribute for higher percentages of buffer absorption due intrinsic film porosity (Batista et al., 2019) and the absorption is stimulated by collagen present in the gelatin, since it is a strongly hydrophilic protein (Rahman et al., 2013), which can facilitate skin-film adhesion and assure a highly liquid environment that is necessary for healing (Ahmady et al., 2022).

The degradation of the matrices is shown in the Figure 5. The weight loss of the 40:60 matrices were around 80% and 75% to the 50:50 ratio matrices. Furthermore, the matrices with more chitosan in the composition present a degradation of nearly 70%, which indicates that the presence of gelatin in higher values can modulate the degradation of the films.

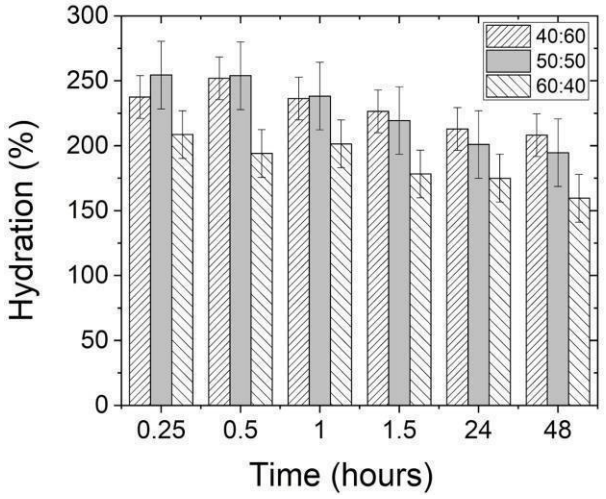


Fig. 4. Percentage of hydration of the swelling test

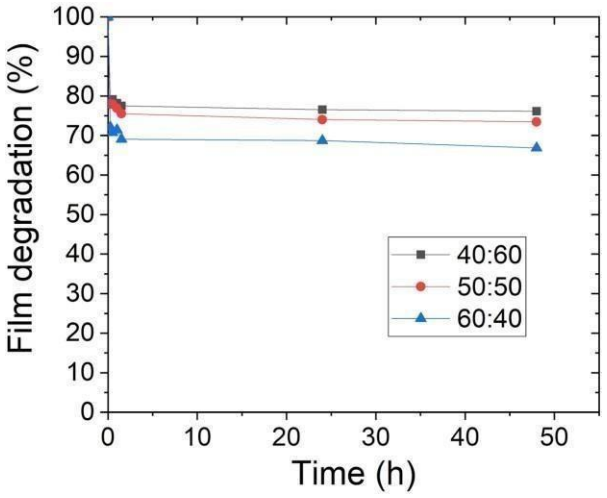


Fig. 5. Percentage of degradation of the matrices

3.4 Release Profile of Insulin-Loaded Liposomes

The *in vitro* release of the insulin encapsulated into liposomes and in free form in the matrices are shown in the Figure 6. The matrices with high quantities of chitosan (60:40) presented less percentage of insulin release (≤ 5%), reaching a plateau profile after 48 hours. Following the same pattern, the matrices with 50:50 ratio presented an initial release percentage bigger than in 60:40, with a sustained release (≤10%) in 24 hours, maintaining the increase of insulin release until 48 hours (≤ 15%), after stabilizing the quantity of insulin released in the buffer.

The films with a bigger percentage of insulin released are the matrices having more gelatin (40:60), that presented a pronounced release up to 24 hours, with 15% of insulin released in the buffer, and after 40 hours, the release was still growing continuously until the final of the experiment, with around 25% of insulin released.

The increase of gelatin concentration in the film was capable of elevating the peptide release. The collagen fibers form hydrogen bonds with chitosan and partially interrupt the intra and intermolecular hydrogen interactions of the chitosan, increasing its flexibility (Mahdizadeh Barzoki et al., 2016).

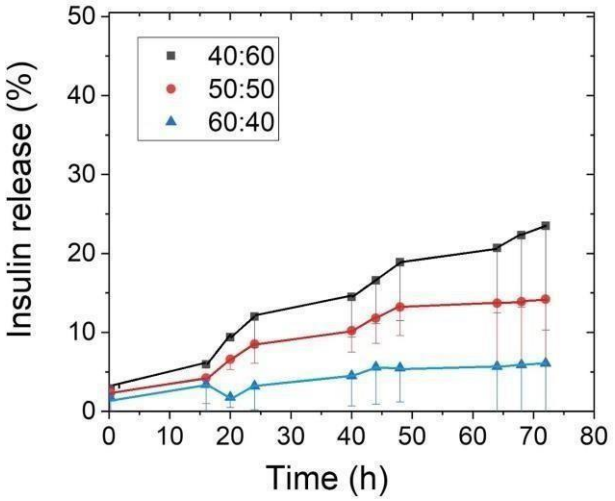


Fig. 6. Concentration of Insulin release determined by high performance liquid chromatography (HPLC)

The higher films swelling observed for samples with more gelatin also corroborates the higher peptide release. The collagen is more hydrodispersible than chitosan in neutral pH, so it will swell more and degrade faster, promoting a higher release. Furthermore, the assay was conducted in PBS buffer (pH 7.35), which is higher than chitosan pKa (6.1) and the isoelectric point of insulin, between 5.0 - 5.33 (Wintersteiner and Abramson, 1933). Therefore, the chitosan free amino groups (NH_3) will not suffer addition of hydrogens atoms (protonation) in this pH, and will not electrostatically interact with the insulin, not interfering in the process (Wang et al., 2006).

The initiation of release probably is from the free insulin (not encapsulated) in the chitosan-gelatin matrices. In another study developed by our group, not yet published, an encapsulation efficiency of insulin-loaded liposomes was observed around 50% to 70%, depending of the preparation method. The peptide encapsulated is probably gradually released, as the matrix erodes in the buffer and the diffusion gradient drives. The release of up to 25% can be increased with more time of analysis, which was not reached by the present study.

Giovino et al. created chitosan polymer matrices with PEG-b-PLA loaded insulin nanoparticles, and described a 70% of insulin release until 360 hours of analysis, which means that the insulin release from the matrix can be longer, demonstrating the efficiency of chitosan in a long-lasting peptide delivery system (Giovino et al., 2013). In other research, Mahdizadeh Barzoki et al. built chitosan-gelatin insulin-loaded nanoparticles patches and evaluated the *in vitro* release, and identified a low peptide release until 8 hours of analysis (≤ 22%), which can be caused by "compacted" eventual formation of polymer around the nanoparticles leading to reduced the release rate. Higher concentrations of polymer in the solution increase the gel viscosity, and promote a higher presence of molecules in the hydrogel as well as in the dry films (Mahdizadeh Barzoki et al., 2016).

Therefore, the peptide coefficient diffusion may be reduced, which can affect the release rate. In other study, Ahmady et al. evaluated a sustained release of Thymol loaded in alginate microparticles into chitosan-gelatin matrices, and observed a high release of the molecules from the films, with or without alginate microparticles, showing the efficiency of chitosan-gelatin matrices as a sustained drug delivery systems, irregardless of the kind of nanostructure that were added (Ahmady et al., 2022).

3.5 Electrospray Ionization Quadrupole Time-of-flight Mass Spectrometry (ESI-QTOF-MS)

The results of the time-of-flight mass spectrometry are shown in

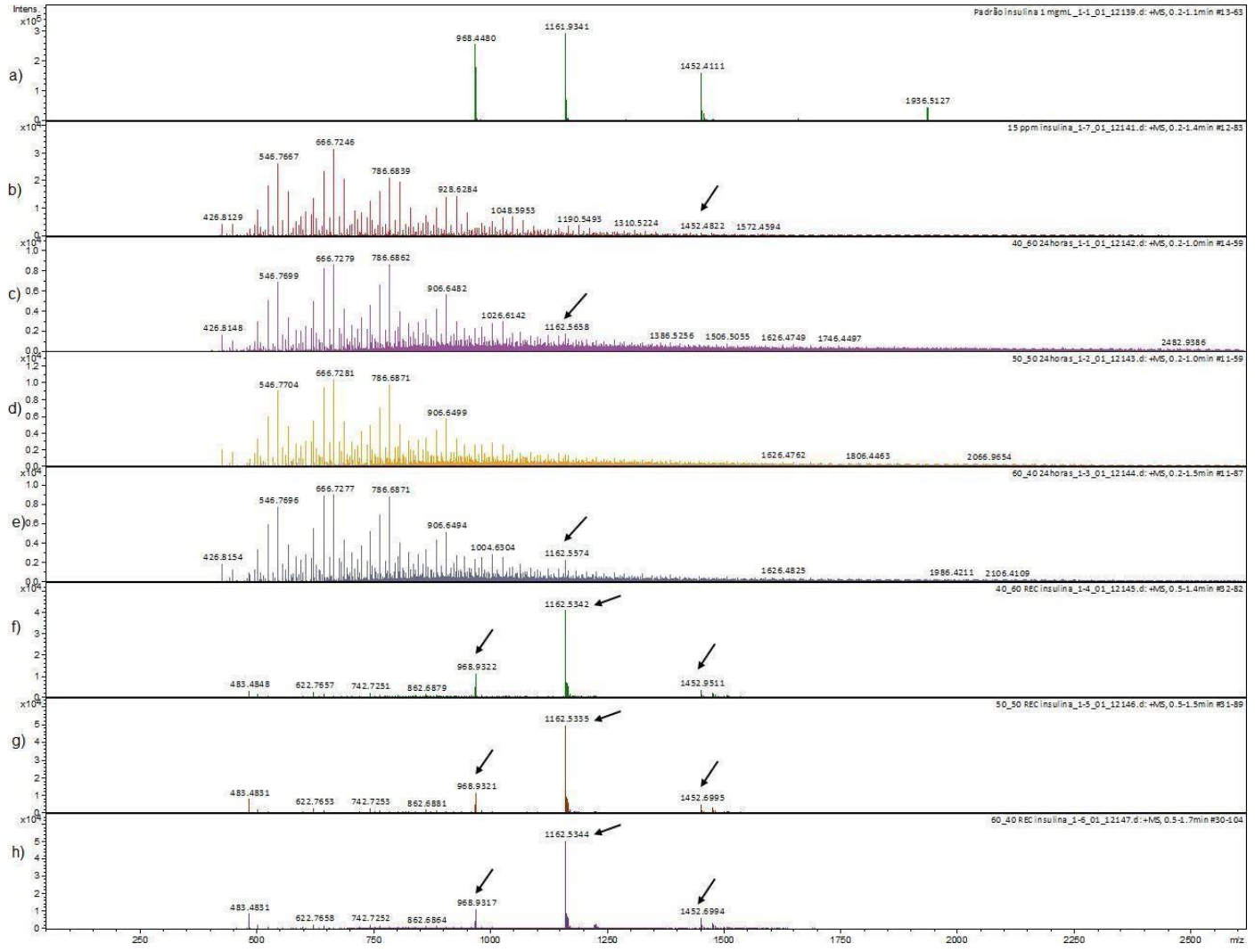


Fig. 7. Image with spectra compiled obtained by time-of-flight mass spectrometry. (a) insulin in distilled water standard (1 mg/mL), (b) insulin in buffer (15 µg/mL), (c) 40:60 release sample, (d) 50:50 release sample, (e) 60:40 release sample, (f) 40:60 spiked insulin (30 µg/mL). (g) 50:50 spiked insulin (30 µg/mL) and (h) 60:40 spíked insulin (30 µg/mL).

the Fig. 7. The spectra (a) and (b) show the standards, distilled water with insulin (1 mg/mL) and insulin in PBS (15 µg/mL), respectively. In (a) we observe four peaks with 968.4480 m/z, 1161.9341 m/z, 142.4111 m/z and 1939.5127 m/z characteristics of the human insulin (Hamidli et al., 2022). In (b) just one peak is seen (1452.4822 m/z), with lower intensity.

The spectra (c), (d) and (e) represent the release samples collected at the point of 24 hours, in the 40:60, 50:50 and 60:40 ratios, respectively. In (c) it is only possible to identify the peak of 1162.5658 m/z in lower intensity; in (d), no peaks relative to insulin are shown; and, in (e), the peak 1162.5574 m/z is present in the spectrum again. The spectra shown in (f), (g) and (h) are relative to the spiked insulin solutions (30 µg/mL) in the 40:60, 50:50 and 60:40 ratios, respectively. In these spectra, it is possible to see the peaks 968 m/z, 1162 m/z and 1452 m/z, referring to the insulin. In (b), although the insulin concentration was 15 ppm, the characteristic peaks of human insulin are not evidenced as in (a). This can be explained by the presence of phosphate buffer in the solution.

Moreover, this factor may be related to the appearance of several peaks present in the lower m/z values. In the release samples, in (c) and (e), the same insulin-related peak appears and in (d) no correlation with the standard is shown. It is worth pointing out that the concentration of insulin in the release samples is much lower compared to the spiked insulin solution, so the intensity of the peaks should be lower. Again, in this spectra several peaks appeared with m/z values in common, as in (b), and this may be related to the buffer and the presense of the other componentes of the matrix, such as phospholips, gelatin, chitosan etc, but perhaps, by the structural changes of insulin itself, since in spectra (f), (g) and (h) the liquid used for the preparation of the solutions is also phosphate buffer and, in the respective spectra, the characteristic peaks of insulin are well evidenced, as in (a).

From the results obtained, it is not possible to infer if there was degradation of insulin released in the buffer. The peptide may be undergoing structural changes due to the insertion into the liposomes and the matrix preparation process, but only with further analyses, like Circular dichroism for example, it is possible to confirm the degradation.

4. Conclusions

The matrices formed by chitosan-gelatin in the ratios 40:60, 50:50 and 60:40 were homogeneous and showed high swelling (≥ 150%) and degradation (≥ 70%). The films with more gelatin absorbed more buffer and suffered a higher degradation in comparison to the films with equal ratio or more chitosan.

The release occurs after the absorption of liquid by the matrix and degradation. As time goes by, the matrix swells and erodes into the buffer, the free insulin in the matrix is released first, and then the peptide-loaded liposomes are released from the material into the buffer. Thus, it is possible infer that the chitosan and gelatin matrices were able to promote peptide

release through a swelling and degradation mechanism.

From this, it is highlighted that the 40:60 films released more peptide in the buffer compared to the other ratios in 72-hour analysis, which demonstrates the modulating ability of gelatin in the matrix constitution. In addiotion, the films with more gelatin do not reach the release plateau, like the 50:50 and 60:40 films until the end of the analysis. Probably the release continues to increase, and a longer release time assay may prove how much insulin can be released into the buffer from the matrix.

The microscopy images showed the formation of microphases in the chitosan-gelatin films and an increased rugosity of the material with the addition of liposomes. The addition of the peptide left the film more opaque, demonstrating changes in the interactions between the components of the matrix.

To better understand the interaction between the matrix components and provide a greater increment *in vitro* release, it is necessary to increase the variety of analyses. Thus, increase the time of the release analysis is critical to know the total peptide that the matrix with more gelatin is able to release into the buffer.

Declaration of Competing Interest

The authors declare no conflict of interests.

Acknowledgements

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