UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Desenvolvimento de formulações nanotecnológicas contendo imiquimode para o tratamento do câncer cervical

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Desenvolvimento de formulações nanotecnológicas contendo imiquimode para o tratamento do câncer cervical

Tese apresentada por Luiza Abrahão Frank
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Eu sei que tudo quanto Deus faz durará eternamente; nada se lhe pode acrescentar, e nada se lhe pode tirar; e isso Deus faz para que os homens temam diante dele. O que é, já existiu; e o que há de ser, também já existiu; e Deus procura de novo o que ja se passou. (Eclesiastes 3.14-15)

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RESUMO

Esta tese se fundamenta na necessidade de novos tratamentos para o câncer do colo de útero visando o aumento da adesão dos pacientes aos tratamentos, assim como à qualidade de vida dos mesmos. Nesse sentido, formulações nanotecnológicas foram desenvolvidas com o objetivo de carrear o fármaco imiquimode para um local específico – a mucosa vaginal – esperando gerar melhores desempenhos nesse tratamento quando comparados com a formulação comercial. Três nanoestruturas com morfologias distintas foram propostas visando potencializar o efeito do fármaco em células de câncer cervical (SiHa). As formulações desenvolvidas compreenderam: nanoemulsões (NE_{imiq}), nanocápsulas poliméricas (NC_{imiq}) e nanocápsulas poliméricas revestidas com quitosana ($NC_{imiq\text{-}chit}$). Observou-se que nanocápsulas poliméricas produzidas com poli(ε-caprolactona) apresentaram efeito mais pronunciado frente às células SiHa. Para tanto, essas formulações (NC_{imiq} e $NC_{imiq\text{-}chit}$) foram incorporadas em hidrogéis de quitosana e de hidroxietilcelulose a fim de possibilitar uma melhor futura aplicação para o paciente. Estudos envolvendo mucosa vaginal suína demonstraram que ambas as formulações são mucoadesivas e permeiam a mucosa vaginal. Porém, a formulação produzida com hidrogel de quitosana (NC_{imiq}) apresentou maior desempenho. Esta foi a formulação escolhida para dar continuidade aos estudos deste trabalho, sendo objeto de estudo posterior em cultura de células SiHa a fim de elucidar o mecanismo de ação da mesma. Esses estudos demonstraram que há uma ocorrência de processos combinados de diminuição da viabilidade celular de maneira tempo-dependente e que mecanismos como apoptose, autofagia e parada de ciclo celular estão presentes. Essa formulação (NC_{imiq}) apresentou porcentagens de morte celular significativas, mesmo utilizando baixas concentrações do fármaco. Portanto, os achados desta tese constataram que nanoestruturas modulam efetivamente a interação do fármaco com as células.

Palavras-chave: Câncer cervical, imiquimode, nanotecnologia, SiHa

ABSTRACT

This thesis deals with the need of new treatments for cervical cancer in order to increase the adherence of patients to the treatment as well as to improve their quality of life. In this sense, nanotechnological formulations were developed to carry imiquimod to a specific site – the vaginal mucosa – expecting to obtain better performance than the commercial drug in the cervical cancer treatment. Three nanostructures with different morphologies were proposed to potentilize the drug effect on cervical cancer cells (SiHa). The developed formulations are: nanoemulsions (NE_{imiq}), polymeric nanocapsules (NC_{imiq}) and polymeric nanocapsules coated with chitosan ($NC_{imiq\text{-}chit}$). It was observed that polymeric nanocapsules produced with poly(ε-caprolactone) presented a stronger effect against SiHa cells. Therefore, formulations NC_{imiq} and $NC_{imiq\text{-}chit}$ were incorporated into hydrogels of chitosan and hydroxyethylcellulose to enable a better future application on patients. The studies of this thesis involving porcine vaginal mucosa demonstrated that both formulations are mucoadhesive and that they provided a good drug permeation. However, the formulation produced with chitosan hydrogel (NC_{imiq}) showed a better performance. This formulation was therefore chosen to follow the next steps of this work, conducted in SiHa cell culture to elucidate its action mechanism. This study demonstrated that there is an occurrence of combined processes of decreasing cell viability in a time-dependent type. The study also showed that mechanisms such as apoptosis, autophagy and cell cycle arrest are simultaneously present. The formulation NC_{imiq} presented a significantly percentage of cellular death, even when low concentrations of the drug were used. Consequently, the findings of this thesis indicate that nanostructures effectively modulate the interaction of the drug with the cancer cells.

Keywords: Cervical cancer, imiquimod, nanotecnology, SiHa

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1 INTRODUÇÃO

A via vaginal apresenta características promissoras para administração de medicamentos. Algumas dessas características citadas na literatura são as seguintes: (i) grande superfície de contato, (ii) boa permeabilidade, (iii) elevada irrigação sanguínea resultando em facilidade de absorção dos fármacos, (iv) evita metabolismo de primeira passagem que os fármacos sofrem no fígado, e (v) redução da incidência e severidade de efeitos adversos reduzidas (VALENTA 2005; BONFERONI et al., 2008; BALOGLU et al., 2009). Por outro lado, o ambiente hostil da mucosa diminui o tempo de contato da formulação com a vagina e, consequentemente, leva a um menor tempo de contato do fármaco com a mucosa (YEN CU et al., 2009).

Por esse motivo, os sistemas mucoadesivos são utilizados como estratégias para aumentar o tempo de contato da formulação com a mucosa vaginal (FRANK et al., 2014; CARAMELLA et al., 2015; FRANK et al., 2017). Alguns polímeros mucoadesivos são utilizados em diferentes formas farmacêuticas visando aumentar o tempo de contato do fármaco, dentre eles: a quitosana, a gelatina, os derivados de ácidos poliacrílicos, o ácido hialurônico, o alginato, dentre outros (VALENTA et al., 2005; CARAMELLA et al., 2015). Essas diferentes formas farmacêuticas desenvolvidas são apresentadas em formas de cremes, géis, pomadas, óvulos e comprimidos (CARAMELLA et al., 2015).

Uma alternativa proposta para entrega efetiva e aumento da adesivadade dos fármacos nesta via é a utilização de sistemas nanoestruturados. Uma recente análise na plataforma Web of knowledge, realizada em 27/07/2017, com a combinação das palavras *nano** and *vaginal* demonstra que o interesse de pesquisa nessa via vem crescendo significativamente (Figura 1). Desde 1997, quando iniciaram as publicações dos primeiros artigos científicos, até a data analisada, já se somam um total de 350 publicações. Ademais, é possível observar que mais de 20% dessa produção bibliográfica encontra-se no ano de 2015, o que reforça o crescente interesse pela área. A maior parte desses artigos considera formas de aumentar o efeito adesivo de formulações, bem como melhorar o desempenho do fármaco buscando um maior tempo de contato fármaco/mucosa.

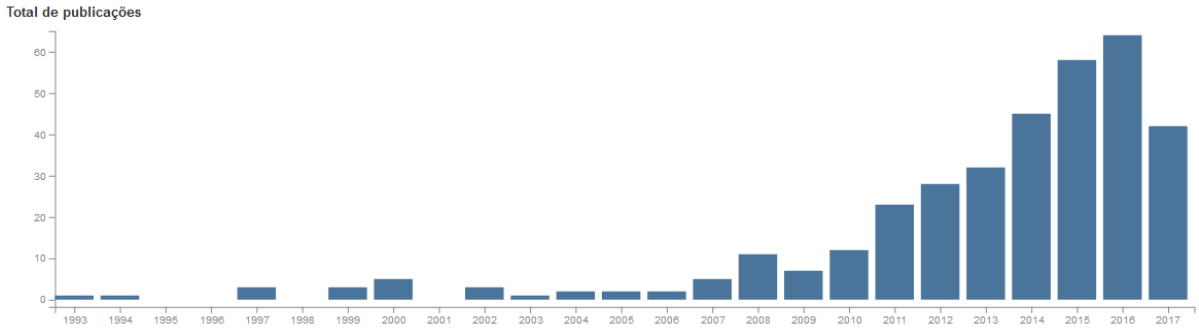

Figura 1 Pesquisa realizada na plataforma *Web of knowledge* no dia 27/07/2017 cruzando as palavras nano* and vaginal. Nota: valores parciais para 2017.

Dentre os trabalhos publicados nesse período encontram-se cinco revisões de literatura relevantes sobre a via vaginal. A primeira é de 2005 (VALENTA 2005) e aborda aspectos físico-químicos relacionados à via vaginal, assim como também faz uma revisão da literatura citando os principais polímeros adesivos utilizados em diferentes formulações. São eles: os poliacrilatos, a quitosana, os derivados da celulose e pectina, os polissacarídeos sulfatados, o amido, o alginato de sódio e a gelatina, todos utilizados visando aumentar a mucoadesividade das formulações. Em 2006, Das Neves e colaboradores (2006) publicaram um trabalho revisando os diferentes tipos de géis utilizados na via vaginal. Mais recentemente, em 2013, Vànic e colaboradores (2013) publicaram um artigo de revisão sobre a terapia de doenças vaginais utilizando formulações nanotecnológicas. Nesse artigo, os autores apresentam sistemas contendo nanotecnologia para tratamento de infecções que acometem a via vaginal e que estão em fase de desenvolvimento em laboratórios e alguns que já se encontram em ensaios clínicos. Os principais sistemas utilizados para encapsulação de fármacos abordados nesse trabalho de revisão compreendem ciclodextrinas, niossomas, micelas poliméricas, nanopartículas lipídicas sólidas e nanoemulsões. Muitos dos trabalhos citados nesse manuscrito visam propor uma forma farmacêutica para prevenção de doenças sexualmente transmissíveis, sobretudo a infecção por HIV.

Posteriormente, no ano de 2015, novamente Das NEVES e colaboradores (2015) publicaram uma revisão abordando a potencialidade de nanocarreadores na entrega de fármacos visando ação farmacológica e de profilaxia na via vaginal. Esses autores abordaram os benefícios e as limitações dos nanosistemas propostos até a data de

publicação. Os nanocarreadores vaginais são comumente conhecidos por apresentarem vantagens tais como: i) proteção de moléculas lábeis, ii) controle da liberação do fármaco, iii) modulação de adesão ao muco, iv) penetração através da mucosa, v) entrega do fármaco em local específico, vi) entrega intracelular. Conforme aponta essa revisão, as pesquisas com nanosistemas para entrega vaginal vêm crescendo tanto para uso terapêutico como para profilático. Dentre as partículas destacam-se os lipossomas, as nanopartículas lipídicas sólidas, os veículos lipídicos nanoestruturados e as nanoemulsões.

O trabalho de revisão mais recente foi publicado por Caramella e colaboradores (2015) e abordou diferentes sistemas mucoadesivos e termogelificantes para a entrega de fármacos na via vaginal visando à melhoria de sua eficácia. Os principais sistemas mucoadesivos abordados compreendem os géis, os óvulos, as micropartículas, as nanopartículas; enquanto os sistemas termogelificantes são baseados em quitosana e poloxâmeros compostos por mais de 30 agentes ativos de diferentes superfícies não iônicos.

Dentre os fármacos utilizados para tratamentos de enfermidades da via vaginal, destacam-se principalmente as classes de antifúngicos, bactericidas, antivirais e antineoplásicos. Os novos sistemas estudados por diferentes autores se dedicam especialmente a proporem uma alternativa à prevenção de doenças sexualmente transmissíveis. Dentre os fármacos estudados estão o tenofovir (ALUKDA et al., 2011), clotrimazol (BACKHAV et al., 2009; SANTOS et al.,2013), imiquimode (DONELLY et al., 2006; RAMINENI et al., 2013), dapivirina (das NEVES et al., 2012), entre outros.

Um fármaco que se destaca é o imiquimode. Esse é utilizado para diferentes doenças que acometem a via vaginal e a pele, como por exemplo: carcinoma basocelular (NETO 2002), neoplasia vulvar intraepitelial (DAVIS et al., 2000), verrugas genitais e perianais externas (TYRING et al., 1998), molusco contagioso (BROWN et al., 2000), ceratoses actínicas (EDWARDS et al., 2000), herpes simplex (CHRISTENSEN e HENGGE, 1999) e câncer de colo do útero (KOUTSKY et al., 1983). Apesar de ser amplamente utilizado no tratamento dessas doenças, o imiquimode apresenta efeitos adversos que envolvem dor, coceira e ulceração, o que leva alguns pacientes a

abandonarem o tratamento (WIELAND et al., 2006; KREUTER et al., 2008). A formulação disponível comercialmente apresenta um custo elevado e deve ser utilizada por um longo período, em torno de 16 semanas. Além disso, trata-se de um sistema não adesivo o que facilita sua remoção antecipada, sobretudo na mucosa vaginal. Nesse sentindo, a nanotecnologia pode ser uma alternativa para contornar possíveis empecilhos causados pelo fármaco bem como pela formulação.

O óleo de copaíba é um composto natural (CASCON e GILBERT, 2000) constituído de uma fração sólida não volátil, formada por ácidos diterpênicos, e uma fração de óleo essencial formada por sesquiterpenos (RIGAMONTE AZEVEDO et al., 2004). Esse composto possui diferentes funções, como por exemplo, na indústria de perfume como fixador de odores, na indústria de vernizes como secativo (VEIGA JUNIOR e PINTO, 2002) e na indústria alimentícia como aditivo aprovado pelo FDA (Food and Drugs Administration). Por apresentar propriedade emoliente, este óleo é utilizado na área cosmética no desenvolvimento de cremes, sabonetes e xampus. Além disso, o mesmo possui ação anti-inflamatória (VEIGA et al., 2007; HUMBERTO et al., 2016) e atividade antifungíca (DIAS et al., 2014), e por esse motivo, possui uma grande aplicação em farmacologia como, por exemplo, no tratamento de melanoma neoplásico e do carcinoma invasivo micropapilar (OHSAKI et al., 1994). Além disso, o óleo de copaíba pode ser um substituto de corticoesteróides no tratamento de dematites, diminuindo coceira, dor, edema e calor (HADJI-MINOGLOU e BOLCATO, 2005).

As formulações relatadas acima que visam aplicação vaginal possibilitam o tratamento de doenças associadas a esta via. Dentre essas doenças destaca-se o câncer do colo do útero, que é o sexto tipo de câncer mais frequente na população em geral e o segundo mais comum entre mulheres. Estudos epidemiológicos têm mostrado uma forte associação entre a infecção pelo papilomavírus humano (HPV), doença viral sexualmente transmissível mais comum e o câncer de colo de útero (AYRES et al., 2010). Os subtipos de HPV de baixo risco, como por exemplo, HPV 6 e 11 são agentes causadores das verrugas genitais, enquanto que os subtipos de alto risco, por exemplo, HPV 16 e 18 estão diretamente ligados ao desenvolvimento do câncer cervical (PINOTTI et al., 2005). O melhor tratamento para esta infecção é aquele que melhora a

resposta imune contra o vírus. Nesse sentido, estudos têm demonstrado que a aplicação tópica de imiquimode induz a produção de citocinas e reduz a carga de HPV em pacientes com verrugas genitais externas (KOUTSKY et al., 1983).

No campo da nanotecnologia farmacêutica, uma abordagem moderna é a possibilidade da co-encapsulação de fármacos em nanocápsulas poliméricas, buscando efeitos terapêuticos associados. Entretanto, para viabilizar esta possibilidade é necessário considerar os materiais essenciais para a formulação das nanocápsulas, um polímero biodegradável, um óleo para a constituição do núcleo das cápsulas, o(s) fármaco(s) e o(s) tensoativo(s) estabilizante(s) da suspensão. Nesse sentindo, a associação de imiquimode e óleo de copaíba em nanocápsulas poliméricas já foi proposta para o tratamento de carcinoma basocelular (VENTURINI et al., 2015), porém os autores não avaliaram a potencialidade dessa nova formulação para tratamentos de doenças que acometem a via vaginal. Esse sistema desenvolvido pelos autores citados acima representa uma potencialidade para ser incorporado em veículos semissólidos objetivando aumentar a mucoadesão e facilitar a sua aplicação na mucosa vaginal. Para esse trabalho a poli(ε-caprolactona) foi selecionada como polímero para ser utilizado na composição das nanocápsulas, bem como o óleo de copaíba e o monoestearato de sorbitano como constituinte de núcleo, e o polissorbato 80 como estabilizante da suspensão.

A incorporação de nanocápsulas poliméricas em hidrogéis de quitosana para aplicação vaginal já foi proposta recentemente por nosso grupo de pesquisa (FRANK et al., 2014). A quitosana é um polímero pseudonatural catiônico, que consiste de glucosamina e unidades de N-acetilglucosamina. É, sobretudo, obtida por desacetilação de quitina derivada do exoesqueleto de crustáceos, tais como camarão e lagosta (NASTI et al., 2009). Esse polímero é considerado um biomaterial atóxico, apresentando biocompatibilidade e biodegradabilidade (SINGLA e CHAWLA 2001). Além disso, apresenta propriedades físico-químicas e biológicas como formação de filme sobre pele e mucosas, boas propriedades de fluxo, bioadesividade, inocuidade e atividade antibacteriana (ALEMDAROGLU et al., 2006; HAMIDI et al., 2008; ZHANG e KAWAKAMI, 2010). Esse polímero também apresenta propriedades mucoadesivas e

atividade antimicrobiana (LUEBEN et al., 1996; KIM et al., 2003) o que o torna uma alternativa interessante para aplicação de formulações na via vaginal.

Em vista do exposto, o presente trabalho tem como objetivo propor uma formulação nanotecnológica mucoadesiva incorporando nanocápsulas poliméricas de poli(ε-caprolactona) contendo imiquimode e óleo de copaíba associados em sistemas mucoadesivos (hidrogel de quitosana) a fim de aumentar a adesevidade da formulação e interação com a mucosa, bem como avaliar o efeito e mecanismo de morte dessa formulação em linhagem de células de câncer cervical contendo cópias do HPV 16 para propor uma terapia inovadora para o tratamento dessas enfermidades.

1.1 TEMA E OBJETIVOS

O tema de pesquisa desta tese contempla as áreas de nanotecnologia farmacêutica e biologia celular. Dentro da área da nanotecnologia, esta pesquisa concentra-se especificamente no estudo de uma formulação contendo o fármaco imiquimode associado ao óleo de copaíba como material estruturante das nanocápsulas poliméricas e também visando seu efeito farmacológico, conforme descrito na literatura. A proposta dessa formulação tem como objetivo o tratamento do HPV e do câncer cervical. Em vista disso, na área de biologia celular essa pesquisa concentra-se na aplicação da formulação nanotecnológica em linhagem celular de câncer cervical, a fim de avaliar seu efeito e propor uma terapia inovadora.

Face ao exposto, o objetivo geral desta tese é desenvolver e realizar a caracterização físico-química de suspensões de nanocápsulas contendo imiquimode e óleo de copaíba, bem como avaliar o efeito, mecanismo de morte e ação após tratamento celular dessa formulação *in vitro* utilizando linhagens de células de carcinoma cervical.

Para que seja possível alcançar o objetivo geral deste trabalho, propõem-se os seguintes objetivos específicos:

a) Preparar e caracterizar suspensões de nanocápsulas poliméricas contendo o fármaco imiquimode e óleo de copaíba, quanto ao tamanho, potencial zeta, pH, morfologia e estabilidade física;

b) Validar um método analítico para determinação quantitativa do imiquimode e determinação do teor e taxa de encapsulação do fármaco nas nanocápsulas poliméricas;
c) Estudar comparativamente duas propostas de formulações mucoadesivas (hidrogel de quitosana incorporado com nanocápsulas poliméricas contendo imiquimode e hidrogel de hidroxietilcelulose incorporado com nanocápsulas poliméricas revestidas com quitosana contendo imiquimode) a fim de determinar a potencialidade de utilização das mesmas para os objetivos gerais propostos;
d) Avaliar a viabilidade e morte celular após o tratamento utilizando as formulações desenvolvidas em linhagem celular (SiHa);
e) Determinar a concentração de interleucina-6 após tratamento em linhagem celular (SiHa) com a formulação realizando o doseamento de citocinas por ELISA;

1.2 REVISÃO DA LITERATURA E JUSTIFICATIVA

1.2.1 Imiquimode

O imiquimode é um fármaco de baixo peso molecular derivado da família amino imidazoquinolina (Figura 2). É um quimioterápico e imuno-estimulante com atividade antitumoral e antiviral. Foi aprovado pelo Food and Drug Administration (EUA) em 2004 para uso tópico e no tratamento de verrugas genitais e perianais (GUPTA et al., 2002; SKINNER 2003), porém, seu uso para doenças benignas tais como molusco contagioso, ceratoses actínicas e doença de Bowen vêm crescendo (PAULA et al. 2008; SAUDER et al., 2000; GUPTA et al., 2002).

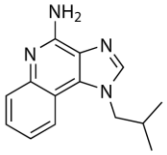

Figura 2 Representação da estrutura química do fármaco imiquimode.

Embora a maioria dos agentes imunomoduladores disponíveis ou em desenvolvimento agem inibindo as vias envolvidas na ativação imune, o imiquimode é o único que ativa a função imune (SAUDER et al., 2000). Sua ação imunomoduladora

se deve à atividade agonista no receptor 7 dos monócitos, macrófagos e células dendríticas (Langerhans) ativando assim a imunidade inata e a celular (Th1), pela indução de citocinas pró-inflamatórias como interferon alfa, fator de necrose tumoral alfa e interleucinas 1, 6, 8 e 12. Além disso, induz apoptose e ativa os linfócitos B, potencializando a resposta imunológica (GUPTA et al., 2002; HENGGE et al., 2004; HENGGEE et al., 2001; MCINTURFF *e*t al., 2005; MEYER et al., 2003).

O imiquimode se encontra disponível no mercado sob a forma farmacêutica de creme a 5% (Aldara®3M). Em 2006 a ANVISA aprovou uma pomada contendo imiquimode para o tratamento do câncer de pele. A mesma pomada já era utilizada no Brasil para o tratamento de verrugas genitais produzidas pelo papilomavirus humano (HPV). A formulação deve ser administrada sobre a lesão três vezes por semana e deve permanecer em contato por no mínimo 6 horas; por isso sugere-se que seja aplicada à noite por um período de 8 a 16 semanas. Alguns efeitos adversos ocorrem em pacientes que fazem uso desse tipo de formulação, sendo a maioria efeitos locais como vermelhidão, queimação, irritação, ulceração e dor. O grau de eritema local que ocorre naqueles pacientes que aderem ao tratamento é em virtude da liberação das citocinas pró-inflamatórias, como parte do mecanismo de ação do fármaco (WIELAND et al., 2006; KREUTER et al., 2008).

Existem pesquisas que foram desenvolvidas a fim de avaliar a eficácia terapêutica ao uso do imiquimode tópico. Por exemplo, Neto (2002) realizou um estudo com 10 voluntários que apresentavam carcinoma basocelular (CBC) do tipo superficial e nodular que se recusaram a fazer intervenção cirúrgica. O objetivo desse estudo foi analisar a efetividade e tolerabilidade do creme comercial disponível comercialmente contendo imiquimode 5% para esse tipo de enfermidade. Os resultados encontrados pelo autor demonstraram uma diminuição visível das feridas características dessa enfermidade. Porém, o autor alerta que cada paciente deve receber uma avaliação e acompanhamento individualizado devido às características específicas de cada lesão. Por sua vez, o trabalho de Pachman e colaboradores (2012) avaliou 56 pacientes com displasia cervical que foram divididos em dois grupos aleatoriamente. Um grupo foi tratado com imiquimode 5% e o outro grupo com o tratamento padrão para essa

enfermidade. Embora o grupo tratado com imiquimode apresentasse maiores efeitos adversos, os autores observaram que o mesmo pode ser usado como complemento ao tratamento da displasia cervical.

Em outro trabalho, Donnelly e colaboradores (2006) preparam adesivos transdérmicos contendo imiquimode. Esses adesivos foram preparados em virtude de que o imiquimode deve permanecer por um tempo mínimo de 6 horas em contato com a região afetada e dificilmente cremes convencionais permanecem este tempo em regiões como o trato genital feminino. Em estudo *in vitro*, foi observado que os adesivos foram capazes de liberar mais fármaco do que o creme convencional. Segundo esses autores, isto pode ser explicado pela característica lipofílica do imiquimode que pode ter prejudicado a liberação do creme para o compartimento receptor aquoso utilizado no experimento. Embora Donnelly e colaboradores (2006) tenham proposto um novo sistema adesivo contendo o fármaco imiquimode, a avaliação do efeito desse novo sistema bem como a avaliação dos possíveis maiores efeitos adversos causados pela maior liberação do fármaco não foram realizados.

Um trabalho mais recente proposto por Venturini e colaboradores (2015) comparou duas formulações nanotecnológicas (nanocápsulas poliméricas compostas de poli(ε-caprolactona) e um nanocarreador lipídico nanoestruturado (composto de manteiga de cupuaçu), ambas contendo a associação de imiquimode e óleo de copaíba, para o tratamento ao carcinoma de pele. Embora ambas as formulações tenham apresentado compatibilidade com células da pele, os autores sugeriram que as nanocápsulas poliméricas poderiam ser utilizadas como o sistema mais promissor porque permitiram uma maior retenção do fármaco nas camadas da pele. Contudo, nesse trabalho os autores não avaliaram a adesividade dessa nova formulação proposta e também não desenvolveram um sistema mucoadesivo visando aumentar o tempo de contato da formulação com a mucosa utilizada.

Sendo assim, observa-se nas pesquisas anteriormente citadas que a formulação comercial contendo imiquimode mostra-se eficaz frente a diferentes patologias. Contudo, não foram identificadas na literatura científica proposições de formulações de base nanotecnológica mucoadesiva que vise melhorar a performance do imiquimode, ou

seja, capazes de aumentar o seu tempo de contato com a mucosa e de diminuir efeitos adversos relacionados ao seu uso. O imiquimode é um fármaco lipossolúvel com recentes aplicações terapêuticas descritas e que deve permanecer por longo período em contato com a mucosa, o que pode levar ao desenvolvimento de efeitos adversos locais, os quais podem ser, em hipótese, reduzidos através de sua inclusão em nanopartículas. Por isso, o imiquimode foi selecionado na presente proposta como fármaco alvo para a nanoencapsulação devido ao fato de preencher os requisitos necessários para justificar o interesse do emprego da nanotecnologia.

1.2.2 Via vaginal

A vagina é um tubo fibromuscular que mede em torno de 7 a 10 cm que se estende desde o exterior do corpo até o útero. A morfologia do tecido vaginal varia em relação à região anatômica (VALENTA 2005; MALLIPEDDI e ROHAN 2010). Esse órgão é um complexo sistema dinâmico que contém fluidos que podem exercer função tanto de barreira à entrada de substâncias quanto alvo de entrega de fármacos. A vagina possui todos os elementos essenciais para uma resposta imune eficaz e fornece um sistema de defesa inato que não deve ser alterado (MALLIPEDDI e ROHAN 2010).

Esta via representa uma excelente alternativa para administração de fármacos por apresentar vantagens como: (i) grande superfície de contato (ii) boa permeabilidade a muitos fármacos, (iii) elevada irrigação sanguínea, (iv) relativa facilidade de administração do fármaco, (v) evita o metabolismo de primeira passagem que os fármacos sofrem no fígado, (vi) redução da incidência e severidade de efeitos adversos gastrointestinais (BONFERONI et al., 2008; VALENTA 2005; BALOGLU et al., 2009).

Porém, o ambiente hostil da mucosa reduz o tempo de residência de fármacos quando estes são administrados (YEN CU et al., 2011). Isto se deve ao fato de haver uma descarga vaginal que envolve fluidos endometriais, secreções das glândulas bortholinas e transudatos através do epitélio, os quais contribuem para a remoção da formulação a partir do local de aplicação. Essa descarga vaginal, muitas vezes conhecida como muco, desempenha um papel importante na capacidade de absorção de fármacos.

A mucosa vaginal é composta pelo muco que tem como função principal a proteção da vagina contra o ambiente externo. Esse muco é formado por glicoproteínas, lipídeos e sais inorgânicos suspensos em água. A concentração desses componentes bem como suas propriedades reológicas são afetados com a idade da mulher, fase do ciclo mestrual e com a relação sexual, influenciando assim a liberação do fármaco quando administrado dentro da vagina (VALENTA 2005, VANIC et al., 2013). Tais variações de volume e viscosidade do muco podem afetar a eficácia dos fármacos. Além disso, a vagina apresenta um pH em entre 3,5 e 4,5 que é mantido pelos lactobacilos que convertem o glicogênio do epitélio em ácido lático (BOSKEY et al., 2001; VALENTA et al., 2005). Alterações do pH do fluido vaginal são fatores limitantes para a administração de fármacos. Por fim, cabe ressaltar que, devido à ação da descarga vaginal (ação de autolimpeza da via), o tempo de residência das diferentes formas farmacêuticas de administração é reduzido, a menos que se utilizem estratégias para que a formulação seja mantida por um período de tempo prolongado como, por exemplo, óvulos, géis, anéis vaginais, comprimidos mucoadesivos, entre outros (CARAMELLA et al., 2015). Portanto, formulações contendo fármacos para serem aplicados nesta via devem ser capazes de contornar todas essas dificuldades, somando-se à necessidade de uma baixa propensão para causar irritação genital e toxicidade sistêmica (VALENTA 2005, VÀNIC et al., 2013).

Apesar dos empecilhos citados, bem como as limitações específicas de cada indivíduo oriundos da sua fisiologia, o interesse pela administração de fármacos através da via vaginal vem crescendo em virtude das interessantes propriedades já descritas anteriormente. Esta via pode oferecer uma alternativa favorável à via parenteral para fármacos como bromocriptina e propranolol (VERMESH et al., 1988; JOSONNI et al., 1991; PATEL 1984) e, ainda, pode ser uma alternativa para a entrega de contraceptivos hormonais, devido à falta de interações medicamentosas observadas no trato gastrointestinal (VALENTA 2005). No entanto, esta via não tem sido extensamente explorada para entrega sistêmica por ser uma via exclusivamente feminina e também por sofrer muitas alterações cíclicas e fisiológicas (VALENTA 2005; PERIOLI et al., 2008). Tradicionalmente, a cavidade vaginal tem sido utilizada para entrega de fármacos

com ação local, tais como antibacterianos, antifúngicos, antiprotozoários, antivirais, agentes espermicidas e esteroides (PERIOLI et al., 2008).

Dois trabalhos de revisão da literatura merecem destaque. Um deles é o trabalho de Cutler e Justman (2008) que apontaram os principais fármacos utilizados nos tratamentos de doenças da via vaginal. De igual forma merece menção o trabalho de Das Neves e colaboradores (2010) que realizaram um levantamento da literatura com todos os sistemas já publicados baseados em nanotecnologia para prevenção e tratamento do HIV. Além disso, como fora explicado anteriormente, o ambiente hostil da mucosa reduz o tempo de residência de fármacos quando estes são administrados. Uma alternativa para aumentar o tempo de contato entre o fármaco e a mucosa vaginal é o desenvolvimento de formulações que apresentem uma maior mucoadesividade tais como géis, filmes e comprimidos. Nesse sentido, trabalhos como os de Valenta e colaboradores (2001) Perioli e colaboradores (2008 e 2009), Fetherston e colaboradores (2013), Villena e colaboradores (2013) e Frank e colaboradores (2014), abordaram questões relativas à mucoadesividade na via vaginal. Estes trabalhos são detalhados a seguir.

O trabalho de Valenta e colaboradores (2001) descreve uma nova matriz para entrega vaginal de progesterona, produzida através de uma ligação covalente de L-cisteína com Carbopol 974P comercialmente disponível. Os autores demostraram uma melhor mucoadesividade desse novo sistema, além de uma liberação controlada do fármaco em comparação com os comprimidos que não possuíam esta matriz proposta.

No trabalho de Perioli e colaboradores (2008) foram produzidos géis de hidroxietilcelulose, combinados com quitosana e com seu derivado 5-metilpirrolidinona. Nesses géis foi incorporado o metronidazol, um fármaco utilizado no tratamento da vaginose bacteriana, e foi comparado com um gel comercial. Os autores observaram que todos os géis contendo quitosana apresentaram melhor desempenho reológico, mucoadesivo e de liberação do que o gel comercial. Sendo assim, esse polímero foi considerado adequado e promissor para uso em formulações para via vaginal.

Em outro trabalho, Perioli e colaboradores (2009) desenvolveram comprimidos vaginais contendo metronidazol. Os comprimidos foram preparados utilizando uma combinação de polímeros mucoadesivos (quitosana, polivinilpirrolidona e policarbofil). Esse trabalho demonstrou que a mistura dos polímeros (quitosana e polivinilpirrolidona) em proporções adequadas (1:1, m/m) favorece a compressão dos mesmos e também representa uma alternativa para administração de fármacos por essa via, podendo levar a uma melhor adesão ao tratamento por parte das pacientes.

Fetherston e colaboradores (2013) desenvolveram um anel vaginal de elastômero de silicone do tipo matriz com o objetivo de controlar a liberação de dois antirretrovirais (dapivirine e maraviroc) com diferentes mecanismos de ação na tentativa de aumentar a amplitude de proteção contra o vírus do HIV e limitar o surgimento de cepas resistentes. Diferentes concentrações dos fármacos foram incorporadas nos anéis vaginais e foram realizados estudos de estabilidade e liberação. O anel vaginal contendo 25 mg de dapivirine e 100 mg de maraviroc foi o que demonstrou melhores resultados. O sistema contendo esses fármacos apresentou uma liberação controlada durante 29 dias e também estabilidade frente a variações de umidade e calor durante dois anos. Esse anel vaginal já se encontra em estudos clínicos de fase I e parece ser promissor na prevenção do HIV.

O trabalho de Villena e colaboradores (2013) propôs três diferentes tipos de micropartículas contendo o fármaco nistatina utilizando três polímeros diferentes (quitosana, alginato e Poloxamer 407®) para administração vaginal no tratamento da candidíase genital. As micropartículas apresentaram liberação controlada do fármaco e adequada mucoadesividade em vagina de porcas. Os autores também demonstraram que esses sistemas apresentaram efeito antifúngico sem apresentarem toxicidade.

Por fim, em trabalho realizado no mestrado (FRANK et al, 2014) propusemos a associação da nanotecnologia como estratégia para aumentar a adesividade de formulações para uso vaginal. Foi demonstrado que o vermelho no Nilo (utilizado como modelo de fármaco lipofílico) associado a nanocápsulas de Eudragit RS® 100 e incorporadas em hidrogel de quitosana na concentração de 2.5% são capazes de aumentar a adesividade da formulação, além de interagirem fortemente com a mucosa

vaginal e, consequentemente, aumentando a permeação do vermelho do Nilo, empregado como sonda.

Sendo assim, é possível observar que muitos estudos têm se dedicado a proporem diferentes formulações tecnológicas para aumentar a mucoadesão das formulações ao tecido vaginal, bem como diminuírem os efeitos tóxicos de alguns fármacos. Alguns estudos inclusive propõem uma nova formulação adesiva e comparam com a formulação comercial (VALENTA et al., 2001; DONELLY et al., 2006; PERIOLI et al., 2008). Além disso, recentes trabalhos demonstraram que nanocápsulas poliméricas apresentam adesividade frente a mucosas. Porém, não se encontrou na literatura estudos comparativos de diferentes formulações nanotecnológicas adesivas a fim de propor uma formulação final que apresente melhor desempenho frente à mucosa desejada.

1.2.3 Modelos para estudo da via vaginal

Alguns modelos animais têm sido propostos para analisar a absorção e mucoadesão de fármacos através da via vaginal (WU e ROBINSON, 1996). Dentre as diferentes mucosas vaginais animais já utilizadas como modelo, podem-se destacar, por exemplo, a de coelhas, de cadelas, de macacas, de ratas e de ovelhas. Em muitos desses modelos, o grau de absorção de fármacos variou como consequência da fisiologia e da anatomia da vagina e também em virtude das alterações durante o ciclo menstrual de cada espécie animal. Essas modificações cíclicas na histologia e na fisiologia da vagina podem afetar a absorção de fármacos tanto em mulheres como também nos modelos animais utilizados para estudos de absorção (RICHARDSON e ILLUM, 1992). Poucos estudos comparam as diferentes espécies entre si. Um deles é o de Hussain e Ritschel (1989) que estudaram a biodisponibilidade do fosfonoformato em cadelas e em coelhas. A biodisponibilidade após administração vaginal foi de 14% e 34%, respectivamente. Considerando que a biodisponibilidade após administração tópica foi de 3% e 12%, respectivamente, este resultado pode ser uma indicação de que também as taxas de absorção em mulheres e animais possam ser diferentes.

Medidas de mucoadesão envolvem o uso de mucosa, normalmente a partir de um animal, sendo que as variações nas propriedades biológicas podem dificultar a

reprodutibilidade dos resultados. Além disso, dependendo da localização da mucosa, a sua remoção é um fator limitante, tornando sua utilização inapropriada. Em vista disto, alguns autores utilizaram disco de mucina, uma glicoproteína responsável pela característica adesiva de muco, como forma de determinar a mucoadesão de formulações (LEITNER et al., 2003; BRUSCHI et al., 2007; SRIAMORNSAK et al., 2010; FONSECA et al., 2014). Porém, um recente estudo (CHAVES et al., 2017) avaliou o desempenho mucoadesivo de formulações compostas por polímeros com cargas de superfícies variadas em relação à mucosa vaginal, mucosa bucal e disco de mucina a fim de verificar a possibilidade de a mucina reproduzir os resultados das diferentes mucosas animais utilizadas. Nesse estudo, os autores concluíram que a forma mais completa de avaliar a mucoadesividade das formulações é utilizando mucosa animal fresca e que a mucina pode ser utilizada como alternativa quando a utilização dessas mucosas não for possível.

Muitos pesquisadores têm utilizado o modelo vaginal suíno para avaliar a mucoadesividade, penetração e absorção de fármacos através da via vaginal (SANDRI et al., 2004; BONFERONI et al., 2008; PERIOLI et al., 2008; VILLENA 2013; FRANK et al., 2014). Para estudos de mucoadesividade alguns autores utilizam mucosa vaginal previamente congelada a -20ºC; no entanto, para os experimentos que envolvem permeação e penetração é necessário que a mucosa vaginal seja fresca para que as propriedades do tecido sejam mantidas integras. Os autores acima citados não compararam a mucosa vaginal suína com outro modelo vaginal, porém há uma concordância de que o modelo vaginal suíno é um dos mais adequados para simular a mucosa vaginal feminina. Com base nisso, na presente tese, foram utilizados modelos suínos para a realização de todos os experimentos envolvendo mucoadesão e permeação.

1.2.4 Mucoadesão

A mucoadesividade de uma forma farmacêutica é medida como o resultado da interação entre o material presente na formulação com o muco, ou seja, é a capacidade dessas estruturas permanecerem em contato por um determinado tempo, através de ligações interfaciais (ANDREWS et al., 2009). A propriedade de mucoadesão vem

sendo alvo de estudos na área farmacêutica para aumentar a interação entre fármaco e mucosa, bem como para potencializar a absorção de substâncias em regiões de interesse. Os alvos desse tipo de sistema compreendem diferentes regiões do organismo que são revestidas por uma camada de muco como a ocular, bucal, nasal, retal, gastrointestinal e vaginal (CARVALHO et al., 2010). Nesse sentido, já foram propostos materiais com diferentes características bioadesivas visando aumentar a adesão com o muco, dentre eles: quitosana, alginato e gelatina (CARAMELLA et al., 2015).

O muco é uma película que reveste a mucosa e tem composição química variável dependendo do órgão e características de cada indivíduo. 95% da sua constituição são representados por água. Sua característica adesiva é devido a presença de uma glicoproteína: a mucina. Sua composição é rica em oligossacarídeos, que são encontrados em seu núcleo proteico, que confere tanto uma carga negativa do muco em pH fisiológico como também uma característica de gel coesivo. Alguns estudos apontam a mucina como a responsável pela característica adesiva do muco (PATEL et al., 2011).

Diferentes teorias foram propostas a fim de tentar elucidar os mecanismos envolvidos no processo de mucoadesão, porém esse fenômeno ainda não é completamente claro. De maneira geral, mais de uma teoria são utilizadas simultaneamente para explicar o mecanismo de adesão envolvendo dois materiais em contato (CARVALHO et al., 2010). Dentre as principais teorias utilizadas destacam-se: teoria eletrônica (atração entre cargas opostas), teoria da adsorção (interações hidrofóbicas, ligações de hidrogênio, forças de Van der Waals), teoria da molhabilidade (interação líquido e camada de muco), teoria da difusão (considera a penetração de moléculas na rede de muco e difusão de mucina na forma farmacêutica), teoria da fratura (dificuldade de ruptura de duas substâncias após a adesão) e teoria mecânica (considera o efeito da rugosidade da superfície no aumento da área de contato) (KHUTORYANSKIY, 2011).

Os polímeros são os principais materiais alvos de pesquisas no desenvolvimento de formulações (DAS NEVES et al., 2013; CARAMELLA et al., 2015). O tipo de interação que cada polímero apresenta quando é colocado em contato com a mucosa depende principalmente de sua carga de superfície. De maneira geral, os polímeros

devem apresentar adequada flexibilidade para penetrar à mucosa, não serem tóxicos, biocompatíveis e, se possível, serem de baixo custo (PATEL et al., 2011). Os polímeros catiônicos são os mais utilizados, pois são considerados bons materiais mucoadesivos. Isso pode ser explicado devido a eles apresentarem carga positiva na sua superfície que são capazes de interagir com as moléculas negativas da mucina através de atração eletrostática. Um exemplo desse tipo de polímero com grande aplicação na área farmacêutica é a quitosana e os polímeros polimetacrilatos (SANDRI et al., 2004; BONFERONI et al., 2008; FRANK et al., 2014). Por sua vez, os polímeros aniônicos possuem grupos carboxílicos em sua estrutura que podem interagir através de ligação de hidrogênio com oligossacarídeos presentes na molécula de mucina. Por esse motivo, podem ser considerados bons materiais adesivos. Em contraposição, polímeros não iônicos não se destacam por apresentar boa capacidade mucoadesiva. Acredita-se que esses materiais interajam com o muco através de difusão e interpenetração na camada mucosal (ANDREWS et al., 2009; KHUTORYANSKIY, 2011).

Diferentes sistemas adesivos vêm sendo propostos para liberação de fármacos, tais como géis (SANDRI et al., 2004), comprimidos (PERIOLLI et al., 2011), óvulos (SOBEL et al., 2001), filmes (DONELLY et al., 2006) e sistemas nanométricos (PERIOLLI et al., 2008; CONTRI et al., 2014; FRANK et al., 2014). Nanopartículas poliméricas podem ser consideradas sistemas adesivos, pois devido ao seu tamanho nanométrico e sua carga de superfície são capazes de interagir com a mucina de maneira eficiente (CARAMELLA et al., 2015).

Os testes *in vitro* podem ser utilizados para avaliar a capacidade mucoadesiva de uma formulação proposta. Dentre os mais utilizados está o método da lavabilidade, que permite avaliar o quanto da formulação é capaz de permanecer em contato com a mucosa quando um fluxo (mimetizando ação do fluido vaginal, suor, saliva, lágrima, água) é aplicado sobre a mesma. Nesse tipo de experimento é avaliada a quantidade da substância ativa que é lavada em função do tempo. Sendo assim, o que não foi lavado representa o conteúdo da formulação que ficou retido na mucosa (BONFERONI et al., 2008; CONTRI et al., 2014; FRANK et al., 2014; CHAVES et al., 2016). Outra forma de avaliar a capacidade mucoadesiva dos sistemas envolvidos é o método que mede o

trabalho (ou a força máxima) necessário para romper ligação entre a mucosa e um sistema adesivo, através da aplicação de uma força externa. Nesse método utiliza-se um equipamento chamado analisador de textura (DAS NEVES et al., 2008; FRANK et al., 2014; FONSECA et al., 2015; CHAVES et al., 2016). Testes reológicos também são utilizados e avaliam alterações nas estruturas dos sistemas após contato com componentes do muco (ROSSI et al., 2001; CARVALHO et al., 2010). Todos esses testes podem ser utilizados conjuntamente para avaliar de forma mais completa a mucoadesividade de um sistema proposto.

1.2.5 Sistemas de liberação de fármacos

Os sistemas de liberação de fármacos vêm recebendo grande atenção devido à necessidade de aperfeiçoar a ação de fármacos no organismo. Dentre esses sistemas destacam-se as micropartículas e os sistemas nanoestruturados, que têm por objetivo controlar a liberação do fármaco no organismo e diminuir a incidência de efeitos adversos (COUVREUR et al., 2002). As micropartículas são estruturas de tamanho micrométrico obtidas por microencapsulação do fármaco. Estas podem ser divididas em dois tipos: partículas poliméricas esféricas (microesferas) ou reservatórios (microcápsulas), as quais diferem entre si pela composição química. Por outro lado, as nanopartículas são sistemas carreadores de substâncias ativas que apresentam diâmetro inferior a 1 μm e que são utilizadas como vetores para carrear substâncias minimizando os efeitos adversos e a possível toxicidade que substâncias bioativas podem apresentar em tecidos ou órgãos saudáveis (SCHAFFAZICK e GUTERRES 2003; CONTRI et al., 2014; VENTURINI et al., 2015). Ainda, é possível controlar a liberação dessas substâncias e sua disponibilidade para agir, quando associadas às nanopartículas (SOPPIMATH et al., 2001). Os sistemas nanopartículados por apresentarem um tamanho menor do que as micropartículas têm uma maior facilidade de transporem as barreiras e penetrarem mais facilmente em tecidos (TORCHELIN 2000; FRANK et al., 2014).

O termo nanopartículas inclui as nanoemulsões e também as nanopartículas poliméricas e lipídicas. As nanopartículas constituem um sistema de entrega de fármaco

versátil, devido à sua capacidade de superar as barreiras fisiológicas e direcionar o fármaco para as células específicas ou para compartimentos intracelulares por diferentes mecanismos (MALLIPEDDI e ROHAN 2010). Dentre as nanopartículas de caráter lipídico encontram-se as nanopartículas lipídicas que são nanoestruturas capazes de veicular substâncias ativas, tendo sua maior aplicação em formulações de uso tópico (FANG et al., 2008). Essas partículas apresentam em sua estrutura um lipídio sólido ao invés de um óleo e isso tem como objetivo diminuir a mobilidade do ativo no lipídio, aumentando assim o controle de liberação do mesmo. Além disso, esse tipo de estrutura apresenta uma matriz polimérica constituída por lipídios fisiológicos, o que diminui o risco de toxicidade dos mesmos (MEHNERT e MÄDER, 2001).

As nanopartículas poliméricas são representadas pelas nanocápsulas e nanoesferas, as quais diferem entre si segundo a composição e organização estrutural. As nanoesferas são formadas por uma matriz polimérica, que pode adsorver ou reter o fármaco. Elas não apresentam núcleo oleoso em sua composição e são formadas por uma matriz polimérica. As nanocápsulas, por sua vez, são constituídas por um invólucro polimérico disposto ao redor de um núcleo oleoso, podendo a substância ativa estar dissolvida neste núcleo e/ou adsorvida à parede polimérica. Neste caso, é fundamental que tanto o óleo quanto a água sejam não solventes para o polímero. (SCHAFFAZICH et al., 2003). Quando a substância ativa é associada às nanopartículas é possível controlar sua liberação e a disponibilidade para exercer sua ação (SOPPIMATH et al., 2001) podendo assim apresentar uma maior eficácia terapêutica. Um dos principais objetivos desse tipo de formulação é manter a concentração do fármaco acima da dose subterapêutica e abaixo da concentração tóxica, por um tempo prolongado (TEDESCO et. al., 2007).

Por ser uma via ainda pouco explorada para administração de fármacos, os artigos que estudam a via vaginal e o uso da nanotecnologia na mesma são poucos e recentes. Alguns tipos de nanocarreadores foram pesquisados como veículos para melhorar a entrega de fármacos na via vaginal. Estes sistemas podem ser geralmente classificados como poliméricos (sintéticos e naturais), inorgânicos ou nanopartículas lipídicas sólidas (VANIC et al., 2013). Dentre as nanopartículas poliméricas já desenvolvidas para

aplicação vaginal, destaca-se, por exemplo, o trabalho de Neves e colaboradores (2012) que desenvolveram nanopartículas de poli(ε-caprolactona) e encapsularam o antirretroviral dapivirine. Os resultados demonstraram a potencialidade dessas nanopartículas de entregarem o fármaco em um local específico, além do controle da liberação do mesmo. Por sua vez, Ham e colaboradores (2009) desenvolveram uma formulação de nanopartículas de ácido poli-lático-co-glicólico (PLGA) contendo o PCS-RANTES (um modulador que reduz a expressão de CCR5 na superfície da célula), como um método para prevenir a transmissão sexual de HIV-1. Nesse estudo, as nanopartículas produzidas não apresentaram efeito tóxico e aumentaram a entrega de PCR-RANTES para os tecidos de forma controlada.

O trabalho de Cu e colaboradores (2011) demostrou que nanopartículas com superfície modificada têm uma melhor capacidade de transpor as barreiras de muco e entregarem mais facilmente fármacos dentro da mucosa vaginal. Os veículos propostos podem facilitar a entrada de siRNA e plasmídeo de DNA dentro das células. Recentemente, Santos e colaboradores (2013) desenvolveram nanocápsulas contendo o fármaco clotrimazol utilizando como polímero o Eudragit® RS100. Os autores demonstraram através de estudo microbiológico que as nanocápsulas mantiveram atividade antifúngica do clotrimazol contra espécies de *Cândida* resistentes ao fluconazol, bem como atividade em contra candidíase vulvovaginal.

As nanopartículas lipídicas sólidas foram propostas como uma alternativa às nanopartículas poliméricas. Elas são partículas de tamanho nanométrico, esféricas e são constituídas de lipídeos sólidos e emulsionantes. Essas nanopartículas também têm potencial para transportarem fármacos para via vaginal como demonstra o trabalho de Alukda e colaboradores (2011), os quais incorporaram tenofovir em SLN para ser administrado na via vaginal como uso tópico. Os autores observaram o potencial das SLN de melhorar a absorção celular de tenofovir e, assim, impossibilitar a propagação do vírus durante o processo de infecção por HIV, possivelmente levando a uma prevenção mais eficaz da transmissão da doença.

Os niossomas são outro tipo de veículo para entrega de fármacos na via vaginal que foram estudados objetivando uma liberação sustentada e direcionada do fármaco

(SANKHYAN e PAWAR, 2012). Niossomas são vesículas que não contém fosfolipídios, são compostos por tensoativos não iônicos como, por exemplo, polioxietileno glicóis. Embora sua morfologia seja semelhante à dos lipossomas, estruturalmente são diferentes. Ning e colaboradores (2005) relataram a incorporação de clotrimazol em um gel niosomal e observaram liberação prolongada do fármaco e atividade antifúngica adequada. Niossomas também foram relatados como promissores para aplicação tópica vaginal em gel contendo o fármaco tenofovir que demonstrou não ser irritante em mucosa vaginal de ratas (PATEL e PATEL, 2011).

Outro tipo de nanopartículas são as nanoemulsões. Estas apresentam características promissoras para entrega de fármacos na via vaginal. Elas podem ser obtidas de forma simples (emulsificação), apresentam estabilidade por um longo período e são capazes de solubilizar diversos fármacos destinados a via vaginal (TADROS et al., 2004). Embora muitas vezes descritas como microemulsões na literatura, parece ser mais apropriado se referir a esses sistemas como nanoemulsões porque o tamanho das partículas é inferior a 200 nm (VANIC et al., 2013). Um estudo realizado por Bachhav e Patravale (2009) comparou nanoemulsões contendo o fármaco antifúngico clotrimazol com um gel comercial contendo o mesmo fármaco. Os autores demostraram que o gel contendo a nanoemulsão apresentou maior atividade antifúngica do que o gel comercial. Outros autores também desenvolveram um gel contendo microemulsão com nonoxinol-9 no contexto contraceptivo e compararam com uma formulação disponível no mercado. O gel desenvolvido se mostrou mais eficaz que o gel comercial e, além disso, não causou irritação nem toxicidade local ou sistêmica, demostrando ser seguro (D´CRUZ et al., 2001).

Um sistema adesivo contendo nanocápsulas poliméricas foi proposto por nosso grupo de pesquisa (FRANK et al., 2014) para entrega de vermelho do Nilo (modelo de fármaco lipofílico utilizado) em mucosa vaginal. Pode-se observar um melhor desempenho do gel (maior adesividade e maior penetração) frente à mucosa vaginal quando o mesmo continha nanocápsulas poliméricas. Recentemente, um estudo realizado por Contri e colaboradores (2014) em humanos, pode-se observar que uma formulação contendo nanocápsulas com capsaicinóides incorporados em hidrogel de

quitosana não produziu sintomas de irritação cutânea quando comparada com a formulação comercial na mesma concentração de capsaicinoides. Essa ausência de irritação está relacionada com o controle de liberação das nanocápsulas utilizadas e também do hidrogel de quitosana. Considerando estes resultados, o presente trabalho segue esta mesma linha de pesquisa e avalia o emprego da nanotecnologia para o fármaco imiquimode como um meio de melhorar alguns possíveis empecilhos para aplicação do mesmo na via vaginal incoporando o fármaco proposto em hidrogel de quitosana.

A quitosana vem sendo utilizada como um adjuvante farmacêutico, e tem sido empregada em diferentes formulações, como pós, comprimidos, emulsões e géis. Além disso, a quitosana garante uma liberação controlada de fármacos (ILLUM 1998; DODANE e VILIVALAM 1998). Por ser de origem natural, a quitosana também apresenta biocompatibilidade e segurança comprovada. A presença de grupamentos OH e NH em sua estrutura pode dar origem a ligação de hidrogênio e garantir uma boa mucoadesividade em formulações (PEPPAS e BURY 1995; ROBINSON e MLYNEK 1995). Hidrogéis de quitosana são obtidos através da interligação entre as cadeias desse biopolímero. Estes apresentam diversas aplicações na área farmacêutica para diferentes vias de administração, como via oral e aplicação de fármacos sobre a pele e mucosas (RINAUDO, 2006; BERGER et al., 2004a, BERGER et al.; 2004b). Quando aplicada em tecidos e mucosas, a quitosana é capaz de permanecer mais tempo no local em contato com o ferimento, uma vez que esta possui propriedade mucoadesiva (PERIOLI et al., 2008). O hidrogel de quitosana apresenta biocompatibilidade com a pele e mucosas, biodegradabilidade e mucoadesividade, além de formação de filme que permite uma liberação homogênea do fármaco, evitando perda do mesmo. Por sua vez, as nanocápsulas garantem uma liberação controlada e prolongada e também proteção do fármaco frente à degradação (SHAFFAZICK e GUTERRES, 2003; VALENTA e AUNER, 2004).

Na atualidade, as mais promissoras aplicações da nanotecnologia incluem o uso dos nanocarreadores para a veiculação de fármacos empregados no tratamento de diferentes tipos de câncer. A nanoencapsulação de fármacos antitumorais como a

doxorrubicina (CHEN et al. 2014), o docetaxel (RATA-AGUIAR et al., 2012), e éster dietílico metotrexato (YURGEL et al. 2014), levaram a maiores efeitos antiproliferativos quando comparados aos fármacos livres (não encapsulados) em linhagens cancerígenas de cultura de células. Apesar da importância dos estudos *in vitro*, estudos envolvendo modelos animais são necessários para confirmar a eficácia do fármaco nanoencapsulado, tendo em vista a alta complexidade dos mecanismos envolvidos (FRANK et al., 2015). Nesse sentido, BERNARDI e colaboradores (2009) demonstraram que após o tratamento de um gliobastoma em ratos que o grupo tratado com nanocápsulas de núcleo lipídico contendo indometacina apresentou um aumento na taxa de sobrevivência dos animais e uma diminuição de tamanho do tumor quando comparado com o fármaco livre na mesma dose administrada. Isso pode ser explicado devido a melhor interação das nanocápsulas com o tecido de tumoral (BERNARDI et al., 2009). Corroborando com esses resultados um recente estudo (EL-GOGARY et al. 2014) demonstrou que nanocápsulas de PLGA contendo em sua superfície ácido fólico conjugado com polietilenoglicol são capazes de interagirem de forma mais eficiente com células de câncer (HeLa) e, assim, entregarem uma maior quantidade do fármaco quercetina em camundongos. Nesse estudo os autores demonstraram que nanocápsulas com modificação na superfície estão mais localizadas no tumor do que em outros órgãos.

A modificação na superfície de nanocápsulas poliméricas vem sendo recentemente proposta para aumentar a captação das partículas pelas células e consequentemente aumentar a internalização do fármaco (EL-GOGARY et al., 2014). Diferentes ligantes podem ser conjugados com a parede polimérica das partículas e isso dependerá do objetivo pretendido. Por exemplo, Bender e colaboradores (2012) propuseram decorar a superfície de nanocápsulas de poli(ε-caprolactona) de núcleo lipídico com quitosana de baixo peso molecular após sua estabilização com polissorbato 80 e lecitina. Dessa forma, os autores observaram inversão do potencial zeta da partícula (de aproximadamente -9 mV para nanocápsulas sem quitosana para +15mV quando a quitosana foi adicionada), o que indica que a quitosana está localizada na interface das partículas, ou seja, ela reveste as nanocápsulas de núcleo lipídico externamente. Nesse mesmo trabalho os autores avaliaram a compatibilidade desse novo sistema utilizando

duas concentrações (2 e 10% v/v) em células de sangue e o sistema foi considerado promissor para administração intravenosa. Essa nova plataforma proposta pelos autores citados, é interessante para aplicação em mucosas, uma vez que a superfície positiva da partícula pode interagir com a superfície negativa de mucosas através de interação eletrostática e aumentar a adesividade das formulações.

Outra abordagem moderna dentro da área da nanotecnologia é a possibilidade da co-encapsulação de fármacos em nanocápsulas poliméricas, buscando efeitos terapêuticos sinérgicos ou combinados. Entretanto, para viabilizar esta possibilidade é necessário considerar os materiais essenciais para a formulação das nanocápsulas, um polímero biodegradável, um óleo para a constituição do núcleo das vesículas, o fármaco e os tensoativos estabilizantes da suspensão.

Considerando as características acima expostas, destaca-se a relevância e inovação dos objetivos propostos no presente trabalho, uma vez que este propõe desenvolver uma formulação associando imiquimode e óleo de copaíba através de sua co-encapsulação em nanocápsulas poliméricas de poli(ε-caprolactona), visando à avaliação dos possíveis efeitos associados em linhagens celulares de câncer cervical. Além disso, o presente trabalho tem por objetivo elucidar o mecanismo de ação do fármaco encapsulado. Outra contribuição deste trabalho é a avaliação da aplicação da formulação proposta em uma área específica, isto é, na via vaginal e estudar se há influência da carga de superfície do nanocarreador sobre as respostas consideradas.

1.3 DELINEAMENTO DO ESTUDO

Para alcançar os objetivos, a condução deste trabalho ocorrerá em quatro etapas, que são apresentadas em formato de artigos. Cada um desses artigos apresenta um objetivo específico necessário para alcançar o objetivo geral da tese. Para cada etapa e para cada objetivo a ser alcançado, utilizar-se-á um conjunto de metodologias experimentais específico. A estrutura do trabalho, com os artigos, seus objetivos e métodos, é apresentada na Figura 3.

Estudos	Objetivos	Questões de Pesquisa	Objeto de estudo	Métodos
Artigo 1 (*)	Revisar de forma abrangente artigos científicos entre os anos de 2004 a 2014 que utilizam nanocápsulas poliméricas e avaliam sua ação biológica	Qual as principais vantagens de utilização de nanocápsulas poliméricas? De que maneira as nanocápsulas poliméricas são capazes de interagir com os sistemas biológicos?	1. Aumento da estabilidade química 2. Modulação da interação com tecidos e células 3. Estudos *in vitro* e *in vivo* 4. Diminuição de efeitos adversos 5. Avaliação da segurança	Pesquisa teórica qualitativa: 1. Análise de conteúdo de artigos científicos visando contribuir de forma atualizada e abrangente com a literatura científica
Artigo 2	Proposta de uma nova formulação nanotecnológica (nanoemulsão) contendo imiquimode visando efeito citotóxico em células de câncer cervical	As nanoemulsões podem ser consideradas formulações adequadas para tratamento do câncer cervical? Qual o comportamento desse sistema em comparação com as nanocápsulas poliméricas?	1. Desenvolvimento de nanoemulsão 2. Estudo de permeação após lavablidade 3. Estudo da permeação 4. Estudo de citotoxicidade 5. Estudo de mecanismo de morte	1. Método de emulsificação 2. Lavabilidade 3. Permeação 4. Avaliação da citotoxicidade *in vitro* 5. Mecanismo de morte
Artigo 3 (*)	Proposta de uma formulação nanotecnológica adesiva (nanocápsulas poliméricas) contendo imiquimode visando efeito citotóxico em células de câncer cervical	Qual formulação nanotecnológica apresenta a melhor performance para uma possível aplicação vaginal? Qual a ação citotóxica das formulações sobre células de câncer cervical (SiHa)?	1. Desenvolvimento das nanocápsulas poliméricas adesivas 2. Estudo dos hidrogéis 3. Avaliar a adesividade 3. Estudo da permeação 4. Estudo de citotoxicidade 5. Seleção da formulação com melhor performance	1. Método de nanoprecipitação 2. Lavabilidade 3. Permeação 4. Avaliação da citotoxicidade *in vitro* 5. Comparação das diferentes formulações (gráfico radar)
Artigo 4	Investigar o mecanismo envolvido na ação citotóxica induzida pelo imiquimode nanoencapsulado com a finalidade de propor uma nova terapia no tratamento do câncer cervical	De que forma as nanocápsulas poliméricas contendo imiquode agem sobre as linhagens de células de câncer cervical ao longo do tempo? Qual o mecanismo de morte envolvido na sua ação sobre essas células?	1. Avaliar a citotoxicidade 2. Investigar o mecanismo de morte induzido nas células após o tratamento 3. Avaliar o mecanismo de morte envolvido 4. Avaliar a liberação de citocinas inflamatórias no meio extracelular	1. Citometria de Fluxo 2. Ensaio clonogênico 3. Ensaio com kit de anexina V/ iodeto de propidio; laranja de acridina 4. Ensaio com kit de ELISA
		Resultados complementares (Apêndice A e B)		
Artigo 5 (**)	Avaliar de maneira comparativa diferentes mucosas com discos de mucina em termos de adesão através da aplicação de três formas farmacêuticas (suspensão, gel e pó) a fim de propor uma adequada metodologia de análise.	Discos de mucina podem substituir o uso de mucosas em experimetnos *in vitro* a fim de avaliar sua adesividade? As formas farmacêuticas apresentam adesividade diferente frente a aplicações diversas?	1. Avaliar a mucoadesividade das formulações propostas	1. nanoprecipitação 2. spray-dryer 3. Analisador de mucoadesão (texturometro)
Artigo 6	Investigar o mecanismo envolvido na ação citotóxica induzida por óleo de copaíba nanoencapsulado	De que forma as nanocápsulas poliméricas contendo óleo de copaíba agem sobre a linhagem de células de câncer cervical? Qual o mecanismo de morte envolvido na sua ação sobre essas células?	1. Avaliar o efeito citotóxico 2. Investigar o mecanismo de morte induzido nas células após o tratamento.	1. Citometria de Fluxo 2. Ensaio com kit de anexina V/ iodeto de propidio; laranja de acridina

Figura 3 Estrutura das etapas da pesquisa desenvolvida. Status: (*) artigos já publicados no momento da defesa desta tese; (**) artigo submetido no momento da apresentação deste trabalho.

1.4 ESTRUTURA DA TESE

Esta tese está organizada em quatro capítulos principais e dois apêndices que estão apresentados na forma de artigos originais no final desta tese e sucintamente descritos a seguir.

No primeiro capítulo foram apresentados a contextualização do trabalho e os objetivos, justificando a importância desta pesquisa do ponto de vista acadêmico e prático. Este capítulo também apresenta o método de trabalho, a estrutura e as delimitações do estudo.

O capítulo dois refere-se ao primeiro artigo da tese já publicado. Trata-se de um artigo de revisão da literatura centrado no tema da presente tese.

O capítulo três traz um artigo que contemplou a incorporação de imiquimode em nanoemulsões visando propor uma nova terapia para o tratamento do câncer cervical.

O capítulo quatro compreende um artigo já publicado que versa sobre o desenvolvimento de diferentes nanocápsulas poliméricas contendo imiquimode e incorporadas em diferentes hidrogéis visando avaliar a performance das formulações considerando a via de aplicação.

O capítulo cinco correponde a avaliação aprofundada da formulação que consideramos a mais promissora para o tratamento do câncer de colo do útero em linhagens de cultura de células.

O capítulo seis apresenta uma discussão geral contemplando os resultados encontrados nesse trabalho, assim como apresenta as conclusões deste trabalho.

Além disso, a presente tese contém dois apêndices que apresentam artigos originais que relatam experimentos complementares cujos resultados contribuíram para os demais artigos desta tese. Neste sentido, o artigo do Apêndice A apresenta um estudo de mucoadesão comparando diferentes formas farmacêuticas avaliadas em diferentes superfícies e modelos, o que permitiu justificar a escolha da mucosa vaginal utilizada nos experimentos do Artigo 3 desta tese. Por sua vez, o artigo do Apêndice B apresenta resultados referentes à utilização de nanocápsulas poliméricas contendo óleo de copaíba (sem imiquimode) em culturas de células de câncer cervical (SiHa). Este artigo contribuiu para concluir que o fármaco utilizado no Artigo 4 desta tese é o maior responsável pelo efeito citotóxico na linhagem celular utilizada.

A estrutura da tese descrita acima é representada esquematicamente na Figura 4 a seguir. Nessa figura observa-se o diagrama de fluxo de desenvolvimento do trabalho

assim como os trabalhos de apoio que foram desenvolvidos e apresentados nos apêndices deste trabalho.

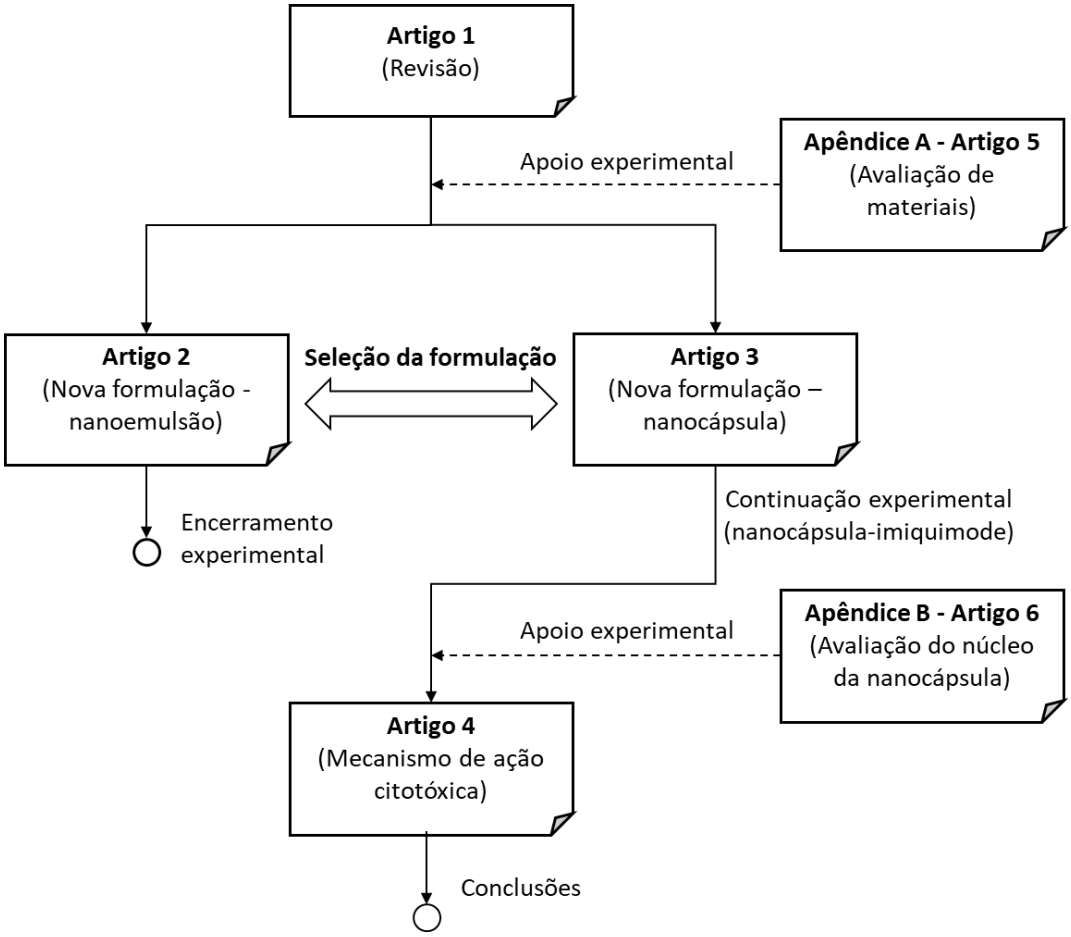

Figura 4 Representação esquemática da estrutura da tese.

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2 ARTIGO 1

2. ARTIGO 1 - Improving drug biological effects by encapsulation into polymeric nanocapsules

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Abstract

This review is based on selected reports from 2004 to 2014 and provides a comprehensive and updated overview of the state of the art related to the biological applications of polymeric nanocapsules, which are a specific type of polymeric nanoparticles used for drug delivery. Special attention is given to the advantages of using nanocapsules to increase the chemical and photostability of drugs, to modulate the interaction with cells and tissues, to reduce adverse effects of drugs, and to increase the drug efficiency and/or bioavailability. Moreover, this review covers in vitro and in vivo studies, highlighting interesting examples of drugs from several therapeutic classes for which efficacy is improved by encapsulation in different types of nanocapsules, especially in lipid-core nanocapsules. We also briefly present the first results obtained so far attesting to the safety of using polymeric nanocapsules for drug delivery.

Keywords: Polymeric nanocapsules, drug delivery, drug stability, tissue interaction, cell interaction, efficacy, adverse effects, safety.

2.1 INTRODUCTION

The first studies about polymeric nanocapsules as drug carriers were published in the 1970s by P. Speiser, J. Kreuter and P. Couvreur using, in general, poly(acrylamide) and poly(alkylcyanoacrylate) as polymers (source: Web of Science, Thomson Reuters). Early reviews exclusively dedicated to nanocapsule technology were published in 1999 and 2002[1,2]. These articles covered the methods of nanocapsule production (interfacial polymerization of a monomer and interfacial deposition of polymer), as well as some examples of the biological applications of the polymeric nanocapsules. In addition to reviewing methods for the production of polymeric nanoparticles, strategies to label nanoparticles intended for in vitro and in vivo investigations were reviewed in 2009[3]. Additionally, this interesting review presented the subsequent operations (concentration, purification, sterilization and freeze-drying) applied after the synthesis of nanoparticles. In 2010, a review updated the preparation methods other than classical nanoprecipitation used to produce polymeric nanocapsules including emulsion-diffusion, double emulsification, emulsion coacervation, polymer coating, and layer-by-layer[4]. Methodological and mechanistic aspects are discussed, allowing the establishment of criteria for selecting a method for a specific use. Furthermore, two reviews specifically focused on the use of poly(ε-caprolactone) to produce microspheres and nanospheres[5] and microcapsules and nanocapsules were also published[6]. The first of these studies, published in 2004, was dedicated to the applicability of microspheres, nanospheres and implants for sustained drug delivery based on the physico-chemical properties of this polymer, such as its very low glass temperature. The second of these two reviews, published in 2013, addressed the methods of synthesis, the mechanism of self-assembly, and some examples of biological applications of poly(ε-caprolactone) microcapsules and nanocapsules. As opposed to other reviews, this article described the development of intermediate and end products (dosage forms), the scale-up process and examples of marketed products, as well.

Since the first published paper on the subject, an exponential growth in the number of documents has been observed for the filters “nanocapsule*” and “nanoc* and

drug", showing total numbers of documents of 3,647 and 9,577, respectively. Figure 5 shows the geometric progression of the number of documents as a function of time. The compound annual growth rates for 1992-2002 and 2003-2013 are 23.3% and 44.0%, showing the dramatic growth in the last decade. In the present review, we selected papers from 2004 to 2014, taking into account that a sharp inflection of the curve of papers published on the subject per year is centered between 2003 and 2005. This review provides a comprehensive and updated overview of the state of the art related to the biological applications of polymeric nanocapsules. Special attention is given to the advantages of using nanocapsules to increase the chemical and photostability of drugs, to modulate the interaction with cells and tissues, to reduce adverse effects of drugs and to increase drug efficiency and/or bioavailability. Moreover, this review covers in vitro and in vivo studies, highlighting interesting examples of drugs from several therapeutic classes for which efficacy is improved by encapsulation in different types of nanocapsules, especially in lipid-core nanocapsules. We also briefly present the first results obtained so far attesting to the safety of using polymeric nanocapsules for drug delivery.

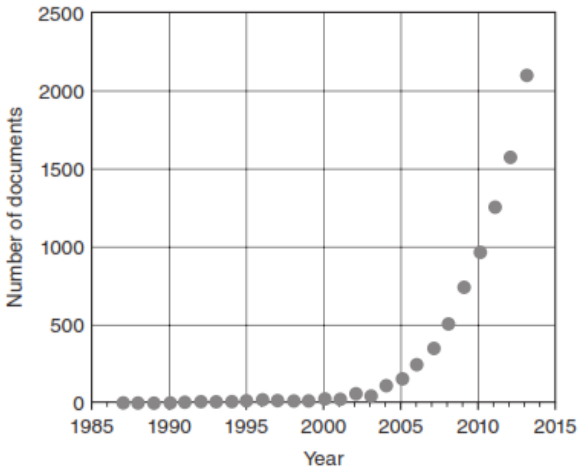

Figura 5 Number of documents per year for the filter "nano* and drug"

2.2 NANOCAPSULES COMPOSITON

In general, polymeric nanocapsules are composed of drug, polymer, oil, surfactant and water (Table 1). The polymeric nanocapsules are produced by different methods with narrow size distributions having diameters smaller than 1 μm. The submicron size distributions render to those colloids a high specific surface area. This characteristic is strongly correlated to their biological responses. For this reason, nanocapsules have been used as substrates for interfacial reaction to have their surface modified. Examples of those strategies providing chemical specificity for molecular recognition[7] or improving physical stability in biological media[8] have been recently reported. The nanocapsules are usually produced in aqueous media as a turbid colloidal solution. This opalescent solution can be used as produced (end product) or it can be further incorporated into gels[9-15] or converted into powders[16] to facilitate their applications and to increase the physical stability of the product.

The polymer forming the nanocapsule wall, which has a great influence in the control of drug release[17], is frequently a biodegradable material. The internal phase is generally composed of oily components or, rarely, of an aqueous core. The oily core can be formed by a liquid lipid, by a mixture of liquid lipids, or by a mixture of liquid lipid and solid lipid (organogel). The latter leads to a specific type of nanocapsules called lipid-core nanocapsules, the core viscosity/consistency of which modulates *in vitro* release of drugs[18]. Hence, the lipid-core nanocapsules have two diffusional barriers for drug release: the polymer wall and the organogel core. Moreover, whatever type of nanocapsule is used, it is important to use a liquid lipid as a non-solvent for the water-insoluble polymer to ensure that the polymer is located at the nanocapsule-water interface[19]. Among the polymers used in the production of nanocapsules, the most frequently used are poly(ε-caprolactone) (PCL), poly(lactide) (PLA) and poly(lactide-*co*-glycolide) (PLGA) (Table 1). The drug is encapsulated in the internal phase (dissolved or dispersed), entrapped in the polymeric wall, and distributed among the pseudo-phases of the system. If the colloidal phase is saturated, the drug might form nanocrystals, which grow and precipitate[6]. In other words, the presence of

nanostructures other than nanocapsules, such as drug nanocrystals, nanoemulsion and nanospheres, should be investigated in pre-formulation studies[20]. Nanostructures other than nanocapsules present in a drug-loaded nanocapsule formulation are considered contaminants that must be avoided.

Six different methods for the production of nanocapsules have been reported: interfacial deposition of preformed polymer (also known as nanoprecipitation, solvent displacement and self-assembling), emulsion–diffusion, double emulsification, emulsion-coacervation, polymer-coating and layer-by-layer[4]. The nanoprecipitation method is the most frequently used, most likely due to its reproducibility and easy handling (Table 1). The physico-chemical and biological properties of polymeric nanocapsules are dependent on both the method of preparation and the raw materials used to produce them. The most important physico-chemical properties to be analyzed include particle sizing and polydispersity, zeta potential, drug loading, and drug release profile.

Tabela 1 Composition and preparation technique of polymeric nanocapsules (cited according to the original papers)

Preparation technique	Active substance	NC core	NC shell	Surfactant/ stabilizer	Reference
Interfacial deposition	Not present	CCT, Sorbitan monostearate	PCL-Rhodamine B	Polysorbate 80	Brum et al. (2015)
Nanoprecipitation	IR-780 cyanine dye	Coconut oil	PLA or PCL	Cremophor® EL	Bazylinska et al. (2014)
Self-assembly and interfacial reactions	Not present	CCT, Sorbitan monostearate	PCL-Chitosan-Zn, PCL-Chitosan-Fe	Polysorbate 80, Soybean phosphatidylcholine	Bender et al. (2014)
Interfacial deposition	Not present	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Bulcão et al. (2014)
UV-induced thiol–ene interfacial cross-linking in transparent miniemulsions	Doxorubicin	Not present (hollow)	Allyl-functionalized cationic polylactide	Not present	Chen et al. (2014)
Interfacial deposition	Capsaicin	CCT	Eudragit® RS100	Polysorbate 80	Contri et al. (2014)
Interfacial deposition	Capsaicin	CCT	Eudragit® RS100	Polysorbate 80	Contri et al. (2014)
Self-assembling	Olanzapine	Medium chain triglycerides, Sorbitan monostearate	PCL	Polysorbate 80	Dimer et al. (2014)
Nanoprecipitation	Quercetin	Castor oil	PLGA- Folic acid	Soybean lecithin, Tween® 80	El-Gogary et al. (2014)
Interfacial deposition	Nile red dye	CCT, Sorbitan monostearate	Eudragit® RS100/ Eudragit® S100	Polysorbate 80	Frank et al. (2014)
Self-assembling by solvent displacement	Tacrolimus	CCT, Span® 60	PCL	Polysorbate 80	Friedrich et al. (2014)
Self-assembling	Pyrimethamine	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Pissinate et al. (2014)
Interfacial deposition	Clobetasol propionate	Rice bran, soybean or sunflower seed oils, Sorbitan monostearate	PCL	Polysorbate 80	Rigo et al. (2014)
Miniemulsion/polyaddition polymerization	Silver	Not present (hollow)	Potato starch- AgNP	Lutensol® AT50	Taheri et al. (2014)

Tabela 1 Composition and preparation technique of polymeric nanocapsules (continuation)

Preparation technique	Active substance	NC core	NC shell	Surfactant/ stabilizer	Reference
Self-assembling	Methotrexate diethyl ester	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Yurgen et al. (2014)
Nanoprecipitation	Vitamin D_3	Miglyol® 829	PLA	Montanox® VG 80	Almouazen et al. (2013)
Interfacial deposition	Vitamin K_1	CCT, Sorbitan monostearate	PCL	Polysorbate 80	da Silva et al. (2013)
Interfacial deposition	Ketoprofen	Medium chain triglycerides, Span® 80	PCL	Tween® 80	da Silveira et al. (2013)
Interfacial deposition	Chloroaluminium phthalocyanine	Miglyol 810N	PLA or PLGA	Poloxamer 188 and Epikuron 170®	de Paula et al. (2013)
Inverse miniemulsion	Dexamethasone	Water	Hydroxyethyl starch	Poly((ethyleneco-butylene)-b-(ethylene oxide)), P(E/B-b-EO)	Fichter et al. (2013)
Layer-by-layer on silica templates	Curcumin	Not present (hollow)	Chitosan, poly(allylamine hydrochloride), and poly(sodium 4styrenesulfonate)	Not present	Goethals et al. (2013)
Layer-by-layer on silica templates	Bovine serum albumin	Not present (hollow)	Tertiary amine and hydrazide grafted polyaspartamide and Carboxyl and aldehyde grafted polyaspartamide	Not present	Gu et al. (2013)
Not mentioned	Recombinant human bone morphogenetic protein-2	Not present	Chitosan	Not mentioned	Lai et al. (2013)

Tabela 1 Composition and preparation technique of polymeric nanocapsules (continuation)

Preparation technique	Active substance	NC core	NC shell	Surfactant/ stabilizer	Reference
Solvent displacement or emulsification solvent diffusion	3,4-Dihydro-12-hydroxy-2,2-dimethyl-2H,6Hpyrano[3,2-b]xanthen-6-one	Miglyol® 840	PLGA	Pluronic® F-68, Tween® 80, Span® 80, PVA	Paiva et al. (2013)
Emulsion-diffusion	Cyclosporine	SEDDS core-forming mixture (oil and surfactant)	Poly-dl-lactide (PDLLA)	SEDDS core-forming mixture (oil and surfactant)	Park et al. (2013)
Solvent displacement	DiD dye or Docetaxel	Miglyol® 812	Epikuron® 170	Poloxamer 188 and PASN	Rivera-Rodriguez et al. (2013)
Nanoprecipitation	Nile red dye or Retinoic acid	Miglyol® 829	PLA or PCL	Montanox® VG 80	Almouazen et al. (2012)
Inverse miniemulsion	Not present	Water	Hydroxyethyl starch or Hydroxyethyl starch - Folic acid	Poly((ethyleneco-butylene)-b-(ethylene oxide)), P(E/B-b-EO)	Baier et al. (2012)
Interfacial deposition	Not present	CCT, Sorbitan monostearate	PCL or PCL – Chitosan	Polysorbate 80, lecithin	Bender et al. (2012)
Interfacial deposition	Haloperidol	Fish oil, Sorbitan monostearate	PCL	Polysorbate 80	Benvegnú et al. (2012)
Interfacial deposition	Not present	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Bulcão et al. (2012)
Nanoprecipitation	Maghemite and selol	Not mentioned	PLGA	Not mentioned	Falqueiro et al. (2012)
Nanoprecipitation	15-deoxy-D12,14-PG J2 (15d-PGJ2)	CCT, Sorbitan monostearate	PLGA	Polysorbate 80	Napimoga et al. (2012)
Double emulsion	Gemcitabine	Water	PLA	DSPE-mPEG2000, Span® 80	Paolino et al. (2012)
Interfacial deposition	Nile red dye or docetaxel	Olive oil	Chitosan - Folate	Epikuron 145V	Rata-Aguilar et al. (2012)

Tabela 1 Composition and preparation technique of polymeric nanocapsules (continuation)

Preparation technique	**Active substance**	**NC core**	**NC shell**	**Surfactant/ stabilizer**	**Reference**
Interfacial deposition	Grandisin	Soybean oil	PLGA	Pluronic F68, Pluronic F127, Phosphatidylcholine	Stecanella et al. (2012)
Interfacial deposition	Baicalein or Benzophenone3	Retinyl palmitate and Span® 60	PLA	Pluronic F68 or Tween® 80	Teixeira et al. (2012)
Interfacial deposition	Haloperidol	Not mentioned	PCL	Not mentioned	Benvegnú et al. (2011)
Nanoprecipitation	Resveratrol	Medium chain triglycerides, Sorbitan monostearate	PCL	Polysorbate 80	Detoni et al. (2012)
Ionotropic gelation	Plasmid DNA	x	Chitosan	x	Gaspar et al. (2011)
Multiple-step process, involving coacervation and O/W emulsification	L-glutamine derivatives	Water	Chitosan and sodium alginate	Polysorbate 80	Grebinisan et al. (2011)
Interfacial deposition	Lipoic acid, Melatonin	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Kullkamp et al. (2011)
Interfacial deposition	Tretinoin	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Marchiori et al. (2011)
Interfacial deposition	Tretinoin	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Ourique et al. (2011)
Solvent displacement	Benzocaine	Mineral oil, isopropyl myristate or Cetiol® V, Span 60	PLGA	Polysorbate 80	Silva de Mello et al. (2011)
Interfacial polymerization	N-(3-aminopropyl) methacrylamide hydrochloride	Water	N-(3-aminopropyl) methacrylamide hydrochloride	Not present	Zhao et al. (2011)

Tabela 1 Composition and preparation technique of polymeric nanocapsules (continuation)

Preparation technique	Active substance	NC core	NC shell	Surfactant/ stabilizer	Reference
Interfacial deposition	Rutin	Grape seed oil, Sorbitan monostearate	PCL	Polysorbate 80	Almeida et al. (2010)
Interfacial deposition	Indomethacin and indomethacin ethyl ester	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Cattani et al. (2010)
Interfacial deposition	Clobetasol propionate	CCT, Sorbitan monostearate	PLA, PLGA 50:50, or PLGA 85:15	Polysorbate 80	Fontana et al. (2010)
Interfacial deposition	Meloxicam	Miglyol 810®, Sorbitan monostearate	PCL	Polysorbate 80	Ianiski et al. (2010)
Interfacial deposition	Curcumin	Castor oil	PLA	Lecithin and Pluronic F 68 or Solutol HS 15	Mazzarino et al. (2010)
Nanoprecipitation	Bupivacaine	CCT, Sorbitan monostearate	PLGA	Polysorbate 80	Moraes et al. (2010)
Interfacial deposition	Tretinoin	CCT, Sorbitan monooleate or Sorbitan monostearate	PCL	Polysorbate 80	Ourique et al. (2010)
Interfacial polymerization	Cyanine IR-768	Iso-propyl myristate, propylene glycol, ethyl oleate, oleic acid, iso-octane	Poly(n-butyl cyanoacrylate)	Tween® 80 or Brij 96	Pietkiewicz et al. (2010)
Interfacial deposition	Retinyl palmitate	Retinyl palmitate, Span® 60F	PLA	Pluronic F68, Tween® 80, Tinogard Q®	Teixeira et al. (2010)
Interfacial deposition	Indomethacin	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Bernardi et al. (2009)
Interfacial deposition	Indomethacin	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Bernardi et al. (2009)
Interfacial deposition	Clobetasol propionate	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Fontana et al. (2009)

Tabela 1 Composition and preparation technique of polymeric nanocapsules (continuation)

Preparation technique	**Active substance**	**NC core**	**NC shell**	**Surfactant/ stabilizer**	**Reference**
Interfacial deposition	Quinine	Miglyol 810®	PCL	Polysorbate 80 and Epikuron® 170	Haas et al. (2009)
Interfacial deposition	Lipoic acid	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Kullkamp et al. (2009)
Interfacial deposition	Benzophenone -3	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Paese et al. (2009)
Interfacial deposition	Indomethacin and indomethacin ethyl ester	CCT, Sorbitan monostearate	PCL	Polysorbate 80	Bernardi et al. (2008)
Interfacial deposition	Tretinoin	CCT	PCL	Tween® 80, Span® 80	Fachinetto et al. (2008)
Interfacial deposition	Melatonin	CCT	Eudragit® S100	Polysorbate 80, , Span® 80	Schaffazick et al. (2008)
Multiple emulsion	Insulin	x	PCL and Eudragit® RS 100 (blend)	PVA	Damgé et al. (2007)
Solvent displacement	Octyl methoxycinnamate	Octyl methoxycinnamate	PCL	Polysorbate 85	Alvarez-Roman et al. (2004)

2.3 ADVANTAGES OF POLYMERIC NANOCAPSULES FOR DRUG DELIVERY

Several studies have evaluated the administration of nanocapsules by different routes, namely oral, ocular, cutaneous, vaginal, and parenteral, demonstrating the versatility of the systems. Many benefits are achieved by encapsulating drugs in polymeric nanocapsules. The main advantages are increased drug stability due to protection against photo- or chemical degradation, increased interaction with tissues and cells, drug targeting and, consequently, increased efficacy and decreased drug adverse effects. These advantages are separately discussed in this section.

2.3.1 Increasing chemical stability and/or photostability

The nanoencapsulation of substances has been described as a way to improve drug chemical stability[21,22]. For instance, nanocapsules composed of PCL led to an increase of lipoic acid stability against oxidation when protected from heat and light compared to formulations containing the non-encapsulated drug[21]. Similarly, nanocapsules composed of PLA (with Pluronic F68 as surfactant) prevented the chemical hydrolysis of curcumin at pH 5, while at pH 7.4 approximately 30% of the nanoencapsulated drug degraded[22]. The nanoencapsulation of tretinoin into lipid-core nanocapsules increased its stability under storage for 4 months compared to conventional polymeric nanocapsules[23].

UVA light is one of the main causes of the photodegradation of topically applied drugs. Several articles report the use of nanoencapsulation to improve the photostability of drugs[10,11,16,22-26]. Different supramolecular structures (liposomes, polymeric lipid-core nanocapsules, nanospheres and solid lipid nanoparticles) encapsulating *E*-resveratrol were compared to determine their ability to increase the photostability of the drug[26]. The lipid-core nanocapsules provided the best *E*-resveratrol photoprotection, reducing its photoisomerization. This protection is due to the ability of nanocarriers to efficiently scatter UVA light[10].

The influence of the type of nanocarrier on the photostability of clobetasol propionate[24] and rutin were also described[27]. Different formulations containing clobetasol propionate (lipid-core nanocapsules, nanospheres and nanoemulsions) were exposed to UVA radiation. All nanostructures increased the photostability of the substance, and the greatest effect was observed for lipid-core nanocapsules, showing the importance of both oil and polymer (PCL) in the composition[24] (Figure 6). However, when comparing nanocapsules with nanoemulsion, encapsulation into PCL-nanocapsules did not significantly increase rutin photostability[27]. Furthermore, the use of amorphous polymers (PLA and PLGA) in the production of nanocapsules to the increase photostability has been proposed[25]. It was concluded that the observed photoprotection is due to the polymer in the nanocapsule interface and not due to the crystallinity of the polymer used because similar profiles were obtained regardless of the polymer crystallinity.

Tretinoin, a retinoid used in the topical treatment of acne, psoriasis and aging skin, is a well-known photoinstable substance. The encapsulation of tretinoin in polymeric nanocapsules, in lipid-core nanocapsules or in spray-dried nanocapsules decreased its photodegradation under UVA and/or UVC lights[11,16,23]. Furthermore, tretinoin-loaded nanocapsules incorporated into a Carbopol® Ultrez 10 NF hydrogel led to even lower photodegradation after exposure to UVA light when compared to the marketed non-encapsulated tretinoin hydrogel[11]. In a similar way, an increase in the photoprotection of benzophenone-3 under UVA light has been shown for a hydrogel sunscreen nanoformulation prepared with Carbopol 940® compared to the hydrogel containing this substance in its non-encapsulated form[10].

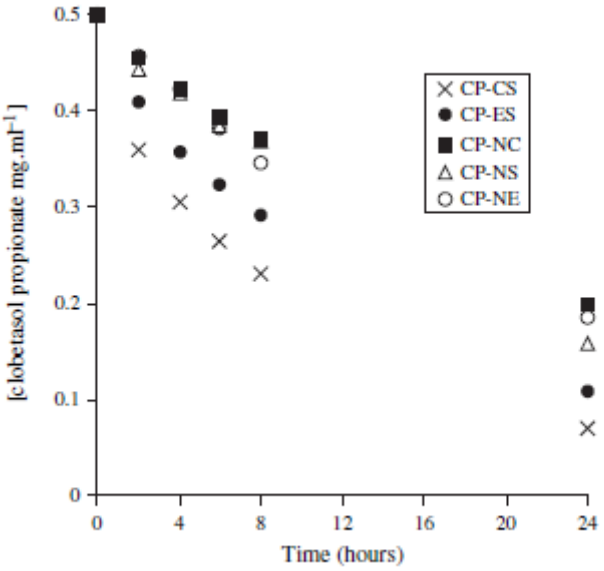

Figura 6 Photostability of clobetasol propionate, encapsulated into different nanostructures, under UVA light (CS = commercial solution, ES= ethanol solution, NC= nanocapsules, NS= nanoshperes, NE= nanoemulsion) (Fontana, 2009, Journal of Biomedical Nanotechnology)

2.3.2 Modulating the interaction with tissues and cells

Nanocapsules can increase substance uptake by cells, as shown for the delivery of doxorubicin into breast cancer cells[28], for dexamethasone into phagocytic liver cells[29] and for quinine into erythrocytes[30]. In the delivery of curcumin into glioma cells, early time points evaluated showed higher uptake for the non-encapsulated drug, whereas for late time points (48 h or more), uptake was increased by nanoencapsulation[31]. Nanocapsules are usually taken up together with the encapsulated substance into the cytosol, in the endosomal compartment or in the micronucleus[29,32-34]. PLA-nanocapsules were described to remain inside cells for a period of at least 7 days without complete degradation[34]. After internalization, the drug is released by simple diffusion, by polymer enzymatic degradation[29], or by redox-responsive degradation of the polymer[33]. The interaction of nanocapsules and cells has been frequently described, but few authors studied the mechanism of cellular uptake. The presence of ligands on the nanocapsule surface, such as folic acid, increases particles uptake by carcinogenic cell lines, which over-express the ligand receptor compared to normal cells[35-37]. Indeed, the chemical

bonding of a ligand to the polymeric wall is the standard method to achieve drug active targeting. In addition to the cellular targeting, a new strategy to provide molecular recognition by ligand-protein interactions has recently been described[7]. The surface of lipid-core nanocapsules was modified by a scFv-anti-LDL(-)-metal-chitosan-lecithin complex to successfully recognize LDL(-), which plays a key-role in atherosclerosis.

Moreover, nanocapsules may also increase the interaction between drug and tissues, leading frequently to an increased drug penetration or to a modified drug biodistribution. Nanocapsules containing indomethacin were intraperitoneally administered to glioma-implanted rats[38] and to Aβ1-42-exposed rats (Alzheimer disease model)[39]. In both works, a higher concentration of the drug was observed in the cerebral tissue compared to the administration of a drug solution. Furthermore, after intraperitoneal administration, the ability of ketoprofen-loaded polymeric nanocapsules to pass the blood brain barrier by a passive transport was observed after *in vivo* treatment of glioblastoma in rats[40]. In addition, an increase in the gemcitabine accumulation into a tumor tissue by an enhanced permeability and retention (EPR) effect was observed after subcutaneous administration of biocompatible nanocapsules[41]. An increase in the interaction of indomethacin ethyl ester (ex vivo) or insulin (in vivo after oral administration) with the gastrointestinal tract due to nanoencapsulation in lipid-core nanocapsules or in Eudragit® RS 100 nanocapsules was also described[42,43]. The lipid-core nanocapsules acted as mucoadhesive drug reservoirs, releasing the drug in a controlled manner[43]. In addition, the cationic surface of Eudragit® RS 100 nanocapsules played an important role in increasing interaction with the negatively charged tissue[42]. More recently, a chitosan hydrogel containing (Eudragit® RS 100 or Eudragit® S 100) nanocapsules increased the in vitro penetration of Nile red, used as an encapsulated fluorescent marker, into a vaginal mucosa tissue compared to the chitosan gel containing the non-encapsulated substance, especially when the nanocapsules presented a cationic surface charge[11] (Figure 7). Regarding cutaneous administration, an increase in the interaction between capsaicin and the skin was induced by the nanoencapsulation into Eudragit® RS 100 nanocapsules, decreasing the amount of drug washed away during

washability experiments. The permanence of the drug in contact with the skin for a longer time led to higher skin penetration into the deeper skin layers[9].

In the development of dermatological products, the localization of the drug in different skin layers is of great importance. Several authors have studied the influence of nanoencapsulation in the drug skin penetration or permeation. An increase in the amount of lipophilic substances, such as ethylhexyl methoxycinnamate[44], vitamin-K1[45] and resveratrol[26], found in the *stratum corneum* layers compared to the non-encapsulated form, is frequently observed, followed by a decrease of the substances in the dermis or receptor medium[11,14,26,45]. The high specific surface area of the nanocapsules allows their interaction with structures such as hair follicles, where the drug is released to penetrate the skin, explaining the observed increased presence[44]. The ability of nanocapsules to penetrate skin layers depends on the unique properties of each carrier, as described for the flexible polymeric nanocapsules composed of a PLA wall, which are able to penetrate into viable skin layers[46], and for the lipid-core nanocapsules that remain in the outer skin layer due to rigidity[47].

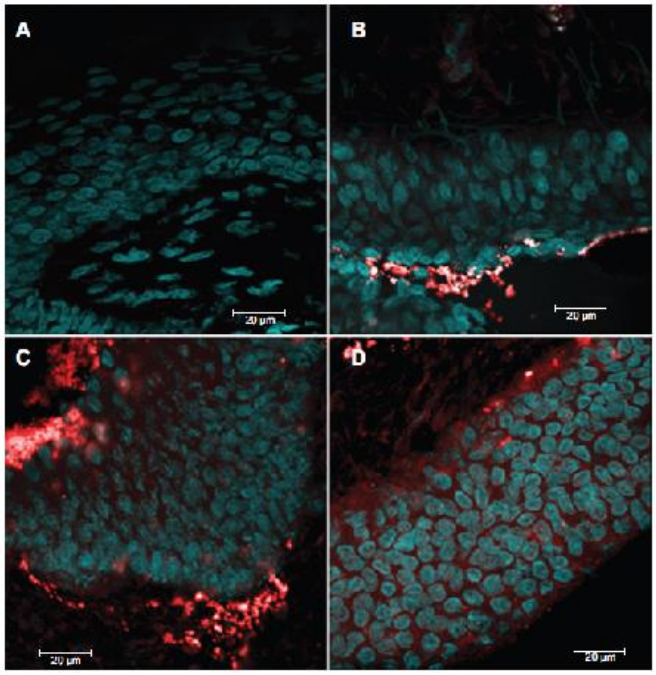

Figura 7 Vaginal mucosae after contact with nanoformulations (A: control of plain mucosae, B: control of free nile red formulation, C: nanoformulation composed of cationic nanocapsules, D: nanoformulation composed of anionic nanocapsules) (Frank et al 2014, International Journal of Nanomedicine).

2.3.3 Increasing efficacy and/or bioavailability

Studies showing increased efficacy and/or bioavailability due to the nanoencapsulation of drugs are discussed in two sections, covering the *in vitro* and the *in vivo* studies. The main focus is the use of polymeric nanocapsules to increase the efficacy of antitumor drugs. Other examples are also described, including the use of polymeric nanocapsules to improve efficacy or bioavailability of antioxidant substances, as well as antipsychotic, anti-inflammatory, antidiabetic, and antimalarial drugs.

In vitro efficacy studies

Although *in vitro* methods present limitations, these studies are important tools to predict the efficacy of substances prior to performing in vivo experiments. In vitro assays have been applied to evaluate the effects of nanoencapsulation of antiproliferative drugs[23,28,31,35,40,41,48-52], antioxidant substances[27,53], and anti-inflammatory drugs[29] on photoactivity[54], phototoxicity[55] and photoprotection[15].

The most common in vitro assay related to nanocapsule efficacy is the antiproliferative effect using cancer cell lines, including glioma cells[31,40,48], breast cancer cells[28,50,52], prostate cancer cells[28], embryonal renal cancer cells[41], leukemia cells[23] and colon adenocarcinoma cells[35]. Non-cancer cells have also been used[49]. The cytotoxicity effect of the substance is frequently measured by assessment of cellular viability (MTT test), by neutral red assay, by cell counting, by nitroblue tetrazolium reduction assay or by propidium iodide assay, which indicates loss of membrane integrity.

Nanoencapsulation of indomethacin[48], gemcitabine[1], vitamin D3[50], ketoprofen[40], doxorubicin[28], methotrexate diethyl ester[52], docetaxel[35], curcumin[31], grandisin[49] and a novel dihydropyranoxanthone[51] led to higher antiproliferative effects compared to the non-encapsulated drug controls. However, the in vitro antiproliferative studies are hardly comparable because different protocols have been used. The effect of gemtabicin-loaded nanocapsules in embryonal renal cancer HEK293 cells was higher than that of the non-encapsulated drug for different concentrations and at different time

intervals studied[41]. In contrast, a greater in vitro antiproliferative effect was observed for the ketoprofen-loaded nanocapsules in a glioblastoma cell line exclusively for the higher concentrations of the drug[40]. Furthermore, an increase in the antineoplastic effect in a resistant breast cancer cell line was observed for methotrexate diethyl ester-loaded lipid-core nanocapsules[52]. In general, lower cellular viability, indicating a greater antiproliferative effect, is a result of a greater interaction of the nanoparticles with the cells and subsequent cellular uptake of the drug. Interestingly, tretinoin-loaded lipid-core nanocapsules did not increase drug efficacy, but prolonged the antiproliferative effect in leukemia cells, highlighting the role of the reservoir property of nanocapsules[23].

Cell cultures have also been used for the analysis of the anti-inflammatory effects of nanoencapsulated drugs. The anti-inflammatory effect of dexamethasone-loaded nanocapsules has been tested with phagocytic cells, analyzing cytokine secretion (IL-6 and TNF-α) after stimulation with lipopolysaccharides[29]. It was observed that these dexamethasone-loaded nanocapsules caused higher suppression of those cytokines than the non-encapsulated drug. In addition, the protective effects of encapsulated indomethacin[39] and resveratrol[56] in neuroinflammation have been evaluated using organotypic hippocampal cultures stimulated by amyloid β peptide. The resveratrol-loaded lipid-core nanocapsules were more efficient than the resveratrol solution, leading to lower ROS formation and higher IL-10 release, while the indomethacin-loaded lipid core nanocapsules were capable of blocking neuroinflammation.

Moreover, the efficacies of nanoencapsulated antimicrobial drugs have being tested in vitro and ex vivo with cultures of microorganisms. Potato starch nanocapsules covered with silver nanoparticles showed efficacy in bacteria cultures of medical interest (*Staphylococcus epidermidis* and *Escherichia coli*)[57]. Chlorhexidine-loaded nanocapsules were incorporated in gel, and its effect against *Staphylococcus epidermidis* was evaluated ex vivo using human skin. A more sustained effect was observed for the gel containing nanocapsules compared to a gel prepared without nanocapsules[14].

The *in vitro* efficacy studies can also be conducted without any living organism. The antioxidant activity of rutin-loaded PCL-nanocapsules was studied[27] using an approach based on the formation of free radical OH^-. The drug-loaded nanocapsules

presented higher antioxidant activity compared either to its non-encapsulated form or to a nanoemulsion formulation, showing the role of the polymeric wall surrounding the oil droplets. Similarly, the antioxidant activity of lipoic acid, evaluated by the lipoperoxidation method induced by ascorbyl free radicals and liposomes as substrate, was improved by its nanoencapsulation[53] (Figure 8). A very interesting property of the lipid-core nanocapsules is the ability to scatter UV light[15], which was demonstrated by the nanoencapsulation of a UV sunscreen, benzophenone-3. Nanoencapsulation provided a delay in sunscreen degradation under UVA radiation, most likely due to light being scattered by the lipid-core nanocapsules. Furthermore, this photoprotection indicated that benzophenone-3-loaded nanocapsules act as a hybrid chemical-physical sunscreen.

Polymeric nanocapsules have also been studied *in vitro* for photodynamic therapy (a new treatment modality for cancer). The photoactivity of nanoencapsulated polymethine cyanine dye was studied by photobleaching and ROS detection analyzing the photooxidation of human serum albumin[54]. Nanocapsulation reduced cyanine photobleaching and increased the oxidation of albumin, enhancing the photodynamic effect of the substance. Previously, chloroaluminium phthalocyanine encapsulated in PLA-nanospheres, PLGA-nanocapsules, PLA-nanocapsules and PLA-PEG-nanocapsules[55] were shown to be more efficient than the free photosensitizer at inducing human fibroblast death after an irradiation dose of $3J/cm^2$. The interaction of the polymeric nanoparticles with the cells might explain the results.

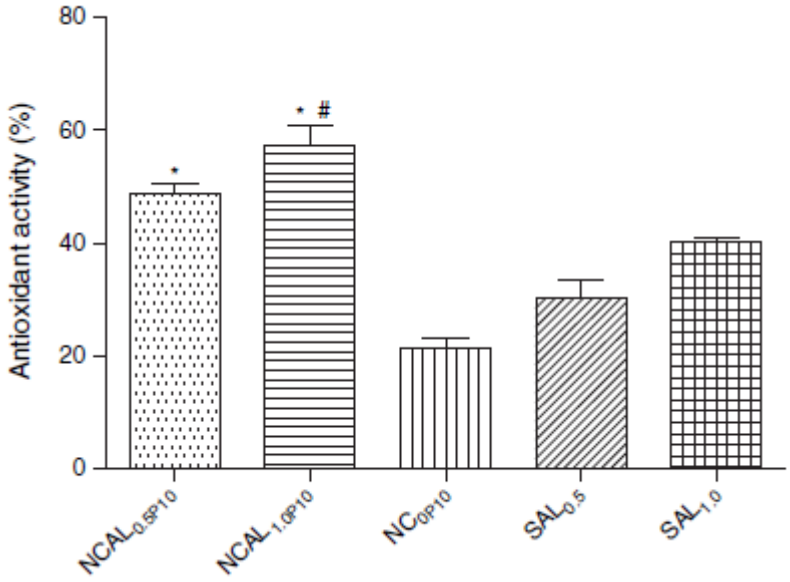

Figura 9 In vitro antioxidant activity of nanoencapsulated lipoic acid (NCAL: lipoic acid-loade, SAL: control drug solution) (Kullkamp et al, 2011, Journal of Biomedical Nanotechnology).

2.3.4 In vivo efficacy/bioavailability studies

Despite the importance of *in vitro* studies, *in vivo* experiments are still necessary to confirm efficacy of nanoencapsulated drugs as well as the advantages of nanoencapsulation, taking into account the high complexity of the mechanisms involved. The majority of in vivo studies are performed with animal models. However, the well-known *in vivo Allium cepa* root-tip cell test has been applied to assess the antiproliferative activity of tretinoin-loaded nanocapsules[58]. In this study, the bulbs were analyzed regarding the mitotic index after the administration of tretinoin-loaded nanocapsules. Nanoencapsulation improved the drug's antiproliferative effect compared to the non-encapsulated drug solution because a lower mitotic index was found, without inducing an increase in the frequency of chromosome alterations in onion root-tip cells.

Several authors have described *in vivo* antiproliferative activity of polymeric drug-loaded nanocapsules using tumor mouse models[31,38,40,41,59]. Tumor cells were implanted in animals to induce glioma[31,38,40], renal tumor[41] and Ehrlich ascetic tumors[59]. The antitumor efficacy can be assessed by tumor volume quantification, pathological analysis and immunohistochemical staining, as well as by body weight, behavior and

motor activity. The nanoencapsulation of ketoprofen[40], indomethacin[38] and curcumin[31] led to higher antiproliferative effects when compared to the drugs administered in the non-encapsulated forms at the same dose. An increase in the survival rate of rats accompanied by a decrease in glioblastoma size was observed after treatment with indomethacin-loaded lipid-core nanocapsules[38] (Figure 9). On the other hand, the antitumor effects of gemtabicin-loaded nanocapsules[41] and a synthesized acyl derivative-loaded nanocapsules[59] were similar to the control formulations of the non-encapsulated drugs using immunodeficient female mice bearing tumors caused by the injection of human embryonic kidney cells and mice with Ehrlich ascetic tumors, respectively. However, other advantages of the nanoformulations were demonstrated, such as the possibility to reduce the dose 10-fold[41] or to obtain a gradual release of the substance[59]. Enhanced interaction of the nanocapsules with tumor tissue is noted as the reason for the increased effect of the nanoformulations[38]. In an *in vivo* uptake study in mice, it was confirmed that nanocapsules presenting folate on their surface are tumor-targeted, leading to higher localization of nanocapsules being observed in the tumor than in other organs[37] (Figure 10).

In addition to antiproliferative efficacy, other properties such as antioxidant, anti-inflammatory, anesthetic and osteogenic activities can also be modulated by drugs incorporation into polymeric nanocapsules. After a single injection of melatonin-loaded Eudragit® S 100 nanocapsules, a reduction in lipid peroxidation in mouse brain and liver tissues was observed, showing dose- and time-dependent antioxidant effects when compared to the drug solution[60]. Regarding acute and chronic inflammation models in rats, indomethacin-loaded lipid-core nanocapsules were compared to the non-encapsulated drug solution[61]. In an acute model, the formulations did not differ when measuring the inhibition of edema induced by carrageenan. However, for the chronic edema model, the drug-loaded lipid-core nanocapsule formulation was more effective than the non-encapsulated drug. A possible explanation could be sustained release to the local site of inflammation by the nanocapsules. It was also observed that only after administration of the nanoformulation, the pro-inflammatory cytokine levels (IL-6 and TNF-α) were decreased while the IL-10 levels were increased[61]. More recently, PLGA-

nanocapsules containing 15d-PGJ_2 (a cyclopentenone-type PG) were studied using a periodontitis mouse model. This formulation decreased bone resorption and inflammatory responses, supporting its immunomodulatory effects[62]. Different benzocaine-loaded nanocapsules were studied, aiming to improve the anesthetic effect of the substance[63,64]. An improvement in the intensity and duration of analgesia (sciatic nerve blockade model in rats) was verified for PLA-nanocapsules containing mineral oil as a core compared to those containing *iso*-propyl myristate and Cetiol V® as cores[63]. Subsequently, PLGA, PLA and PCL were compared as polymeric walls of nanocapsules, showing that the benzocaine encapsulation in PLA-nanocapsules enhanced its anesthetic effect[64].

Moreover, the treatment of protozoa using polymeric nanocapsules has also been described[30,65]. Quinine-loaded PCL-nanocapsules, developed for antimalarial treatment, increased the survival of *Plasmodium berghei* infected Wistar rats with a reduction of approximately 30% in the effective dose[30]. Similarly, pyrimethamine-loaded lipid-core nanocapsules, developed to treat tachyzoites of *Toxoplasma gondii* infected mice, increased the survival rate of infected mice and reduced the effective dose, indicating a likely reduction in the adverse effects of the drug[65]. The increase in drug bioavailability is noted as the reason for the increased efficacy of pyrimethamine against toxoplasmosis.

The influence of the nanoencapsulation on the effects of drugs acting in the central nervous system, such as haloperidol[66] and olanzapine[67], has recently been studied. Although the drugs studied belong to different antipsychotic classes, the authors observed an increase in the efficacy of the drugs incorporated into PCL-nanocapsules[66] and lipid-core nanocapsules[67] compared to the free drugs using the stereotyped behavior model induced by D,L-amphetamine to test antipsychotic efficacy. The authors also observed a prolonged effect due to nanoencapsulation.

Nanocapsules containing meloxicam[13], indomethacin[39] and curcumin[68] were proposed as possible treatments for Alzheimer disease, testing the formulations against the damage caused by amyloid β peptide in mice. In the first study, the nanocapsules, after gavage administration, had a positive effect against memory and learning impairments, preventing cerebral alterations including oxidative status and attenuating

neuronal loss. On the contrary, non-encapsulated meloxicam did not act against the brain injury caused by the peptide[13]. Similarly, after intraperitoneal administration, indomethacin-loaded lipid-core nanocapsules increased the neuroprotective effect of the drug compared to the effect of free drug solution[39], while curcumin-loaded lipid-core nanocapsules allowed a 20-fold dose reduction[68].

Regarding oral administration, polymeric nanocapsules can help to overcome the gastrointestinal tract barrier, which limits the absorption of some drugs/substances. For instance, besides the improvement of drug delivery across the oral barrier, which provided a dose reduction of 40%, tacrolimus-loaded lipid-core nanocapsules showed a similar percentage of lymphocyte reduction after oral and after intraperitoneal (i.p.) administrations[69]. Additionally, insulin, which is used as an antidiabetic substance, has been successfully encapsulated into nanocapsules formed by a blend of PCL and Eudragit® RS100[42]. A prolonged in vivo hypoglycemic effect of the drug was observed for 12 h after subcutaneous injection. After oral administration, the insulin-loaded nanocapsules were absorbed and a glycemic reduction was observed, while this effect was not observed for the free drug.

The increased efficacy of drug-loaded polymeric nanocapsules is frequently attributed to a higher bioavailability or to the maintenance of blood levels for more prolonged times, but few reports have studied the pharmacokinetic profiles of these formulations. The nanoencapsulation of insulin into nanocapsules[42] composed of a blend of PCL and Eudragit® RS 100 and the nanoencapsulation of gemcitabine into PLA-nanocapsules[41] led to an increase in the area under the curve of the pharmacokinetic profiles, data which are related to an increase in biological activity or with the possibility of dose reduction. Cyclosporine-loaded nanocapsules showed increased drug bioavailability after oral administration, with the maintenance of drug levels for at least twice the time observed with the free drug[70]. On the other hand, no alteration in the pharmacokinetic parameters after intravenous administration was observed comparing quinine-loaded nanocapsules to free quinine. The high interindividual variability was suggested as the main reason for the similar drug pharmacokinetic profiles observed[30].

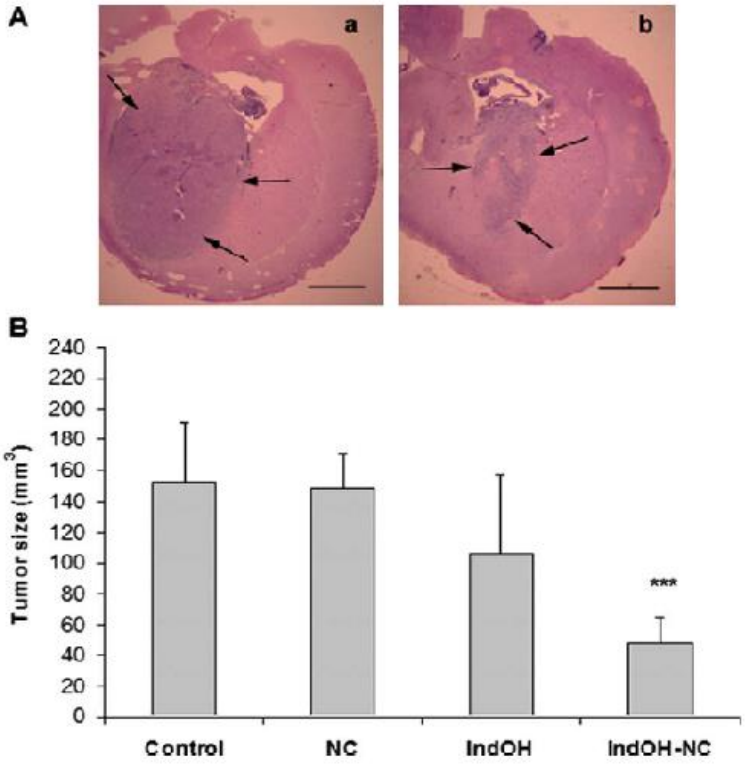

Figura 10 Tumor size observed in glioma-implanted animals after treatment with indomethacin-loaded nanocapsules (NC: nanocapsules, IndOH: indomethacin) (Bernardi et al, 2009, Cancer Letters)

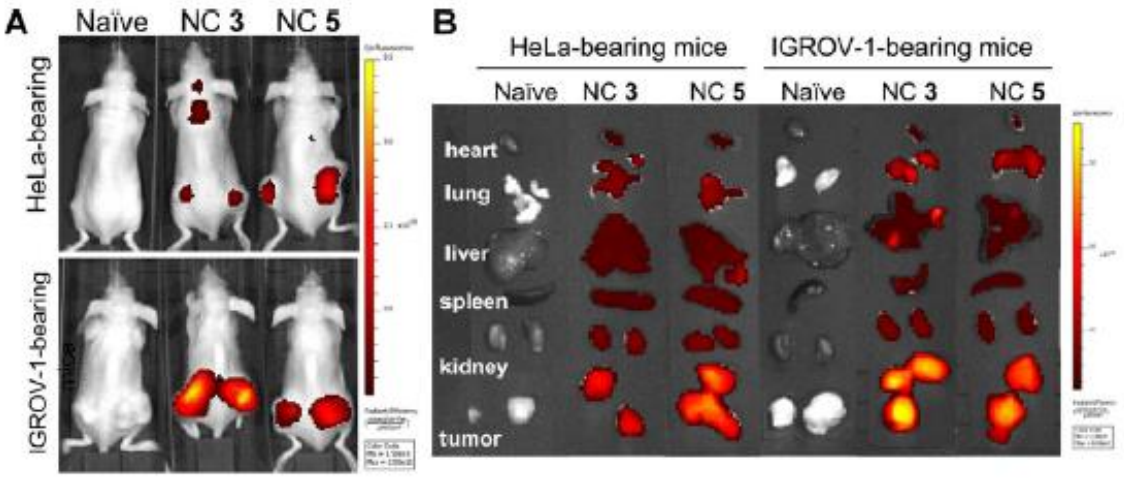

Figura 11 *In vivo* imaging of the fluorescent marker DiR loaded into nanocapsules with (NC5) or without (NC3) folate modified surface, two different tumor types – IGROV-1 and He-La- were analysed (El-Gogary et al, 2014).

2.3.5 Decreasing adverse effects

The nanoencapsulation of drugs into polymeric nanocapsules has been shown to be an alternative to decrease their adverse effects[13,61,66,67,71]. For more than twenty years, the nanoencapsulation of indomethacin and other anti-inflammatory non-steroidal drugs in polymeric nanoparticles has been studied to decrease the gastrointestinal adverse effects of these drugs. One of the most recent reports is the long-term systemic treatment with indomethacin-loaded lipid-core nanocapsules in rats (1 $mg \cdot kg^{-1}$ i.p. every 12 h for 8 days) in a chronic inflammation model[61]. In addition to an improvement of the pharmacological effect, the animals showed a reduced gastrointestinal lesional index compared to the group receiving indomethacin solution. This protective effect was attributed to a reduction of TNFα and IL-6 and an increase of IL-10 levels.

Other examples are haloperidol and olanzapine. Animals treated with haloperidol-loaded nanocapsules showed a decrease in the immobility time and an unaltered behavior related to oral dyskinesia[66]. Dissimilar results were observed for the free drug, proving the effect of the nanoencapsulation in reducing the haloperidol extrapyramidal effects. In a subsequent study, the secondary motor effects induced by acute haloperidol administration were studied in rats[71]. The administration of nanocapsule suspensions (blank and drug-loaded) led to unaltered orofacial movements and no catalepsy. After subchronic administration, similar results were observed, confirming the almost complete absence of adverse effects after the administration of the nanoencapsulated form of the drug. Olanzapine is an atypical antipsychotic drug associated with weight gain and cardio-metabolic diseases, such as hypercholesterolemia and diabetes, after chronic use. Animals treated with olanzapine-loaded lipid-core nanocapsules showed lower weight gain and lower total cholesterol levels when compared to animals receiving the non-encapsulated olanzapine[67]. In this case, according to the authors, the benefits of the nanoencapsulation might be due to medium chain triglycerides, a core component in the lipid-core nanocapsules, which prevent weight gain and metabolic diseases[72].

A recent study reported the effects of the nanocapsules on the decrease of skin irritation caused by an irritant substance (capsaicin) in humans[9] (Figure 11). Capsaicinoids-loaded nanocapsules reduced skin irritation measured by an erythema probe and, in parallel, the volunteers described less irritation sensation after their application. Although the nanocapsules were able to decrease skin irritation, they did not prevent skin penetration, suggesting that the adverse effects of capsaicinoids can be reduced without altering their effect on the skin.

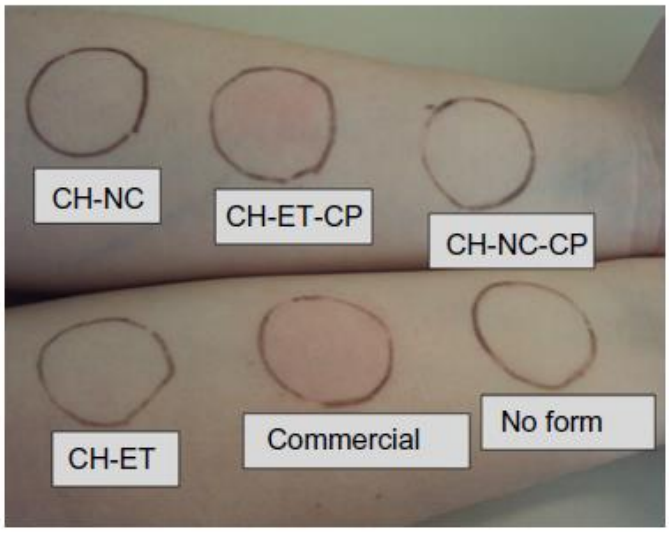

Figura 12 Skin irritation after application of capsaicin-loaded nanocapsules incorporated into a chitosan gel (CH: chitosan gel, ET: ethanolic solution, NC: nanocapsules) (Contri et al, 2014, International Journal of Nanomedicine).

2.4 SAFETY EVALUATION OF POLYMERIC NANOCAPSULES

The assessment of the safety of nanocapsule formulations has become an important issue due to the high number of studies describing the benefits of their use for drug delivery. Regarding in vitro studies, which are more frequently described compared to in vivo studies, the safety of nanocapsules can be evaluated using cytotoxicity, phototoxicity, hemocompatibility or genotoxicity assays. The cell lines used should be in accordance with the desired administration route, although this is not always the case. Several studies have also determined the cytotoxicity of blank nanocapsules (without drugs) in cell lines.

Breast cancer cells were used to evaluate PLGA-nanocapsules[51] and allyl-functionalized cationic PLA-nanocapsules[28], prostate cancer cells were used to evaluate allyl-functionalized cationic-PLA-nanocapsules[28], myeloid leukemia cells were used to evaluate PCL-nanocapsules[23], fibroblasts were used to evaluate PLA-nanocapsules[73] and keratinocytes were used to evaluate PLA-nanocapsules[73]. No considerable cytotoxicity was observed except for PLGA nanocapsules[51]. The authors of these studies have evaluated the correlation between the toxic potential of nanocapsules and their composition and properties. Among the studied formulations, nanocapsules composed of PLGA, PVA and Mygliol® 812 did not present cellular toxicity. On the other hand, the use of surfactants Tween® 80 and Span® 80 were considered responsible for the observed cytotoxicity of some formulations, most likely due to cellular permeabilization effects. Additionally, the lower zeta potential might have influenced to the observed lower cytotoxicity because nanocapsules' surface charge has been reported to be related with toxic effects to cells[51].

To predict dermal safety of polymeric nanocapsules composed of PLA, sorbitan monostearate and retinyl palmitate coated with Pluronic F68, cytotoxicity and phototoxicity assays were conducted using human keratinocyte cell line (HaCaT) and murine BALB/c 3T3 fibroblasts under UVA irradiation[73]. The nanocapsules showed cytotoxicity only at high concentrations and elevated phototoxic potential in BALB/c 3T3 fibroblasts, most likely due to the presence of retinyl palmitate. It should be emphasized that nanocapsules prepared without retinyl palmitate or the non-encapsulated retinyl palmitate, as controls, were not tested.

The lipid-core nanocapsules coated with polysorbate 80-lecithin and funcionalized or not with chitosan were evaluated at two different concentrations in human blood (2% and 10%, v/v) showing hemocompatibility suitable for intravenous administration[74]. Similarly, no hemolytic potential for poly(*n*-butyl cyanoacrylate) nanocapsules has been observed[32]. Additionally, using a vegetal *in vivo* model (*Allium cepa* root-tip), PCL-nanocapsules composed of vegetable oils[75] or caprylic/capric triglycerides[58] showed no genotoxic potential.

Few works evaluated the in vivo toxicity of polymeric nanocapsules after systemic distribution of the particles. To achieve trustworthy results, it is important to follow international guidelines such as the "Safety of Manufactured Nanomaterials Guidelines" from the Organization for Economic Cooperation and Development (OECD). It has been described that nanocapsules obtained with alginate-chitosan showed low toxicity, as evaluated by the LD_{50} after intraperitoneal administration in rats[59]. Acute and subchronic toxicity studies conducted with lipid-core nanocapsules in rats (using an administered i.p. volume 10 times higher than that used in efficacy studies) showed no mortality or permanent body weight changes during these experiments[76]. Furthermore, compared to the reference values and controls, no difference regarding hepatotoxicity and nephrotoxicity markers was observed. At the highest dose evaluated (7.21×10^{13} nanocapsules/kg), an accumulation of material was visualized in the abdominal cavity, and a granulomatous reaction was observed in the liver and spleen by histology. These results highlight the need to find the optimal dose for clinical trials. Recently, we evaluated the toxicity of lipid-core nanocapsules after single and repeated intradermal injections in Wistar rats[77]. No abnormal clinical findings, local inflammation, or histopathological alterations in organs were found. Furthermore, no alterations were observed in blood or urinary markers. The genotoxicity was evaluated by a comet test, and no significant difference was observed for the lipid-core nanocapsules compared to the negative controls. In addition, early renal injury markers indicated no nephrotoxicity after administration of the lipid-core nanocapsules. Regarding cutaneous use, in vivo studies showed that unloaded Eudragit® RS 100 nanocapsules caused no irritation to human skin[9], and lipid-core nanocapsules showed no cutaneous sensitization in mice[15].

2.5 CONCLUSION

Polymeric nanocapsules composed of different polymers and internal phases have shown interesting advantages such as increased drug stability, increased tissue/cell interaction, and decreased adverse effects, frequently leading to a more efficacious and

less toxic treatment. In the last decade, polymeric nanocapsules have been produced mostly using aliphatic polyesters as walls, triacylglycerols as cores, and combinations of surfactants (lecithin, sorbitan derivatives, poloxamers and polysorbate 80). One of the most frequently used types of nanocapsules is the lipid-core nanocapsule, obtained by self-assembling (solvent displacement method). The small size and surface charge of nanocapsules allow the carrier to penetrate or greatly interact with tissues and mucosae, leading to a higher or more sustained effect. Surface modification allows maintenance of the particles in the blood circulation, prolonging the effect of the drug. In addition, surface functionalization provides recognition by specific targets, creating a higher affinity at the site of action. The core-shell structure of the nanocapsules allows a controlled release of the encapsulated substance and avoids direct contact of the drug with the tissue, decreasing local adverse effects. In addition to these interesting benefits, nanocapsules have been described as safe both in vivo and in vitro. Although a great number of scientific works have been published, few products are available on the market.

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3 ARTIGO 2

3. ARTIGO 2 - Imiquimod-loaded nanoemulsion: a new formulation for the treatment of cervical cancer

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Abstract

Cervical cancer is the second most frequent cancer among women and its appearance is associated with the human papilloma virus (HPV). One of the treatments for this disease is based on the application of imiquimode. Some strategies have been used to increase the penetration and the cellular uptake of drugs through the vaginal mucosa. A promising strategy consists in the incorporation of drugs into nanostructured systems. This paper hypothesizes that the use of imiquimod in nanoemulsion results in a better performance than the drug in its free form for the treatment of cervical cancer. Aiming this, permeability studies in vaginal mucosa and studies involving cervical cancer cells (viability, clonogenic and assays for cell death analysis) were performed. The results showed that low amount of imiquimod-loaded nanoemulsion permeated the vaginal mucosa. However, a higher percentage of cells died after the treatment with low amount of the formulation compared to the drug in its free form (control). In addition, the proposed formulation presented a mechanism of differentiated cell death when compared to the control. Finally our results demonstrate that the proposed formulation can be proposed as an alternative in the treatment of cervical cancer validating the hypothesis that the use of imiquimod in nanoemulsion results in a better performance than free imiquimod.

Keywords: nanoemulsion; imiquimod; HPV; SiHa.

3.1 INTRODUCTION

Cervical cancer is the leading cause of cancer death among women in developing countries (THULER et al., 2008). The appearance of this disease is strongly related to human papilloma virus subtypes (HPV) 16 and 18 (AYRES et al., 2010; PINOTTI et al., 2005). Prior research has demonstrated that one effective way to treat this disease is by applying drugs directly on the vaginal mucosa as a drug delivery route (VÀNIC et al., 2013; CARAMELLA et al., 2015). Imiquimod is drug used to treatment of cervical cancer (KOUTSKY et al., 1983) and also HPV (STANLEY et al., 2002), as well as other gynecological diseases (TYRING et al., 1998; BROWN et al., 2000; CHRISTENSEN e HENGGE, 1999). Imiquimod is an immunomodulatory drug with antitumor (ADAMS et al., 2012) and antiviral (SAUDER et al., 2000) activities widely used. Its commercial formulation is presented in the form of cream and the literature has reported severe adverse effects of it such as pain, itching and ulceration, provoking the abandon of the treatment by many patients (WIELAND et al., 2006; KREUTER et al., 2008).

Recently, imiquimod has been incorporated into polymeric nanocapsules and showed promising results in the pharmaceutical area for vaginal application (FRANK et al., 2017). The application of nanotechnology in medicine introduced a variety of new particles with several different structures and chemical compositions for cancer treatment (WANG et al., 2010). These nanoparticles have advantages over conventional drug delivery systems, since the assure the release control of the encapsulated active, reduce the adverse effects decrease and target the delivery at the tumor site (BERNARDI et al., 2009; FRANK et al., 2017). One of these systems are the nanoemulsions, which are submicrometric systems that can be defined as oil-in-water (o/w) emulsions with mean droplet diameters ranging from 50 to 300 nm (BHALODIA et al., 2010; SEVERINO et al., 2013). These nanoemulsions can be obtained by emulsification, and are capable to solubilize various lipophilic drugs (TADROS et al., 2004; VÀNIC et al., 2013). Differently from the microemulsions, which are thermodynamically stable, nanoemulsions exhibit kinetic stability and long-term stability (TRADOS et al., 2004).

Clinically, nanoemulsions are well accepted marketed systems (BENITA et al., 1993). Their fluidity due to reasonable oil concentrations, as well as the absence of thickeners in the formulations can provide a wellbeing feeling when applied to the body (TRADOS et al., 2004). These facts underline the promising therapeutic properties of such colloidal drug carriers, however an adequate characterization of such systems is required (BENITA et al., 1993, VÀNIC et al., 2013).

Several studies have been dedicated to propose the vectorization of lipophilic drugs using nanoemulsions for delivery in the vaginal route. For example, D´cruz and coworkers (2001) have developed a versatile system designed to solubilize lipophilic antiviral and antimicrobial agents. It is a gel containing a microemulsion to be used for the prevention of sexually transmitted diseases or it can be used as contraceptive improving the bioavailability of poorly soluble antimicrobial agents. Other work (KAUR et al., 2017), reported the development of a carbopol gel containing nanoemulsion with clobitasol propionate (CP) and calcipotriol (CT) for the treatment of psoriasis and demonstrated that the proposed system has a greater uptake in HaCaT cells, but it did not show detectable amounts of CP and CT permeated through the pig ear skin during the period of exposure of skin. Nanoemulsions containing clotrimazole showed higher antifungal activity and higher bioadhesion than commercial gel containing the same drug (BACHHAV e PATRAVALE 2009). *In vivo* experiments have also demonstrated the potential of nanoemulsions, such as the work of Kakumano and co-workers (2011) who demonstrated that darnabazine-loaded nanoemulsions administered in mice (0.1mg/kg - intramuscular injection) were able to decrease significantly the tumor epidermoid carcinoma compared to animals receiving the same treatment in solution form. Similarly, Tagne and co-workers (2008) demonstrated the increasing efficacy of darcabazine in nanoemulsion in a mouse melanoma model.

Many studies have been dedicated to the targeting of drugs for treatments of diseases that affect the vaginal route, but none of them was specifically focused on nanoemulsions as carriers to deliver drugs. Additionally, in order to treat cervical cancer, the association in the same formulation of a structural component of the nanoemulsion with antitumor activity (copaiba oil) and an antitumor drug (imiquimod) for a combined

effect is an unprecedented strategy. In this sense, the present work has the objective of studying the association of copaiba oil and imiquimod in a nanoemulsion formulation planned to treat cervical cancer cells. To achieve our goal, studies of drug permeation after washability test into porcine vaginal mucosa, cell viability, clonogenic survival assay and assays to verify the mechanism of cell death have been done.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Sorbitan monostearate (Span 60®) was purchased from Sigma-Aldrich (Steinheim, Germany). Polysorbate 80 (Tween 80®) was purchased from Henrifarma (São Paulo, Brasil) and copaiba oil was kindly donated by Inovam-Da Lamarta & Cia Ltda. Imiquimod (IMIQ) was purchased from Chemical Goods (Guangdong, China). The cervical carcinoma cell line SiHa was purchased from American Type Culture Collection, Rockville, MD. Annexin V, and propidium iodide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All solvents and reagents were of analytical or pharmaceutical grade.

3.2.2 Methods

3.2.2.1 *Production of imiquimod-loaded nanoemulsion*

The nanoemulsion was developed by spontaneous emulsification method (KATZER et al., 2013). The organic phase consisting in copaiba oil (334 µL), Span 60® (38.4 mg) and the drug imiquimod (5 mg), dissolved in acetone (27 mL). After its complete solubilization, the organic phase was injected into an aqueous phase (53 mL) containing polysorbate 80 (76 mg). Posteriorly, the turbid solution was evaporated under reduced pressure in a rotative evaporator at 37ºC (Büchi, Switerland) to approximately 10 mL. This formulation obtained was named NE_{imiq}.

3.2.2.2 *Characterization of nanoemulsion*

The NE_{imiq} was characterized in terms of particle size was measured by different techniques (Laser diffraction: Mastersizer 2000, Nano ZS, Malvern and Dynamic light scattering: Zetasizer Nano ZS, Malvern) by dilution of nanoemulsion in bidestilled water. For determination of the zeta potential, the NE_{imiq} were diluted in NaCl solution (10 mM) and analyzed by electrophoretic mobility (Zetasizer, Nano ZS, Malvern). The pH analysis was performed by direct measurement using potentiometry (B474 Micronal).

The drug content (n = 3) was determined by High Performance Liquid Chromatography (HPLC-UV, Series 200, PerkinElmer, Waltham, MA, USA) as proposed by De Paula and co-workers 2008 and adapted by Frank and co-workers (2017). Calibration curves (n = 3) were made to determine the drug concentration showing linearity (r = 0.999) in the range of 1 to 25 µg mL^{-1}.

3.2.2.3 *Permeation after washability test into porcine vaginal mucosa*

The effect of vaginal flux on permeation of NE_{imiq} was evaluated after 1 h of contact with the vaginal mucosa. The samples NE_{imiq}, as well as imiquimod dissolved in acetate buffer pH 3.7 (VENTURUNI et al., 2015) (IMIQfree) were subjected to permeation measurements with a modified manual Franz diffusion cell (FRANK et al., 2017).

The measurements were made using fresh mucosa to assure the properties and viability of the tissue. The vaginal mucosa (0.9 cm^2) was placed between the donor and the receptor compartment, which was filled with 50 µL of the formulations. After 1 hour of interaction (CONTRI et al., 2014), acetate buffer pH 4.0 (37°C) was fluxed to simulate action of vaginal flux (VALENTA et al., 2005). The flux was set at 0.4 mL min^{-1} (FRANK et al., 2014) (HPLC pump; Gilson; Minipuls 3, France). The receptor was an acetate buffer pH 4.0 previous filtered using a hydrophilic membrane (0.45 µm, Millipore®). After passing the flow through the mucosa (with the formulations) for 180 minutes, the permeate imiquimode content was evaluated by the HPLC-UV method as

mentioned above. The porcine vaginal mucosa was obtained from a local slaughterhouse (Santo Ângelo Ltda., Brazil), for all experiments involving vaginal mucosa.

3.2.2.4 *Imiquimod permeability into porcine vaginal mucosa*

The permeation of imiquimod was evaluated using the same formulations described in the previous subsection (Section 2.3.3) with the same experimental conditions as before. The donor compartment received 100 µL of the NE_{imiq} or IMIQfree and after 24 hours, 40 µL of the receptor medium was withdrawn and directly analyzed by HPLC-UV method.

3.2.2.5 *Microbiological contamination test*

Prior to the initiation of the experiments involving cell culture the NE_{imiq} was tested for its microbiological loading to ensure that the experiments were performed under sterile conditions. Briefly, the detection of fungi and bacteria in the formulations was performed by inoculating 20 µL of each formulation NE_{imiq} for 48 h at 37 ± 1 and 35 ± 1°C in, respectively, a blood agar plate for the bacterial growing and in a Sabouraud late for the fungal growing (PAESE et al., 2016).

3.2.2.6 *Cell culture*

For the experiments involving analysis of the cytotoxicity of the formulations, cervical carcinoma cell line SiHa containing integrated HPV 16 was used (American Type Culture Collection, Rockville, MD). Cells were maintained in low glucose DMEM supplemented with 10% FBS and penicillin/streptomycin antibiotics (0.5 U·mL/95% air at 37°C).

3.2.2.7 *Cell treatment*

Cells were seeded and treated after 24 hours with the formulation NE_{imiq} and IMIQfree. The cultures were exposed to the formulations for 24 hour with concentrations of 3.0 µmol L^{-1} of nanoemulsion dispersions in culture media. All

materials were previously sterilized, and the formulations were prepared under aseptic conditions.

3.2.2.8 *Cell viability and clonogenic survival assay*

Cell lines (20,000 cells/well) were seeded on 24-well plates and 24 hours later they were treated with formulations according to described in Section 2.3.7. At the end of treatment, the medium was removed, cells were washed with 1× PBS, 200 µl of 0.25% trypsin/EDTA was added to detach the cells and 400 µl of DMEM + 10% FBS was added to inactivate trypsin. The viable number of cells were then counted by flow cytometry using FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA). Negative controls were used by treating cells with DMEM supplemented with 10% FBS. Results were expressed in percentage values regarding the control.

After the viability experiment the cells were evaluated for the cytotoxic effect of imiquimod after the cell survival according to established methods for clonogenic assay (FRANKEN et al., 2006; MELLO et al., 2015). Therefore, after the treatments in the same conditions already mentioned, the surviving adherent cells were washed with PBS preheated to 37°C, trypsinized, counted, and replated in six-well plates (100 cells/well). After 10 days of incubation in complete culture medium, the colonies, formed from each cell plated, were stained with crystal violet after fixation with methanol and counted manually. In each case results are expressed as survival fraction, which was obtained by dividing the number of colonies that arise after treatment of cells by the number of cells seeded and plate efficiency (PE: number of colonies formed by untreated cells/ number of cells seeded), multiplied by 100.

3.2.2.9 *Labeling the nucleus of cells with hoechst dye*

Cell nuclei were stained with Hoescht 35565665 (1500 µg/mL) according to the manufacturer's instruction. The dye labeling was performed after treatment, at 24 hours, by fluorescence microscopy.

3.2.2.10 *Annexin V and propidium iodide staining*

Phosphatidylserine externalization was determined by the annexin fluorescence signal of an annexin V–fluorescein isothiocyanate conjugate (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) according to the manufacturer's protocol. Cell cultures were treated, trypsinized, and centrifuged for 6 min at 1600 rpm, and the supernatant was discarded. The pellet was suspended with 150 µl of annexin binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), incubated with annexin V at 0.75 µl/sample and PI at 15 µl/sample for 15 min at room temperature in the dark, and analyzed in a Guava EasyCyte flow cytometer, using Guava EasyCyte software for analysis (Millipore, Billerica, MA). Cisplatin, 40 µM, was used as positive control for apoptosis, and 0.5% Triton X-100 was used as a positive control for necrosis.

3.2.2.11*Detection of autophagic using acridine orange (AO)*

The development of acidic vesicular organelles (AVO) was quantified for autophagy evaluation. For this experiment, cells (20,000 cells/well) were seeded on 24-well multiwell plates and exposed to autophagy inhibitors or inducers. Cells were treated with 3µM NE_{imiq} and IMIQfree for 24 hours. Then cells were trypsinized and incubated with AO (2.7 mM) for 15 min at room temperature, and fluorescence emission was analyzed by flow cytometry using a FACSVerse flow cytometer and FACSVerse software. Rapmicyn (200nM) was used in parallel as a positive control of autophagy inducer.

3.2.2.12*Cell cycle analyses*

After treatment with NE_{imiq} and IMIQfree in the same concentration, the cells were centrifuged at 1500 rpm for 5 min. Subsequently, the cells were resuspended in 200 µL of PBS and centrifuged at 1500 rpm for 5 min. When it was ready, the cells were fixed in 70% ethanol and stained 2 hours at 4ºC. After, the cells were washed and centrifuged under the same conditions as before and, finally, propidium iodide (12 µg/ml), 0.1% triton X-100 and RNAase (50 µg/ml) were added. Cells were incubated

for 30 minutes at room temperature and subsequently analyzed by flow cytometry (FACSVerse). Mitomycin C (5 μg/mL) was used in parallel as positive control of cell cycle arrest.

3.2.3 Data analysis

Statistical analyses were performed by means of Analysis of Variance (ANOVA) followed by the post-hoc Tuckey's test for multiple comparison of means ($\alpha = 0.05$). The software SPSS statistics 17.0® was used for the statistical analyses.

3.3 RESULTS AND DISCUSSION

3.3.1 Production of imiquimod-loaded nanoemulsion

Laser diffraction analysis showed unimodal size distributions for imiquimode-loaded nanoemulsion (NE_{imiq}). The mean diameters (D[4,3]) by volume, diameter values by the dynamic light scattering and the values of SPAN and PDI can be observed in Table 1.

Tabela 2 Particle size distribution and polydispersity indices (Span and Polydispersity index) of formulation measured by laser diffraction (LD), dynamic light scattering (DLS) and drug content.

Formulation	LD		DLS		Drug content
	D(4,3) ± SD (nm)	Span ± SD	Z-average ± SD (nm)	PDI ± SD	(mg mL^{-1})
NE_{imiq}	220.3 ± 23	1.58 ± 0.35	190 ± 8	0.11 ± 0.01	0.49±0.08

The techniques used to evaluate the diameter of the formulation showed the presence of only nanometric particles. In addition, the low values of PDI and SPAN demonstrated high homogeneity in this new formulation. The pH value for the NE_{imiq} aqueous dispersions was 6.04±0.10. The drug content showed 0.49±0.08 mg mL^{-1} of imiquimod in NE_{imiq}. Thus, this new nanoemulsion was characterized, showing properties suitable for the further studies.

3.3.2 Imiquimod-loaded nanoemulsion reduces the imiquimod permeation in porcine vaginal mucosa when compared to the free drug formulation.

The amount of imiquimod permeated through the porcine vaginal mucosa was evaluated after fluxing it for 3 hours with an acetate buffer (pH 4.0) (washability test) as well as in the presence of the vaginal fluid only. The results were expressed in percentage of imiquimode permeated per mm^2 of vaginal mucosa (Figure 12). It is possible to observe that, in both cases, the amount of $IMIQ_{free}$ permeated through the vaginal mucosa was greater than that measured for the imiquimod-loaded nanoemulsion. According to our data NE_{imiq} formulation leads to 6.18±0.50% of imiquimod permeation after the washability test, while $IMIQ_{free}$ presented almost the double of this value (11.31±4.00%). This higher amount of permeate imiquimode caused by $IMIQ_{free}$ was also observed when the vaginal flow effect was excluded (IMIQfree = 50.13±4.98; NEimiq = 22.89±4.46). This means that the vaginal flow strongly influenced the imiquimod permeation. This can be explained by the fact that the nanoemulsion controlled the drug release because the presence of oil in its composition (CRUZ et al., 2006).

Our previous study has already pointed an increased in the vaginal mucosa permeation profile for imiquimod free drug in comparison with imiquimode release from polymeric nanocapsules. (FRANK et al., 2017). Moreover, imiquimod incorporation into chitosan hydrogel significantly increased its permeation profile. In addition, this formulation has the potential to be incorporated into semi-solid vehicle for more adhesive vaginal delivery.

Supporting our findings, Chaves and co-workers (2017) observed that free solution of carvedilol (54.3%) permeated the sublingual mucosa more than the free drug when release from two different nanostructures: polymeric nanocapsules NC (32.4%) and lipid core nanocapsules LNC (8.1%). The authors attributed those permeation differences to the property of nanostructures in controlling the drug release as well as to the fact that for the free drug the mucosa is the only barrier for its permeation. On the contrary, the amount of carvedilol permeated after the washability experiment was

higher for both nanostructures than the free drug (NC: 0.72µg.mL^{-1}; LNC 0.52 and FREE: 0.10). This difference can be explained by fact that both polymeric nanocapsules have adhesive polymer in their composition, increasing the formulation-mucosa interaction. In the present work this interaction was not observed because the imiquimod nanoemulsion tested by us presents no polymeric wall on its composition.

Differently, Kong and co-workers (2011) demonstrated that nanoemulsion-loaded with vitamin E (1mg/mL) presented higher capability to permeated rat skin than the vitamin E solution in the same concentration. Moreover, this increased permeation was kept during all period times analyzed (from 0.5 to 24 hours). Various factors including test duration, sampling frequency, sink condition, stirring rate, diffusion membrane material, etc., may explain such differing phenomena. In this work, the type of membrane used was vaginal mucosa, which presented different layers than the skin. Besides that, Mirza and co-workers (2013) incorporated nanoemulsion containing itraconazole in three different thermo-sensitive gels with different polymer concentrations (carbopol 934 and polaxomer 407) and evaluated their permeation compared with conventional formulation. The authors observed that gels presented similar permeation profile to the control formulation. However, the gel with lower concentration of polymer, which means the lowest adhesion value, had the higher permeation index. This higher permeation value was attributed to the nanometric size of this formulation, which increases the interfacial area and influences the drug transportation profile. However, the authors assumed that the low interfacial tensions could also be responsible for slowing down the drug permeation. This assumption might justify our findings, since our nanoemulsion presented lower permeation values in both experiments.

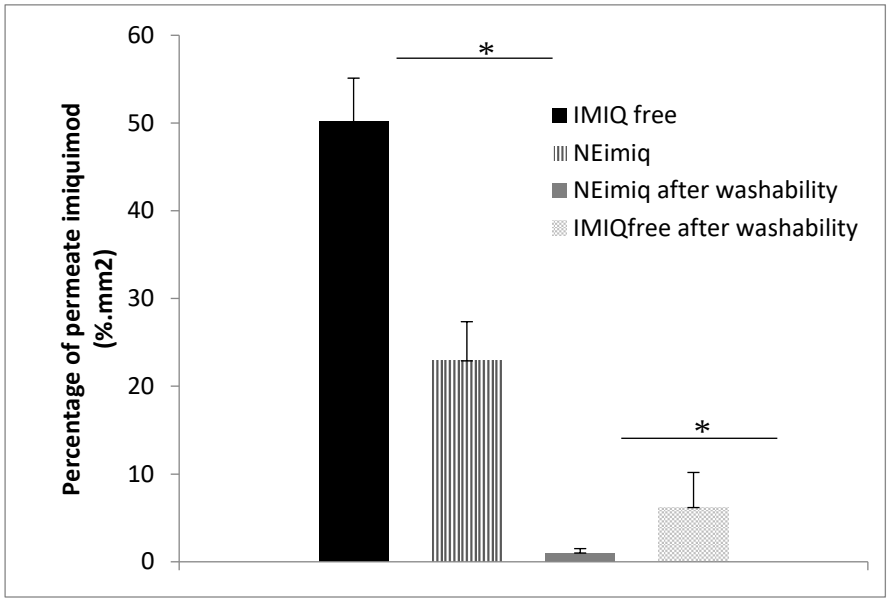

Figura 13 Permeation of imiquimod. Left: Imiquimod permeation into vaginal mucosa after 24 hours of treatment with formulation IMIQfree and NEimiq. Right: Imiquimod permeation into vaginal mucosa after 3 hours of washability for both formulations. Statistical differences were considered for $p<0.05$ (n=3).

Despite the distinct permeation profile related to nanoemulsion formulations in the literature, we observed that the amount of imiquimod permeated from nanoemulsion is lower than that from the free drug formulation. In fact, depending on the context, this effect may be desired, since imiquimod exposure could cause several topic adverse effects such as skin irritation, itching and pain (GUPTA et al. 2000) as well as systemic adverse reactions including fatigue, headache, fever, peripheral nervous system disorders and diarrhoea (WIELAND et al. 2006; KREUTER et al. 2008).

3.3.3 Microbiological contamination test

In order to guarantee NE_{imiq} formulation sterility before starting experiments with cell culture, we tested NE_{imiq} microbiologial contamination and no microorganisms (fungi or bacteria) could be noticed when the formulation was prepared under sterile conditions. As a positive control, we tested a formulation prepared without sterility conditions showed significantly microbiological contamination (Figure 13).

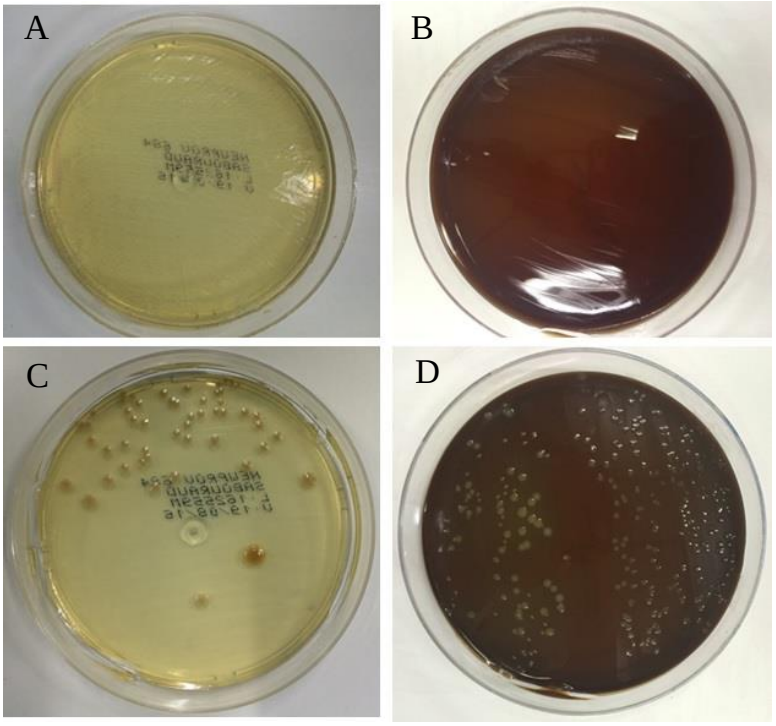

Figura 14 Contamination test in Sabouraud (A and C) and blood (B and D) agar plate. Formulation prepared under sterile (A and B) and normal conditions (C and D) were plate and letting to growth for 48 h at 35 ± 1°C (Sabouraud agar) and 37 ± 1°C (blood agar).

3.3.4 NE_{imiq} potentiates imiquimod-related cytotoxicity

Figure 14B shows that the NE_{imiq} formulation was more effective against cervical cancer cell line (SiHa), significantly reducing cell viability after 24 hours of treatment when compared with $IMIQ_{free}$ (35% NE_{imiq} and 16%$IMIQ_{free}$). Moreover, NE_{imiq} exibited increased capability to inhibit SiHa cells that survival to its acute cytotoxic effect to proliferate and form new colonies (Figure 14A). We have already demonstrated that imiquimod-loaded nanocapsules improved imiquimod-related cytotocixity and ability to block colony formation in cervical cancer cell lines in the same extent as shown here (FRANK et al., 2017). Altogether, our results reinforce the advantage of the nanoencapsulation in potentiating the imiquimod-associated effect and suggest that the polymer wall has no influence on the drug delivery potency. As a rule, nanostructures -for being small structures with nanometric size- are easily captured by the cells,

allowing greater amount of the drug to be released in into the cell as compared to the free drug.

Other works have also shown that nanoemulsion are promising systems for cytotoxic drug delivery in glioblastoma and human ovarian adenocarcinoma cells (DESAI et al., 2007; GANTA et al., 2009). According to Desai and co-workers (2007) nanoemulsion with two combined drugs (paclitaxel + C6-ceramide) boost paclitaxel cytotoxicity against glioblastoma cells (U-118). Different amount of drugs were analyzed and the nanoemulsion that reached the highest cytotoxic effect was composed by 100nM of paclitaxel and 10μM of C6-ceramide. This formulation led to the death around 60% of the cells after 6 days incubation, while their respective control (aqueous solutions with drugs) triggers 50% of the cell death, Similarly, Ganta and co-workers (2009) demonstrated that nanoemulsion containing paclitaxel and curcumin potentiated paclitaxel cytotoxicity against human ovarian adenocarcinoma cells (SKOV3) after 6 hours of incubation (IC_{50} for the nanoemulsion: 0.7nM of paclitaxel+5μM of curcumin and for drug in solution IC_{50}: 4.1nM of paclitaxel +5μM of curcumin). Moreover, the efficiency of the nanoemulsion cytotoxictity was increased when rodamine was conjugated to the formulation. Taking together, these findings support the notion that nanoemulsions containing drugs present better performance against tumor cell lines compared to the free drugs and represent a promising approach to increase the therapeutic efficacy of drugs while decrease the side effects caused by higher chemotherapy dosage.

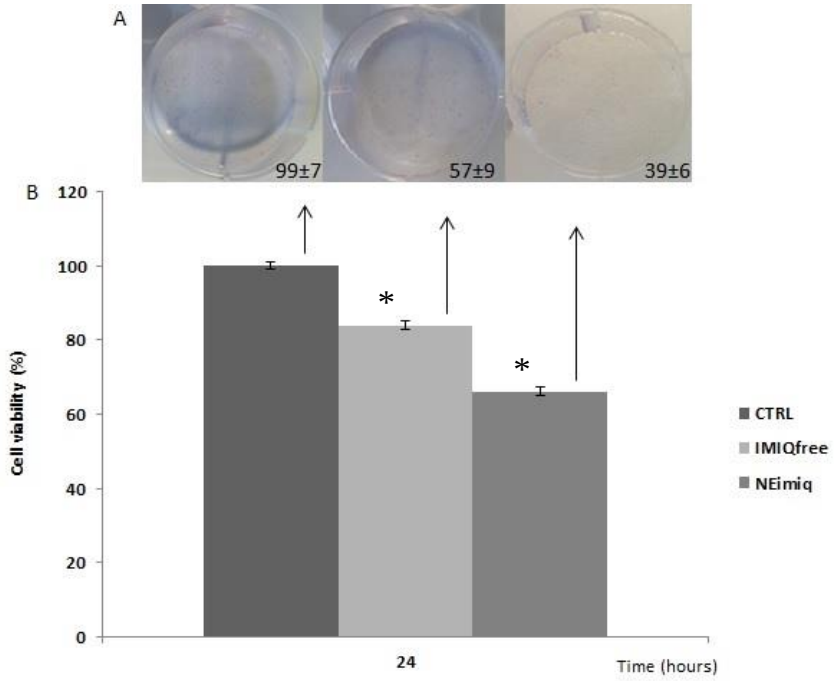

Figura 15 Cell viability. A- 100 viable cells that survival the imiquimod acute cytotoxicity were seeded and let to growth for 12 days in complete cell culture medium (DMEM + 10% FBS). Then, colony formation was evaluated and the number of survival fraction was calculated according to described on the clonogenic assay section. B- Number of viable cells after 3µM NEimiq and IMIQfree treatment for 24h. Arrows represent the population of survival cells used to perform the clonogenic assay. *p < 0.05 compared with control (one-way ANOVA, followed by Tukey's test).

3.3.5 NE_{imiq} increases cervical cancer cell shrinkage, membrane blebbing and chromatin condensation

In Figure 15, the amount of fluorescence for both formulations can be observed. However, it is possible to verify a higher fluorescence intensity for $NE_{imiq.}$ This means that a larger number of cells treated with the NE_{imiq} formulation are involved in apoptotic processes when compared to $IMIQ_{free}$. The untreated cells (CTRL) did not show blue color nuclei, suggesting no apoptosis in these cells.

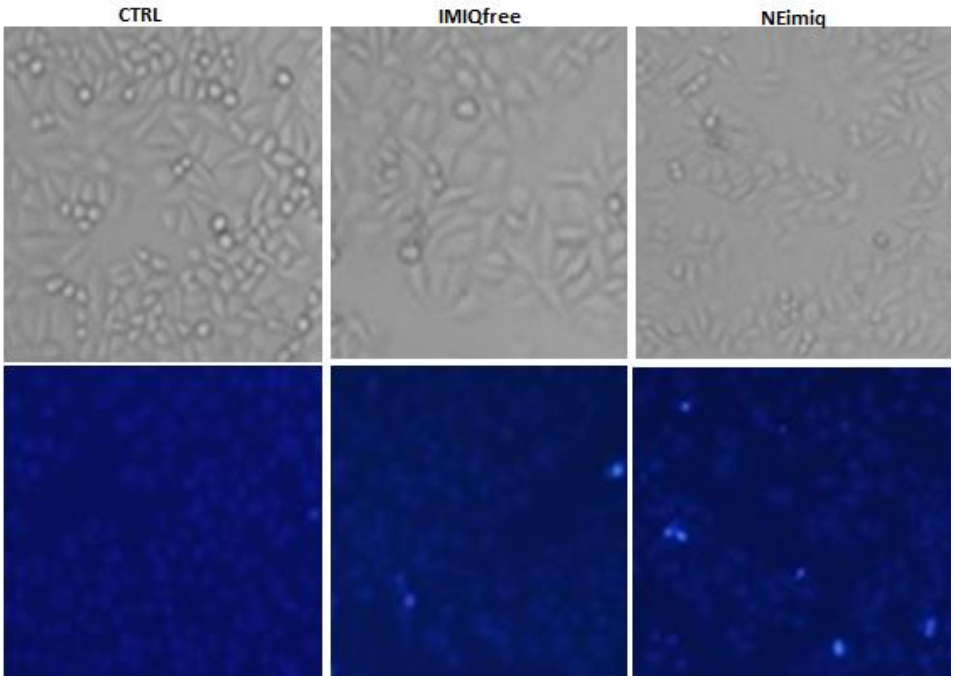

Figura 16 Images of SiHa cell treatment with 3.0µM NEimiq and IMIQ for 24 h. Cell nuclei was stained with Hoescht 35565665 according to manufacturer's instruction. Note apoptotic features such as cell shrinkage and blebbing and fragmented nuclei when cells were treated with NEimiq and IMIQfree. Scale bars, 10 µm; magnification, 10×.

3.3.6 NE_{imiq} induces death by apoptosis

Figure 16 shows that the NE_{imiq} formulation presented higher percentage of cells involved in apoptotic processes compared to CTRL and $IMIQ_{free}$. It is possible to note that the control group (cells untreated) and the cells treated with $IMIQ_{free}$ presented similar percentage ($p>0.05$) and no significant differences was found. Differently, the SiHa cells treated with NE_{imiq} presented significant values ($p<0.05$) of death by apoptosis compared to CRTL and to $IMIQ_{free}$ and no significant values of necrotic death were found ($p>0.05$).

Desai and co-workers (2007) reported an increase (around 10%) in the percentage of cells in apoptosis when both drugs were administered in aqueous solution against U118 human glioblastoma cells line. In this sense, Ganta and co-workers (2009) showed that the treatment with combined therapy of paclitaxel (PTX) and curcumin (CUR) in nanoemulsion (5 nM PTX + 5 µM CUR for SKOV3 and 2 µM PTX + 5 µM CUR for $SKOV3_{TR}$) enhances the apoptotic response when compared to the same treatment

without the nanoemulsion system. These results reinforce the assumption that nanoemulsions play a role in enhancing the activity of the drug by effective cell internalization.

In previous studies (HAM et al., 2013; SHON et al. 2014; HUANG et al., 2016), it has been shown that imiquimod causes death through apoptosis mechanism, However, the drug concentration used was higher than that used in the present work. For example, imiquimod was able to induce the death by apoptosis (8.32%) in prostate cancer cells (TRAMPC-2) after 48 hours of treatment at the concentration of 20µg/ml (HAM et al., 2013). The concentration used by these authors was 5.3 times greater than the used in this study and the time of exposure was twice used here. In other study conducted by Wang and co-workers (2015) for the cells death by apoptosis, the value of imiquimod used was 13 times greater than the used in this study, and also the drug concentrations used were until 40 times greater (SHON et al., 2014). In all the prior works cited above the percentages of apoptosis found were higher, but this can be due to the higher concentration of drug used in the treatment cells.

The high concentrations of imiquimod are being associated with adverse effects such as itching, burning and pain (GUPTA et al., 2000). Some patients who were treated for HPV had erythema, ulceration, tenderness and edema. Systemic reactions include fatigue, fever, flu symptoms, peripheral nervous system disorders, headache and diarrhea (WIELAND et al., 2006; KREUTER et al., 2008). In this context, the innovative formulation proposed in this work presents advantages over the already developed treatments because with less concentrations of the formulation is able to act on cancer cells, allowing a lower incidence of adverse effects.

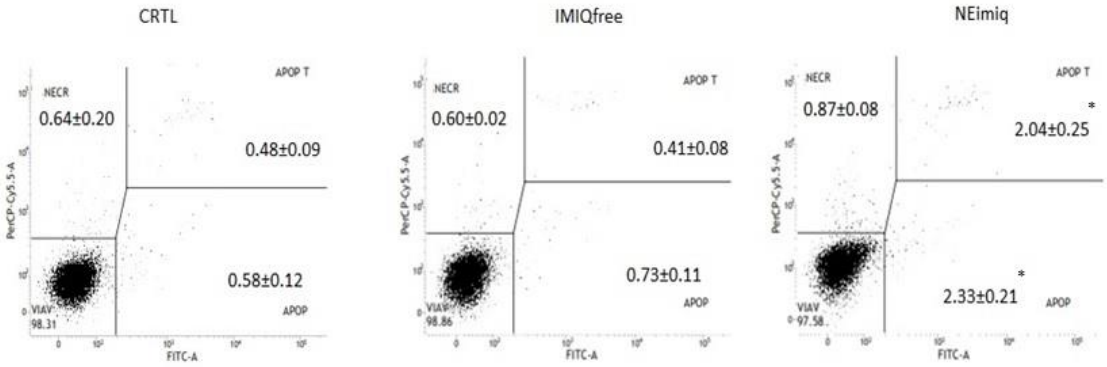

Figura 17 SiHa was exposed or not for 24 hours with 3µM NEimiq and $IMIQ_{free}$. Apoptosis and necrosis were measured according to annexin V/PI binding. n=3 and *p < 0.05 compared with control (one-way ANOVA, followed by Tukey's test). Note: PerCP = annexin V-FITC, FITC-A = propidium iodide.

3.3.7 Imiquimod-induced autophagy process

Marking of the cells with AO dye indicates that autophagy death is occurring in the cellular environment. (KLIONSKY et al., 2008). Our results demonstrated that NE_{imiq} has high values of AO^+ cells, indicating that there is an autophagy process (Figure 17). This formulation showed a significant difference of the other experimental groups ($p<0.05$; $IMIQ_{free}$ and CTRL). At the same time it was not possible to show a significant difference between $IMIQ_{free}$ and CRTL groups ($p>0.05$). This means that imiquimod-loaded nanoemulsion changes the way the cells die after treatment with the drug. These findings are in agreement with the results found for imiquimod-loaded polymeric nanocapsules, and the authors (FRANK et al., 2017) attributed this evidence to the fact that imiquimod concentration is very low to trigger such process in to the cells, which is, on the other hand, potentiated by the nanoencapsulated formulation.

The autophagy pathway is related to pathologies such as cancer, neurodegeneration and infectious diseases and occurs as a consequence of stress conditions that the cells can present (HE et al., 2009). Autophagy is a self-degrading process involving autophagosomes resulting in fusion with lysosomes (HE et al., 2009; LEVINE et al., 2011; ZHI et al.,2015). Recent developments reveal a crucial role for the autophagy pathway and proteins in immunity and inflammation (LEVINE et al., 2011) and both pathologies have been associated with cancer. The current consensus is that

autophagy has a dual role in cancer. On the one hand, autophagy functions as a tumor suppressor mechanism by preventing the accumulation of damaged organelles and aggregated proteins. On the other hand, autophagy is a key cell survival mechanism for established tumors; therefore autophagy inhibition suppresses tumor progression (ZHI et al., 2015).

Previous studies have shown that imiquimod at high concentrations induces autophagy in cell carcinoma (BCC) (WANG et al., 2015; HUANG et al., 2016). In this work, it was demonstrated that low concentrations of imiquimod was also able to promote autophagy, but only after being encapsulated in nanoemulsion indicating the the nanostructure's potential to interact with cervical cancer cells.

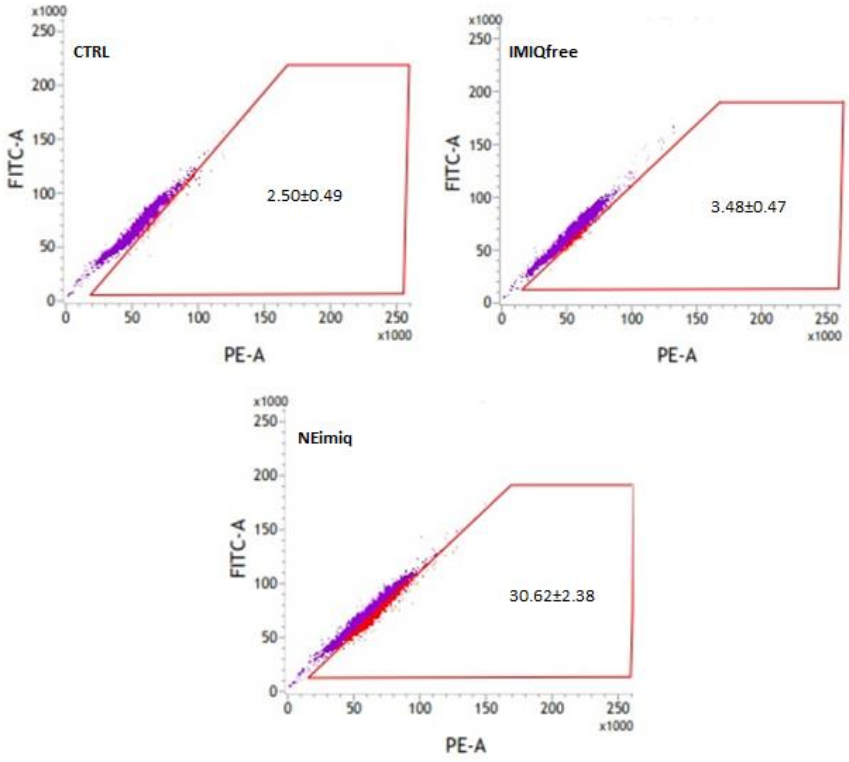

Figura 18 SiHa was exposed or not for 24h with 3µm NEimiq and IMIQ. Autophagy were measured to evaluation and acridine orange (AO) staining. Results are mean values±SD (n=3).

3.3.8 Cell cycle

The Figure 18 demonstrated that, for all phases of cellular cycle, $IMIQ_{free}$ presented similar values to that found for the control group (cells without treatment). Different results were demonstrated for the cells treated with the NE_{imiq} formulation. This formulation increases the percentage of SubG1 and G2 subpopulation and decrease the percentage of G0/G1 of SiHa cells after 24h.

Studies have shown that high concentrations of imiquimod (50µg/ml) induced an increment of subG1 subpopulation in the basocellular carcinoma cells (CCB and A375) (WANG et al., 2015). In this work, it has been shown that with low concentrations of imiquimod-loaded nanoemulsion, it is possible to have similar results to those with high concentrations, but only in its nanoemulsion form, since $IMIQ_{free}$ did not present these results. Ham and co-workers (2013) observed that 20µg/ml of imiquimod increased the amount of TRAMP-C2 cells in G2, and these results are in agreement with the findings of this paper. In the present study, similar results were found with a 6-fold lower concentration. Therefore, the present results induce cancer cell death through cell cycle arrest and the nanoformulation have a better efficiency in producing this effect.

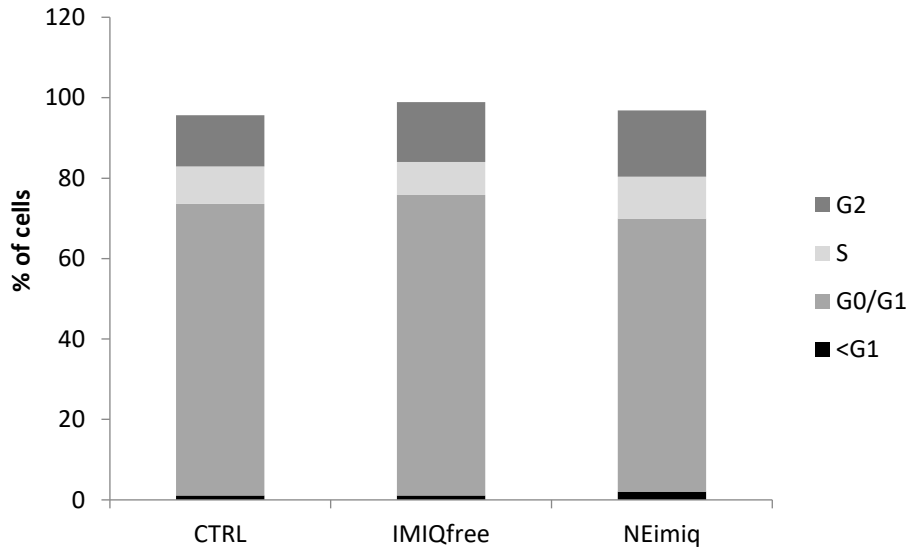

Figura 19 Cell cycle distribution after 24 h of treatment of SiHa cells. Results are mean values±SD (n=3).

3.4 CONCLUSIONS

Imiquimod-loaded nanoemulsions were successfully prepared using a spontaneous emulsification technique. The developed formulation was able to penetrate the vaginal mucosa. When administered in the nanoemulsion formulation, imiquimod was delivered inside the SiHa cells resulting in a significant enhancement of cytotoxicity. The results of this study demonstrated a significant therapeutic benefit of imiquimod in nanoemulsions for the treatment of cervical cancer, once the enhanced cytotoxicity was attributed to an increase in apoptotic activity and autophagy activity after the treatment with imiquimod-loaded nanoemulsion.

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Disclosure of interest

The authors report no conflicts of interest.

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4 ARTIGO 3

4. ARTIGO 3 - The use of chitosan as cationic coating or gel vehicle for polymeric nanocapsules: increasing penetration and adhesion of imiquimod in vaginal tissue

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Abstract

The human papillomavirus (HPV) infection, which is strongly related to cervical cancer, can be reduced by the topical application of imiquimod. Some strategies have been used to increase the adhesion and penetration of drugs through the vaginal mucosa. Two of them are the development of mucoadhesive semisolid formulations and the development of polymeric nanocarriers. In this paper, we hypothesize that the combined use of these two strategies results in a better performance of the formulation to retain imiquimod into the vaginal tissue. Aiming this, two different systems are proposed: a) chitosan-coated poly(ε-caprolactone)-nanocapsules incorporated into hydroxyethylcellulose gel (HEC-NCimiq-chit), and b) poly(ε-caprolactone)-nanocapsules incorporated into chitosan hydrogel (CHIT-NCimiq). These formulations were submitted to three main tests: mucoadhesivity by interaction, permeation and washability test (or retention test). We developed an integrative index that allows comparing the global performance of the proposed formulations by considering jointly the results of these three tests. When considered the integrative indexes for the formulations, our results show that CHIT- NCimiq presents the best performance for the treatment of HPV.

Keywords: Polymeric Nanocapsules; imiquimod, mucoadhesion, chitosan

4.1 INTRODUCTION

Cervical cancer, the second most common neoplasia among women, is strongly associated with the infection caused by the human papillomavirus (HPV) (AYRES et al., 2010). HPV infection in patients with external genital warts can be reduced by the topical application of imiquimod (KOUTSKY et al., 1983). Imiquimod, an immune response modifier, induces cytokines and enhances the antiviral activity in vivo in guinea pigs (MILLER et al., 1999 and DAHL, 2000). However, some adverse effects are related to the use of commercial imiquimod ointments, such as erythema, ulceration, swelling, and edema in addition to systemic reactions like fatigue, fever, flu-like symptoms, peripheral nervous system disorders, headache and diarrhea (WIELAND et al. 2006; KREUTER et al., 2008).

The treatment of local diseases has several advantages when performed directly on the vaginal tissue (VALENTA et al., 2005). For instance, it allows achieving good permeability of many drugs and a high blood supply, it avoids first pass metabolism in the liver, and to decrease the incidence and severity of gastrointestinal adverse effects (BALOGLU et al., 2009). On the other hand, a limitation of this via is the low residence time of the drug due to the vaginal flow that removes the formulation from the mucosa, resulting in lower drug absorption and distribution levels (VALENTA et al., 2005; CARAMELLA et al. 2015). Therefore, the more mucoadhesive a formulation is, the more it should resist to the presence of such flow, maintaining a drug for more time in interaction with the mucosa (Das NEVES et al., 2006; PERIOLI et al.,2008).

Conventional formulations, such as gels (SUTTON et al., 2012), tablets (CEVHER et al., 2014) and suppositories (GERAGHTY et al., 1996) for vaginal application were already developed and analyzed regarding their muchoadhesion. Additionally, some new strategies have been proposed to deal with such limitation in order to increase the adhesion and penetration of drugs through the vaginal mucosa (VÀNIC et al., 2013; GARG et al., 2010), like the development of semisolid mucoadhesive formulations and the development of polymeric nanocarriers (PERIOLI et al., 2008; PERIOLI et al.,2009; FRANK et al., 2014).

Semisolid mucoadhesive formulations are able to prolong drug residence within the vaginal cavity by means of physical and chemical bonds with the mucosa (VÀNIC et al., 2013; CARAMELLA et al ., 2015), while the use of polymeric nanocarriers increases the interaction of drugs with the mucosa (ALUKDA et al., 2011; SANTOS et al., 2013; FRANK et al.,2014). Submicrometric polymeric nanocapsules are drug delivery systems that have been widely studied (SOPPIMATH et al., 2001; BRIGGER et al., 2002., GARCIA-GARCIA et al, 2005, WONG et al., 2007). Those nanocarriers have advantages over conventional formulations of controlling the drug release (SOPPIMATH et al., 2001), reducing adverse effects related to the drug (CONTRI et al, 2014), increasing penetration and adhesion of the encapsulated drug (FRANK et al., 2014; CONTRI et al., 2014). Lipid-core nanocapsules loading imiquimod demonstrated to be promising to treat basal cell carcinoma (VENTURINI et al., 2015), besides being non-toxic to keratinocytes cell lines (HaCaT). In order to increase the interaction of nanocarriers with negatively-charged surfaces, the use of nanoparticles having positive surface charge has been proposed for tenofovir and clotrimazole delivery (MENG et al., 2011; SANTOS et al., 2013). The ability of those systems to adhere to mucosal surfaces is desirable because it increases the residence time of drugs and their bioavailability (RAMINIENI et al., 2013).

Considering the above mentioned contributions of both strategies to increase the adhesion and penetration of drugs through the vaginal mucosa, i.e. the development of mucoadhesive semisolid formulations and the development of polymeric nanocarriers, we hypothesize that the combined use of both strategies could result in a better performance of the formulation to retain and delivery imiquimod into the vaginal tissue. To verify this hypothesis, the present study proposes to develop two different systems for vaginal application of imiquimod: a) chitosan-coated poly(ε-caprolactone)-nanocapsules incorporated into hydroxyethylcellulose gel, and b) poly(ε-caprolactone)-nanocapsules incorporated into chitosan hydrogel. We also studied the aqueous dispersion of chitosan-coated poly(ε-caprolactone)-nanocapsules and the aqueous dispersion of poly(ε-caprolactone)-nanocapsules to compare the performance of the formulations. As a result, this investigation presents an innovative way to compare the

proposed formulations in terms of drug mucoadhesion and penetration into the vaginal tissue. Moreover, this work also shows, by evaluating the formulation effect on HPV cells in vitro, which of them presents the best effect to treat HPV.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Poly(ε-caprolactone) (PCL) (Mn 80 kg mol^{-1}), sorbitan monostearate (Span 60®) and capric/caprylic triglyceride (CCT) were purchased from Sigma-Aldrich (Steinheim, Germany). Polysorbate 80 (Tween 80®) was purchased from Henrifarma (São Paulo, Brasil) and Copaiba oil was kindly donated by Inovam-Da Lamarta & cia Ltda. Imiquimod (IMQ) was purchased from Chemical Goods (Guangdong, China). Soybean lecithin (Lipoid S75®) was obtained from Lipoid (Germany). Chitosan (medium molecular weight, deacetylation degree of 77%) and hydroxyethylcellulose (high molecular weight) were obtained from Sigma-Aldrich (São Paulo, Brazil) and Embacaps (Porto Alegre, Brazil), respectively. All solvents and reagents were of analytical or pharmaceutical grade.

4.2.2 Methods

4.2.2.1 *Production of chitosan-coated imiquimoid-loaded nanocapsules dispersed in water and incorporated in hydroxyethylcellulose gel*

The nanocapsules were produced by the interfacial deposition of preformed polymer method (VENTURINI et al., 2015). Briefly, an organic phase consisting of imiquimod (5 mg), PCL (100 mg), copaiba oil (334 mg) and Span 60® (38.4 mg) dissolved in acetone (25 mL) was added of an ethanolic solution (3 mL) containing 60 mg of Lipoid S75®. The solution was maintained under magnetic stirring at 37ºC during 15 minutes. Then, the organic phase was injected into an aqueous phase (53 mL) containing polysorbate 80 (76 mg). After 10 min, the turbid solution was evaporated under reduced pressure in a rotative evaporator at 37ºC (Büchi, Switerland) to

approximately 9 mL. The white bluish dispersion of nanocapsules was adjusted in a volumetric flask (10 mL) to reach an imiquimod concentration of 0.5 mg mL^{-1}. Nine milliliters of the white bluish dispersion of nanocapsules were dropwise added of 1 mL of a solution at 1% chitosan in 1% acetic acid under stirring. The reaction was maintained for 4 hours (BENDER et al., 2012). The chitosan-coated imiquimod-loaded nanocapsules were named $NC_{imiq\text{-}chit}$. In parallel, a nanocapsule formulation prepared without imiquimod was similarly produced and used as control in the cytotoxicity experiment. This formulation was named NC_{chit}.

The hydroxyethylcellulose gel incorporating nanocapsules was prepared using the methodology proposed by Contri and co-workers (2014). Briefly, $NC_{imiq\text{-}chit}$ (10 mL) was added of 200 mg of hydroxyethylcellulose. The mixture was maintained under refrigeration at 4ºC for 48 hours before performing a manual homogenization and gel formation. The formulation was named HEC-$NC_{imiq\text{-}chit}$ (hydroxyethylcellulose gel containing chitosan-coated nanocapsules).

4.2.2.2 *Production of imiquimoid-loaded nanocapsules dispersed in water and incorporated in chitosan hydrogels*

Imiquimoid-loaded nanocapsules dispersed in water were also produced by interfacial deposition of polymer. Briefly, an organic phase consisted of imiquimod (5 mg), PCL (100 mg), copaiba oil (334 mg) and Span 60® (38.4 mg) dissolved in acetone (25 mL) was injected into an aqueous phase (53 mL) containing polysorbate 80 (76 mg). After 10 min, the turbid solution was evaporated under reduced pressure in a rotative evaporator at 37ºC (Büchi, Switerland) to approximately 9 mL. The white bluish dispersion of nanocapsules was adjusted in a volumetric flask (10 mL) to reach an imiquimod concentration of 0.5 mg mL^{-1}. This nanocapsule formulation was named NC_{imiq}. In parallel, a nanocapsule formulation prepared without imiquimod was similarly produced and used as control in the cytotoxicity experiment. This formulation was named NC.

The chitosan hydrogel containing nanocapsules was prepared as previously proposed by Frank et al. (2014) adding with manual mixing 115 µL of 85% lactic acid

and 250 mg of chitosan in nanocapsule aqueous dispersion (10 mL). The formulation was named CH-NC_{imiq} (chitosan hydrogel containing imiquimod-loaded nanocapsules).

4.2.2.3 *Characterization of nanocapsules and hydrogel formulations*

The nanocapsule aqueous dispersions were evaluated regarding their pH, particle size distribution and zeta potential immediately after production. The pH values determined by potentiometry (B474 Micronal), directly inserting the electrode into the formulation without dilution. Particles sizing were carried out by laser diffraction (Mastersizer 2000, Malvern) inserting a sample of the nanocapsule aqueous dispersion into the wet sample dispersion unit (Hydro 2000SM - AWM2002, Malvern) containing about 100 to 150 mL of distilled water (2,000 rpm), and by dynamic light scattering (Zetasizer Nano ZS, Malvern) after diluting the sample (500-times) using pre-filtered ultrapure water. Zeta potential was determined by electrophoretic mobility (Zetasizer, Nano ZS, Malvern) after diluting the formulation (500 times) in pre-filtered 10 mmol L-1 NaCl aqueous solution.

The hydrogel formulations had their characteristics evaluated by potentiometry (B474, Micronal), rheology (rotatory viscosimetry LVDV-II +Pro, spindle SC4-25, Brookfield, USA) and laser diffraction (Mastersizer 2000, Malvern). The pH values were measured by mixing the gels with ultrapure water (1:10, w/v). The viscosity values were determined at 37ºC, and the presence of nanocapsules in the hydrogels was evaluated after diluting 200 mg of hydrogel in 150 ml of distilled water.

The drug content (n=3) was determined after the imiquimod extraction from the nanocapsule aqueous dispersions and from the hydrogels by High Performance Liquid Chromatography with detection in the ultraviolet (HPLC-UV, Series 200, PerkinElmer, Waltham, MA, USA). The quantification method was adapted from De Paula and co-workers (2008), and validated according to our purposes. A C18 reversed phase column (Merck & Co, Inc, Whitehouse Station, NJ, USA) was used as stationary phase and acetonitrile:acetate buffer (pH4.0; 100mM):diethylamine (30:69:85:0.15 v/v), as mobile phase. An injection volume of 20 µL was used. Calibration curves (n = 3) were made to

determine the drug concentration showing linearity (r = 0.998) in the range of 1 to 25 µg mL-1.

$$EE\% = \frac{C_t - C_{free}}{C_t} \times 100 \quad (1)$$

were C_t is the drug content and C_{free} is the imiquimod concetration determined by HPLC-UV in the ultrafiltrate, which was obtained by using the ultrafiltration/centrifugation technique (Ultrafree-MC 10,000 MW, Millipore, Billerica, USA) at 4,120 ×*g* for 10 min.

4.2.2.4 *Cytotoxicity using SiHa cells*

For the cytotoxicity study (*in vitro* antiproliferative study), 9.0×10^3 SiHa cells per well were cultured in 96-well plates in culture medium for 48 hours to achieve semi-confluence. The formulations were maintained in contact with the cells for a period of 24 hours (n=3). Then, the treatments were removed and an MTT solution (0.5 mg·mL^{-1}) was added followed by a 3 hour incubation at 37°C (FONTANA et al., 2014; VENTURINI et al., 2015). The MTT salt was reduced to formazan crystals by the viable cells. Then, the formed crystals were solubilized in DMSO and measured at 570 and 630 nm in a microplate reader (Spectramax M2e and v 5.4.1; SoftMax Pro Software Interface; Molecular Devices, Sunnyvale, CA, USA). The values were converted to cellular viability, considering 100% as the control with no treatment. The cytotoxicity was analyzed by the MTT assay after 24 hours of incubation. For the MTT assay, we considered two levels of concentration (1.5 µmol L^{-1} and 3.0 µmol L^{-1}) of NC_{imiq} and $NC_{imiq\text{-}chit}$, as well as a free concentration after 24 hours of treatment. The cell viability for the treated groups was calculated based on the cells with no treatment, which represented 100% of viability (FONTANA et al., 2014; VENTURINI et al., 2015). To test the differences between these formulations, an ANOVA test and pairwise comparisons were used.

4.2.2.5 *In vitro studies using vaginal mucosa*

Nanocapsule aqueous dispersions and hydrogels containing nanocapsules were analyzed in terms of *in vitro* mucoadhesion, washability and imiquimod penetration into the vaginal mucosa. The porcine vaginal mucosa was obtained from a local slaughterhouse (Santo Ângelo Ltda., Brazil). All measurements were made using fresh mucosa to assure the properties and viability of the tissue.

4.2.2.6 *Mucoadhesion measurements*

The samples (NC_{imq}; $NC_{chit\text{-}imiq}$; CH-NC_{imq}; HEC-$NC_{chit\text{-}imiq}$) were subjected to mucoadhesion measurements by means of a tensile stress tester (TA.XTplus Texture Analyzer; Stable Microsystem, Godalming, UK). The samples were placed in a container and the discs of porcine vaginal mucosa (10 mm) were attached to the movable probe with double-sided tape. Each piece of mucosa (n=3) was put in contact with the samples using a force of 0.29N during 180 seconds (Frank et al., 2014). The probe was then removed by a constant rate of 0.10 mm s^{-1} until achieving a complete detachment. Peaks of force (mN), displacement (mm) and work of mucoadhesion (mN mm) were registered by the equipment software.

4.2.2.7 *Washability test into porcine vaginal mucosa*

The effect of vaginal flux on adhesion of nanocapsules containing imiquimod was investigated by means of a washability test in pig vaginal mucosa (n=3) (Frank et al., 2014; Chaves et al., 2016). The samples (NC_{imiq}; $NC_{chit\text{-}imiq}$; CH-NC_{imiq}; HEC-$NC_{imiq\text{-}chit}$), as well as imiquimod dissolved in acetate buffer pH 3.7 (Venturuni et al., 2015) ($IMIQ_{free}$) were subjected to washability measurements with a modified manual Franz diffusion cell (FRANK et al., 2014; CHAVES et al., 2016). The vaginal mucosa (0.9 cm^2) was placed between the donor and the receptor compartment, which was filled with 50 µL of the formulations (nanocapsules, $IMIQ_{free}$ or hydrogels). After 1 hour of interaction (CONTRI et al., 2014; CHAVES et al., 2016), acetate buffer pH 4.0 (37°C) was fluxed to simulate action of vaginal flux (VALENTA et al., 2005). The flux was set at 0.4 mL min^{-1} (FRANK et al., 2014) (HPLC pump; Gilson; Minipuls 3, France) and

was collected at predetermined time intervals (5, 10, 15, 20, 25, 30, 60, 120 and 180 minutes). The receptor was an acetate buffer pH 4.0 previous filtered using a hydrophilic membrane (0.45 µm, Millipore®). IMIQ was determined by the HPLC-UV method mentioned above. For drug content, samples of imiquimod solution were directly analyzed and samples of nanocapsules were subjected to extraction process with acetonitrile and methanol (1:1 v/v).

4.2.2.8 *Imiquimod permeability into porcine vaginal mucosa*

The permeation of imiquimod was evaluated using the same formulations described in the previous subsection (washability test). Similarly, to the previous experiment, a modified Franz diffusion cell was used with a receptor volume of 2.5 mL and diffusional area of 0.9 cm^2. The vaginal mucosa was placed between donor and receptor compartment. The receptor was acetate buffer pH 4.0 previous filtered using a hydrophilic membrane (0.45 µm, Millipore®). The donor compartment received 100 µL or 100 mg of the formulations. The cells were maintained in a bath at 37°C under mild agitation. A sink condition was maintained during the experiment. At predetermined time intervals (2, 4, 6, 8, 12 and 24 hours), 40 µL of the receptor medium was withdrawn and directly analyzed by HPLC-UV method as for the washability test.

4.2.2.9 *Efficacy study using cervical carcinoma cell line*

The cervical carcinoma cell line SiHa (American Type Culture Collection, Rockville, MD), was used to verify the capability of the formulations proposed to act on cancer cells. The cells were maintained in low glucose DMEM supplemented with 10% FBS and penicillin/streptomycin antibiotics (0.5 U·mL/95% air at 37°C). The cell cultures were treated after achieving sub-confluence with the formulation NC_{imiq} and $NC_{imiq\text{-}chit}$. As controls, formulations without the presence of the drug (NC and NC_{chit}) were used. The free drug (drug solution in DMSO at the same concentration of the nanoformulations) and DMSO were also used as controls. The cultures were exposed to the formulations for 24 hour at two different concentrations (15 and 30 µmol L^{-1} of

nanocapsule aqueous dispersions in culture media). All materials were previously sterilized, and the nanocapsule suspensions were prepared under aseptic conditions.

4.2.2.10 *Data analysis and comparison procedures*

Statistical analyzes were performed by means of Analysis of Variance (ANOVA) followed by the post-hoc Tuckey's test for multiple comparison ($\alpha = 0.05$). The software SPSS statistics version 17.0® was used for the statistical analyses.

Moreover, after obtaining the statistical results for the experiments performed, a comparison of the formulations based on the three main experiments (mucoadhesivity, imiquimod retained and permeability) was conducted. This comparison aimed to identify which one of the formulations has the best overall performance for the treatment of HPV. However, the three experiments are evaluated in different scales according to their own nature (Mucoadhesivity: mN.mm; IMIQ retained: % of retention; Permeability: %.mm^2). Consequently, those scales cannot be directly summed to obtain an integrative index that allows comparing the overall performance of each formulation. Hence, it is necessary to convert the values into normalized scales, e.g. ranging from 0 to 1 each one, while maintaining a form of proportionality with the original scales. This was made based on similar suggestions from other fields (e.g. KRAJNC and GLAVIČ , 2005; FRANK et al., 2016), using a linear scaling transformation (normalization) according to Equations 2, 3 and 4.

$$p'_i = \frac{p_i}{\sum_{i=1}^{4} p_i} \quad (2)$$

$$m'_i = \frac{m_i}{\sum_{i=1}^{4} m_i} \quad (3)$$

$$IM'_i = \frac{IM_i}{\sum_{i=1}^{4} IM_i} \quad (4)$$

where:

p'_i; m'_i and p'_i represent the normalized values of the three tests: permeability, mucoadhesivity and IMIQ retained, respectively for each one of the four *i* formulations.

p_i; m_i and p_i represent the original values of the three tests: permeability, mucoadhesivity and IMIQ retained, respectively for each one of the four *i* formulations.

According to these equations (Eq. 2, 3 and 4), the sum of all normalized values of the four formulations in each one of the experiments is equal to 1 (or 100%). This means that, for each experiment, the original values of the four formulations were transformed to a factor of contribution to the total range of outputs obtained, showing how high or low is a value compared to the others in the same normalized scale. Moreover, the normalization resulted in three scales, one for each test, with the same range of variation (0 to 1). This means that when the scales are integrated, all them have the same weight in the sum, avoiding biased results (FRANK et al., 2016). Illustrating this, if the original nominal values would be summed, the experiment with higher nominal values would weight strongly in the integrative index. On the other hand, when using the proposed normalized values, it is assumed that the three tests are equal in terms of relevance to define the overall performance, since they have the same weight in the sum.

After transforming the scales, the normalized values for the four formulations were represented in a radar plot. This plot helps to the visual comparison of the performance in three axes representing the three experiments. Finally, the integrative index of the overall performance for each formulation was calculated as a vector sum of the three axes of the radar plot (Equation 5):

$$r_i = \sqrt{p_i'^2 + m_i'^2 + IM_i'^2} \quad (5)$$

where:

r_i represents the integrative index for the formulation *i.*

p_i represents the normalized value of permeability for the formulation *i.*

m_i represents the normalized value of mucoadhesivity (interaction) for the formulation *i.*

IM_i represents the normalized value of IMIQ retained for the formulation *i.*

Equation 5 provides an integrative index (r_i) for each proposed formulation: HEC-NCimiq-chit, CHIT-NC_{imiq}, NC_{imiq} and $NC_{imiq\text{-}chit}$. The four final indexes can be compared to conclude which formulation presents the best overall performance. It is important to highlight that the best performer does not mean that it shows the best result for all dimensions (tests), but that it is the best balanced in the three dimensions evaluated.

4.3 RESULTS AND DISCUSSION

4.3.1 Production of nanocapsule aqueous dispersions

Imiquimod-loaded nanocapsule formulations coated or uncoated with chitosan (NC_{imiq} and $NC_{imiq\text{-}chit}$) were produced as white bluish liquids presenting homogenous macroscopic aspect. Control formulations (NC and NC_{chit}) were also produced with similar aspect. Laser diffraction analysis showed unimodal size distributions for all formulations (NC, NC_{chit}, NC_{imiq}, $NC_{imiq\text{-}chit}$). The volume-weighted mean diameters (D[4,3]) and the diameters at percentiles 10, 50 and 90 under the size distribution curves by volume and by number of particles were plotted in a radar chart (Figure 19). The shape of the curves in the radar chart are fingerprint characteristic of unimodal size distributions, as previously determined for different polymeric nanocapsules (BIANCHIN et al., 2015). All the formulations had d(0.9)v lower than 800 nm and d(0.5)n lower than 300 nm. For the nanocapsules prepared using an ethanolic solution of lecithin, D[4,3]v were 204 and 286 nm, while for the nanocapsules produced without lecithin and using only acetone as organic phase, D[4,3]v were 339 and 408 nm. The use of lecithin and ethanol affects the size distribution profile, as previously observed for chitosan-coated nanocapsules (prepared using CCT), which D[4,3]v was 134 nm (BENDER et al., 2012). The higher D[4,3]v observed for NC_{chit} and $NC_{imiq\text{-}chit}$ compared to the previously reported study (BENDER et al., 2012) is a consequence of the PCL molecular weight, respectively Mn of 80 kg mol^{-1} and 10 kg mol^{-1}, which affects the viscosity of the organic phase. The higher is the molecular weight the higher is the viscosity of the solution, which produces higher mean diameters of particles.

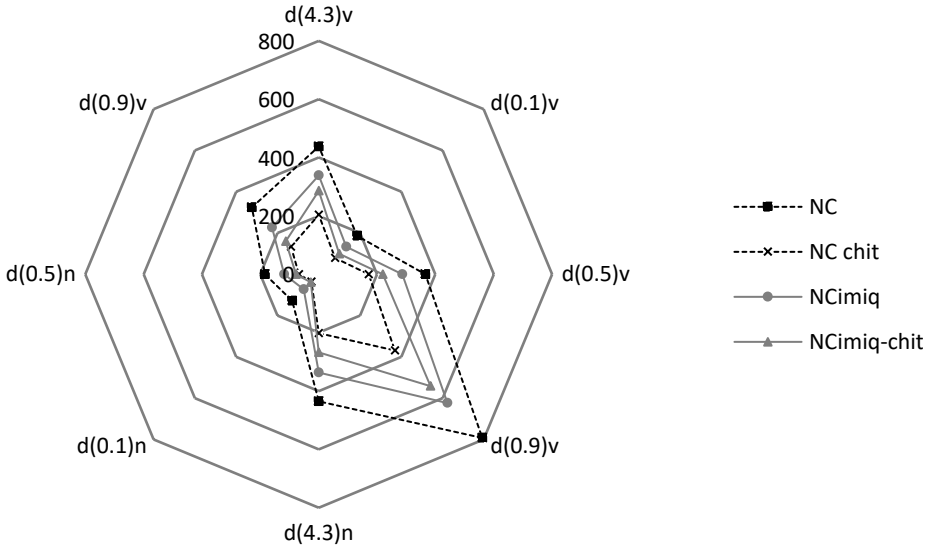

Figura 21 Radar chart presenting the volume-weighted mean diameters (D[4,3]) and the diameters at percentiles 10, 50 and 90 under the size distribution curves by volume and by number of particle.

Dynamic light scattering showed hydrodynamic mean diameters of 199.3±16 nm (NC_{imiq}), 213±10 nm ($NC_{imiq\text{-}chit}$), 223±11 nm (NC), and 209±13 nm (NC_{chit}) with polydispersity indexes of 0.12±0.09, 0.13±0.08, 0.15±0.09 and 0.14±0.07, respectively. The results demonstrated that all nanocapsule aqueous dispersions have narrow unimodal size distributions (data not shown) with high homogeneity of sizes. The encapsulation of imiquimod did not affect the size distributions of uncoated or chitosan-coated nanocapsules. The zeta potential of NC_{imiq} was -10.2±0.3 mV, which value is in accordance with other formulations prepared with poly(ε-caprolactone), as wall, and polysorbate 80, as coating material (CHAVES et al., 2017). The non-ionic surfactant (polysorbate 80) stabilizes the colloidal dispersion by a steric hindrance mechanism (MORA-HUERTAS et al., 2010). On the other hand, the zeta potential of $NC_{imiq\text{-}chit}$ was +15.3±0.4 mV, which reflected the chitosan coating by interaction with Lipoid S75® (BENDER et al., 2012). The zeta potential NC and NC_{chit} were similar to those determined for the imiquimod-loaded nanocapsules.

The pH values for the nanocapsule aqueous dispersions were 6.3±0.05 for NC_{imiq} and 6.2±0.03 for NC. For the chitosan-coated nanocapsule suspensions, the pH values

were 5.0±0.07 for $NC_{imiq\text{-}chit}$ and 5.1±0.04 for NC_{chit}. The lower pH values observed for the latter formulations are due to the use of acetic acid aqueous solution to dissolve chitosan for the coating step of nanocapsules synthesis.

The drug content showed 0.48±0.06 and 0.49±0.08 mg mL^{-1} of imiquimod in NC_{imiq} and $NC_{imiq\text{-}chit}$, respectively. Regarding the encapsulation efficiency, NC_{imiq} presented 97±0.9% of imiquimod retained in the dispersed phase, while $NC_{imiq\text{-}chit}$ presented 57±0.7%. The lower EE% determined for the latter is probably due to the difference of pH of the formulations, since the logarithm of imiquimod distribution (log D) at pH 6 is 1.15, whereas log D at pH 5 is 0.97. Consequently, the mechanism of encapsulation can be affected and the drug is differently partitioned in the formulation (OLIVEIRA et al., 2013). Log D shifting from a value higher than 1 to another lower than 1 means that the molecules of drug are more partitioned for the aqueous phase in the second case.

4.3.2 Cytotoxicity using SiHa

According to the results of cytotoxicity study shown in Figure 20, the comparison between NC_{imiq} at 1.5 (66%) and 3.0 µmolL^{-1} (60%) did not show significant statistical difference (p=0.623). This means that no increase of the cell viability percentage was observed when the drug concentration was augmented from 1.5 to 3.0 µmolL^{-1} in the treatment. However, when both concentrations of NC_{imiq} are compared to the drug-unloaded nanocapsules (NC) it is possible to verify statistical differences: p=0.016 for the comparison between NC (93%) and NC_{imiq} 1.5 µmolL^{-1} (66%); and p=0.006 for the comparison between NC (93%) and NC_{imiq} 3.0 µmolL^{-1} (60%). This indicates that there is a significant decrease in the cellular viability when the polymeric nanocapsules are loaded with imiquimod. Similar results are observed for $NC_{imiq\text{-}quit}$ with no significant differences between the concentration levels evaluated (p=0.164 for the comparison between 1.5 and 3.0 µmolL^{-1}). However, when compared both concentrations of $NC_{imiq\text{-}quit}$ with the drug-unloaded nanocapsule (NC_{quit}), the concentration of 3.0 µmolL^{-1} showed statistical difference (p=0.072 between $NC_{imiq\text{-}quit}$ 90% and NCimiq 3.0 µmolL^{-1} 60%), while the concentration of 1.5 µmolL^{-1} did not show significant differences

because of a higher level of variance (p=0.189 between $NC_{imiq\text{-}quit}$ 90% and NCimiq 1.5 µM 69%). Additionally, a comparison of means between both formulations (NC_{imiq} and $NC_{imiq\text{-}chit}$) was performed for each level of concentration. This was made by means of a T-test. In this case, the analysis did not report statistical significance between both formulations at each concentration level (1. $\mu molL^{-1}$, p=0.398; 3.0 $\mu molL^{-1}$, p=0.211).

Consequently, in a general sense, these results indicate that in terms of cytotoxicity the formulations NC_{imiq} and NC_{quit} present similar characteristics and that in both cases, the results show that there is a decrease in the cells viability when imiquimod is incorporated to the nanocapsules. In addition, it is important to observe that the polymeric nanocapsules without drug did not decrease significantly the cellular viability when compared to the control group. Also, the free drug in the highest concentration used was not able to decrease the viability of the tumor cells in the same way as observed for the encapsulated imiquimod. Therefore, we demonstrate that the nanoencapsulation increases the drug performance in cervical cancer cells.

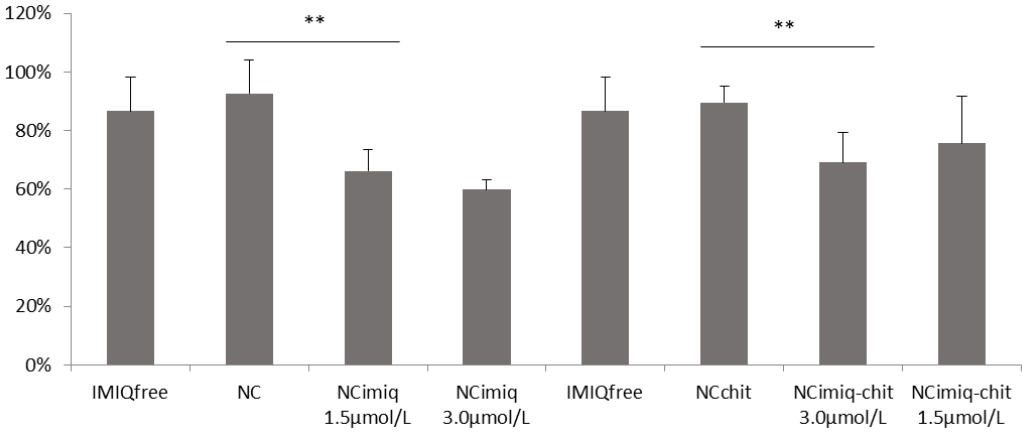

Figura 22 SiHa cell viability after 24 hours of treatment using MTT assay. Statistical differences were considered for p<0.05 (n=3)

Previously, we demonstrated that NC_{imiq} did not show cytotoxicity in HaCaT keratinocytes cell line (VENTURINI et al., 2015), and at the present study we showed that NC_{imiq} is a promising formulation to decrease the viability of vaginal tumor cells. Both cytotoxicity studies are complementary, and taken together they suggest that this formulation is safe and efficient to be study in further preclinical trials.

4.3.3 Production of hydrogel formulations containing nanocapsules

Both hydrogel formulations (CHIT-NC_{imiq}; HEC-$NC_{imiq\text{-}chit}$) were characterized by means of pH, size distribution and viscosity immediately after preparation. The pH value was 4.5±0.1 for CHIT-NC_{imiq}, as previously shown (FRANK et al., 2014), while the pH of 5.8±0.1 was determined for HEC-$NC_{imiq\text{-}quit}$, which corroborates previous results (CONTRI et al., 2014). No particle agglomeration or aggregation occurred during the process of hydrogels production since the particle diameter profiles maintained the same nanometric sizes (Figure 21).

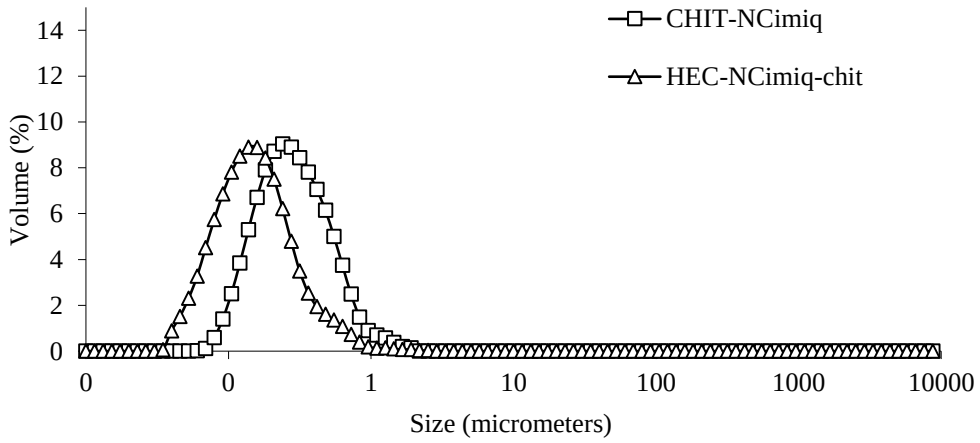

Figura 23 Particle diameter profiles determined by laser diffraction for hydrogels diluted in water: (A) HEC-NCimiq-chit and (B) CHIT-NCimiq

The viscosity of the hydrogels is an important property that must be taken into account in the development of drug delivery systems for the vaginal tissue, since the formulation should not be easily removed (FRANK et al., 2014; CARAMELLA et al., 2015). In this sense, the viscosity of the semisolid formulations was evaluated showing that similar viscosity was observed for both formulations (CHIT-NC_{imiq}=13857 pa.s and HEC-$NC_{imiq\text{-}chit}$=9898 pa.s for a shear rate of 1.76) corroborating previous findings (CONTRI et al., 2014). In addition, it was verified that the viscosity decreased when the shear rate was increased, suggesting pseudoplastic behavior as already described (CONTRI et al 2014).

4.3.4 Evaluation of the mucoadhesion properties

The mucoadhesion of the formulations can be verified by different ways. At first, it is important to verify the interaction between the formulation and the mucosa, meaning how adhesive the formulation is when in contact with the mucosa (CARAMELLA et al., 2015). However, the first analysis does not consider the removal of the formulations when administered in the vaginal tissue by the vaginal fluid. So, the washability is a second form to evaluate the mucoadhesion of the formulations when the flow is applied on the formulation after it was applied on the vagina. The different ways mentioned provide complementary results.

The mucoadhesion of the imiquimod-loaded nanocapsules (NC_{imiq} and $NC_{imiq\text{-}chit}$) were evaluated with the vaginal mucosa, as well as the mucoadhesion of the chitosan and hydroxyethylcellulose hydrogels (CHIT-NC_{imiq} and HEC-$NC_{imiq\text{-}chit}$). For the first mucoadhesion analysis the formulations were submitted to a tensile stress tester that measures the force, displacement distance and work needed to detach the nanocapsules and gels from the vaginal mucosa. Figure 22 shows the average force required to separate the formulations from the mucosa, as well as the formulations displacement distance achieved until they were detached from the mucosa. The area under the curve in each curve represents the work (*i.e.* the product of force and distance) necessary to detach a formulation from the mucosa, which represents the mucoadhesive properties of each formulation (FRANK et al., 2014). Regarding the nanocapsule dispersions, NC_{imiq} presented a longer distance of detachment, as well as a stronger average force required to achieve the detach point when compared to $NC_{imiq\text{-}chit}$. For the imiquimod-loaded nanocapsule aqueous dispersions (NC_{imiq} and $NC_{imiq\text{-}chit}$), the values of work (W) were 22.3 mN mm and 17.1 mN mm, respectively, while for the hydrogels (CHIT-NC_{imiq} and HEC-$NC_{imiq\text{-}chit}$) the values of work (W) were 53.0 mN mm and 78.0 mN mm, respectively. The statistical analysis of the work calculated for NC_{imiq} and $NC_{imiq\text{-}chit}$ did not show significant difference between the formulations ($p=0.613$). Therefore, we concluded that the particle coating with a cationic polymeric (chitosan) did not increase the adhesivity within the period of the experiment.

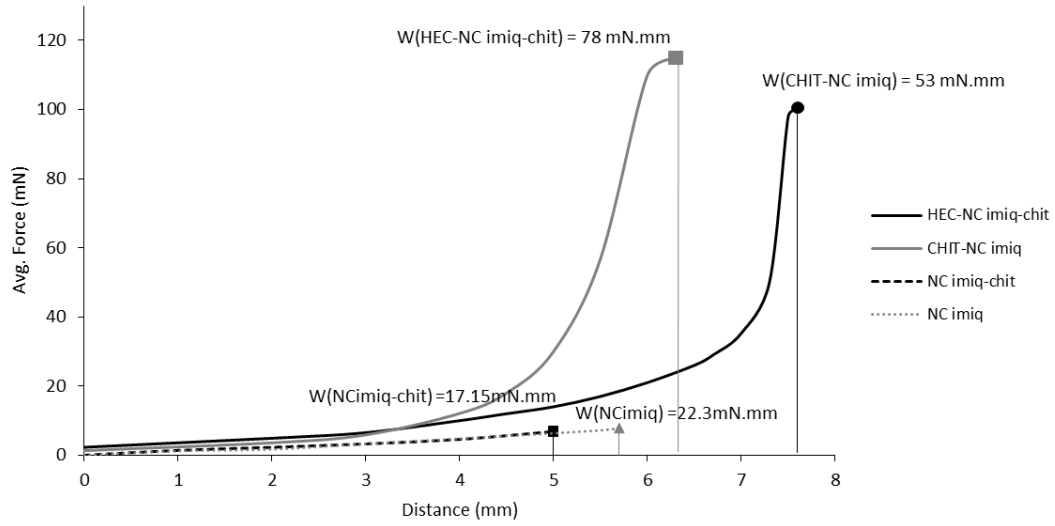

Figura 24 Distance of stretching to detach from the mucosa after 180 seconds of contact mucosa/vagina. Statistical differences were considered for $p<0.05$ (n=3)

Considering the hydrogels behavior, HEC-$NC_{imiq-chit}$ had a shorter detach distance but the required force of detach was stronger than that observed for CHIT-NC_{imiq} (Figure 22). Furthermore, there is a significant difference ($p=0.012$) between the hydrogels: HEC-$NC_{imiq-chit}$ presented a higher mucoadhesion than CHIT-NC_{imiq}. This means that, in terms of material properties, chitosan has a ductile behavior because a lower force was necessary to separate the formulation and because greater stretching distances were observed. On the other hand, hidroxyetylcelullose showed a less elastic behavior with a stronger permanence of contact with the vaginal tissue. Comparing the behavior among the hydrogels and the nanocapsules dispersed in water, the former formulations are significantly more adhesive to the vaginal mucosa than the latter formulations (more than twice). The result can be explained by the highest viscosity of the hydrogel formulations compared to the nanocapsule dispersions. Even though CHIT-NC_{imiq} has less adhesiveness, this formulation is more ductile than HEC-$NC_{imiq-chit}$, being a positive aspect for its application as a formulation for vaginal treatments. This is because this material characteristic will provide more adaptability of the formulation to the vaginal mucosa surface, while a lower elastic behavior as shown in HEC-$NC_{imiq-chit}$ may be less comfortable because it is not very malleable inside the vagina when submitted to natural movements of the body.

The washability experiment was the second study to analyze the mucoadhesive properties of the formulations. This experiment was performed for 180 minutes with the same formulations described above (NC_{imiq}, $NC_{imiq\text{-}chit}$, CHIT-NC_{imiq} and HEC-$NC_{imiq\text{-}chit}$). The analysis of hydrogels and nanocapsules dispersed in water are described in cumulative percentage of formulation washed during the considered time (Figure 23). In other words, the less percentage of formulation washed during a given time-interval the more adhesive the formulation is against the effect of the vaginal fluid.

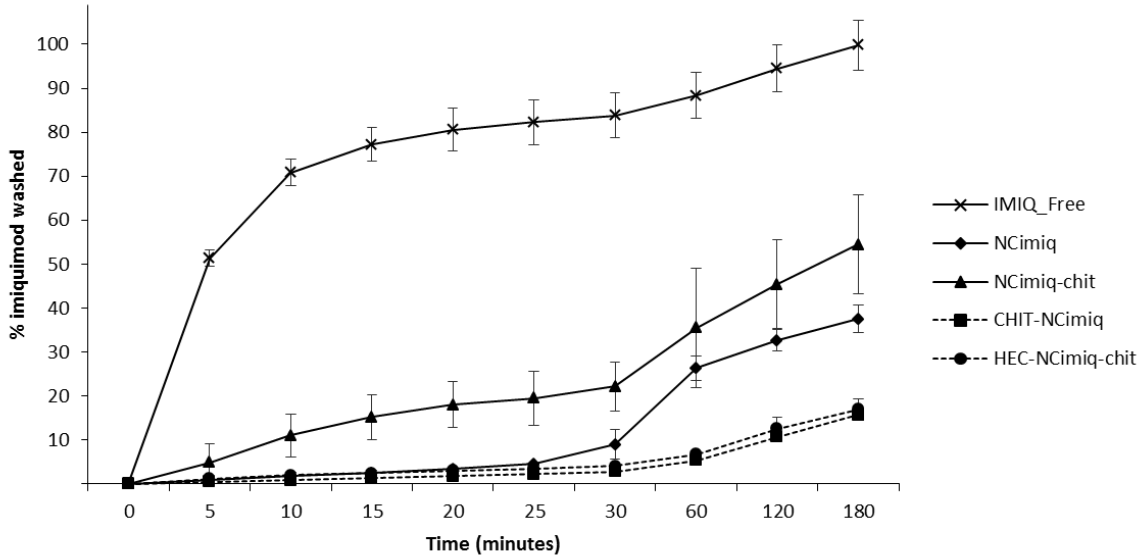

Figura 25 Imiquimod washability profiles after contact by 1 hour with vaginal mucosa. Statistical differences were considered for $p<0.05$ (n=3)

At first, aligned with the previous experiment of tensile stress, washability results (Figure 23) show that both hydrogels presented lower percentages of drug washed when compared with the nanocapsule aqueous dispersion. Indeed, hydrogels are more mucoadhesive against the vaginal fluid effects. Moreover, our results also showed that whether the drug is nanoencapsulated or in hydrogel, the formulations presented better performance than the free imiquimod ($IMIQ_{free}$), since for latter we observed the drug totally washed from the mucosa at 180 minutes.

NC_{imiq} and $NC_{imiq\text{-}chit}$ showed statistical difference within the entire period of experiment. At 5 and 60 minutes, those formulations showed differences at $p=0.005$,

while for the other time-intervals the differences are stronger ($p<0.001$). In this sense, NC_{imiq} presented a lower percentage of imiquimod washed, which means that NC_{imiq} is more mucoadhesive than $NC_{imiq\text{-}chit}$ against the effects of the simulated vaginal fluid. Thus, we discussed above that those formulations did not show differences in terms of stress, meaning that they can be similar when submitted to the same vaginal movements; on the other hand, there are differences in the time of permanence of both NC_{imiq} and $NC_{imiq\text{-}chit}$ submitted to the vaginal fluid. Therefore, NC_{imiq} is apparently the one presenting the highest mucoadhesion among the liquid formulations. Nevertheless, this formulation, prepared without chitosan-coating, presented lower washability probably because of the higher encapsulation efficiency of the drug, which reflected in the lower availability of the drug in the outer pseudo-phase of the formulation (aqueous phase).

These results corroborate prior findings regarding the adhesivity of polymeric nanocapsules (FRANK et al., 2014; CHAVES et al., 2016). It is interesting to observe that, for the free drug (imiquimod solution), after 30 minutes, imiquimod was washed at more than 80%. On the contrary, in the same time-interval, there was still possible to find more than 80% of the drug in the vaginal mucosa when formulations containing the nanocapsules were applied, *i.e.* less than 20% of imiquimod was removed. The results allow to conclude that the encapsulated drug might lead to a better efficacy than the free drug in the treatment of diseases using the vaginal via, since such encapsulated drug remain for longer time in contact with the mucosa.

On the other hand, comparing both hydrogels (HEC-$NC_{imiq\text{-}chit}$ and CHIT-NC_{imiq}), there was no moment where they showed statistical differences over the period of experiment. Consequently, in terms of behavior against the vaginal fluid effect they could be observed as similar, showing a good adhesitivity performance. However, as showed above, in terms of the behavior against simulated movements inside the vaginal mucosa, formulation HEC-$NC_{imiq\text{-}chit}$ has a better performance than CHIT-NC_{imiq}, which may be more appropriate in terms of application due to its ductility, resulting in a better applicability and patient compliance.

4.3.5 Influence of the imiquimod permeation through the vaginal mucosa

Imiquimod permeation using Franz cells are shown in Figure 24. We also calculated the areas under the curves shown in Figure 24, which represent the total amount of imiquimod permeated in 12 hours for each formulation: CHIT-NC_{imiq} (243.44 %.mm^2.h), $IMIQ_{free}$ (99.58 %.mm^2.h), NC_{imiq} (64.48 %.mm^2.h), $NC_{imiq\text{-}chit}$ (40.32 %.mm^2.h) and HEC-$NC_{imiq\text{-}chit}$ (15.94 %.mm^2.h). As observed in this figure and in the calculated total amount of permeated imiquimod for 12 hours, CHIT-NC_{imiq} showed the best performance, which is significantly higher than all the other formulations ($p<0.000$) during the complete period of 12 hours.

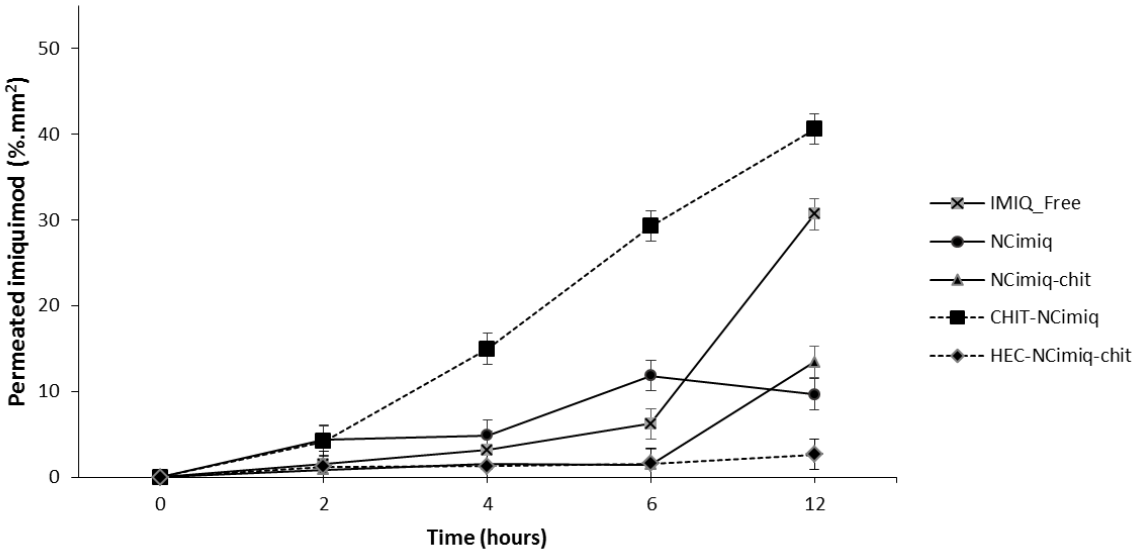

Figura 26 Imiquimod permeation into vaginal mucosa until 12 hours. Statistical differences were considered for $p<0.05$ (n=3)

It is important to highlight that CHIT-NC_{imiq} is composed by the drug in its encapsulated form (EE 97%). This can be the reason for the increment of the permeation, since the nanoparticles interact with the mucus present in the vaginal mucosa (FRANK et al., 2014; CARAMELLA et al., 2015). Moreover, the results suggest that the capacity of the chitosan to bind the mucosa can also be a reason for higher permeation of the drug for CHIT-NC_{imiq}.

These results are aligned with previous findings (FRANK et al., 2014), in which it was demonstrated that chitosan used as vehicle for the nanocapsules is capable of

increasing the Nile red penetration into the vaginal tissue compared to the free drug formulation, since chitosan interacts by electrostatic forces with the vaginal mucus. Frank and co-workers (2014) had already proposed the application of chitosan hydrogel for vaginal administration and observed that the gel was inducing the penetration of the incorporated Nile red specially when the dye was encapsulated into EUGRAGIT® RS 100 nanocapsules. In the present investigation, we observed that the chitosan gel containing nanocapsules is capable to sustain the permeation of imiquimod. This is desirable, since imiquimod is related to several adverse effects such as irritation, itching and pain (GUPTA et al., 2000) and, in more severe cases, to systemic reactions including fatigue, headache, fever, peripheral nervous system disorders, and diarrhea (WIELAND et al., 2006; KREUTER et al., 2008).

4.3.6 Comparison of the different formulations for the application in the vaginal tissue

The obtained results described in the previous subsections are summarized in the radar plot of Figure 25. This radar plot resumes three main results for the four formulations studied in this paper: mucoadhesivity by interaction, permeation and IMIQ retained (washability test). IMIQ retained was calculated as the complement percentage of the washability outputs. The original values presented in the past sections were the following: (i) Mucoadhesivity: HEC-$NC_{imiq\text{-}chit}$ (78 mN.mm), CHIT-NC_{imiq} (53 mN.mm), NC_{imiq} (22 mN.mm) and $NC_{imiq\text{-}chit}$ (18 mN.mm); (ii) Permeability (at 12h): HEC-$NC_{imiq\text{-}chit}$ (2.7%), CHIT-NC_{imiq} (41%), NC_{imiq} (10%) and $NC_{imiq\text{-}chit}$ (13.4%); (iii) IMIQ retained: HEC-$NC_{imiq\text{-}chit}$ (83%), CHIT-NC_{imiq} (84%), NC_{imiq} (37%) and $NC_{imiq\text{-}chit}$ (54%). In order to turn these values of the three dimensions comparable, we normalized the original values according to Equations 1 to 3, so that all scales range from 0 to 1 as shown in Figure 25.

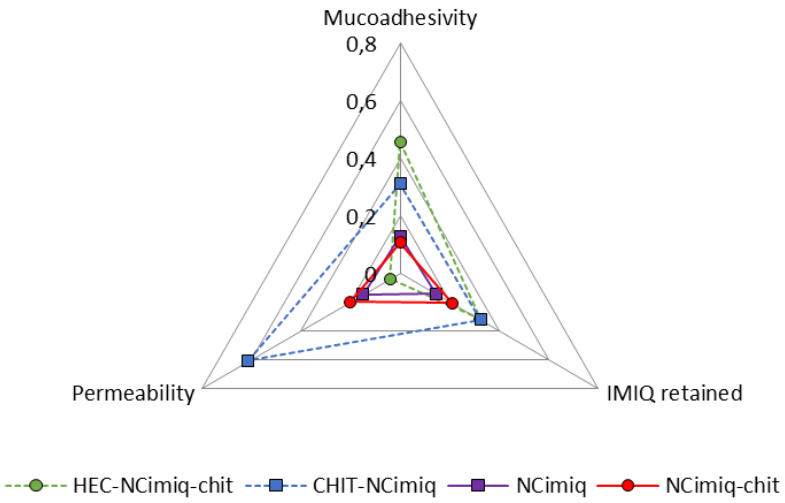

Figura 27 Radar plot presenting the relative mucoadhesivity, imiquimod retained after washability and permeability after 12 hours of experiment.

Considering these results (Figure 25), it is possible to see that HEC-$NC_{imiq\text{-}chit}$ is the best performer for both kinds of mucoadhesion (by interaction or called only as mucoadhesion and by IMIQ retained), while it is the worst in terms of permeability. On the other hand, the nanocapsules in suspension (NC_{imiq}; $NC_{imiq\text{-}chit}$) presented the lowest performances. However, CHIT-NC_{imiq} is the best balanced since it occupies the first place for mucoadhesion by washability and permeability as well as the second place in terms of mucoadhesion performance by interaction.

The normalized values presented in Figure 25 allow calculating a dimensionless integrative performance index that indicates the global performance of each formulation when the three dimensions are integrated. Using Equation 4 (Section 2.2.10), we obtained the following integrative indexes for the formulations: HEC-$NC_{imiq\text{-}chit}$ (0.559); CHIT-NC_{imiq} (0.758), NC_{imiq} (0.243) and $NC_{imiq\text{-}chit}$ (0.308). The calculated indexes corroborate that when the three dimensions are considered jointly, the formulation CHIT-NC_{imiq} showed the best performance for the treatment of HPV.

4.4 CONCLUSIONS

In the present investigation, we successfully developed imiquimoid-loaded nanocapsules uncoated or coated with chitosan. The demonstrated that nanoencapsulation increases the drug performance against tumor cells. The semisolid formulations prepared aimed to combine the strategies for increasing the drug contact with the vaginal tissue and our results showed that, indeed, they can increase mucoadhesion and drug penetration when compared to water dispersed formulations. Furthermore, hydroxyethyl cellulose gel containing coated nanocapsules was considered the most suitable for increasing mucoadhesion, while the chitosan-coated imiquimoid-loaded nanocapsules chitosan presented also suitable mucoadhesion and led to higher values of permeated drug into the vaginal tissue. Therefore, when permeation, mucoadhesion and drug retention are simultaneously considered, the formulation based on chitosan gel and drug-loaded polymeric nanocapsules showed the best promising performance for HPV treatment.

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Conflict of interest: The authors have no conflict of interest.

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5 ARTIGO 4

5. ARTIGO 4 - Imiquimod-loaded nanocapsules improves cytotoxicity in cervical cancer cell line

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Abstract

Nanotechnology can be used to improve drugs performance against tumor cell lines. Thus, this paper reports the development of imiquimod-loaded polymeric nanocapsules as a promising formulation against cervical cancer. This work evaluates the mechanism of death involved in the reduction of the cell viability of this novel formulation, as well as it evaluates the production of an inflammation marker (IL-6) after the treatment in cell line SiHa. The developed formulation has significantly decreased the viability of the cells in a time-dependent manner, after 24, 48 and 72 hours. Results showed a cellular decrease of almost 80% of the cells after 72 hours of treatment. The formulation induced death by apoptosis, necrosis, autophagy, and increased the percentage of SubG1subpopulation of SiHa cells after 72h. In addition, after the same time interval the formulation significantly prevented the appearance of colonies, showing effectiveness against SiHa. Finally, the formulation stimulated SiHa cells to release IL-6. These findings open new possibilities for the development of aqueous nanosuspension containing imiquimod as a novel strategy for the treatment of cervical cancer.

Keywords: Polymeric nanocapsules; imiquimod; HPV; cervical cancer.

5.1 INTRODUCTION

Cervical cancer is the second most common cancer among women (PARADKAR et al., 2014). Epidemiological studies have pointed a strong correlation between cervical cancer incidence and human papillomavirus (HPV) infection, which represents the most common sexually transmitted viral disease (AYRES et al., 2010). HPV is subdivided in low and high-risk according to their malignant potential and cell-transforming capacity in vitro. The subtypes of HPV low-risk, for example HPV 6 and HPV 11, are causative agents of genital warts, while the high-risk subtypes HPV 16 and HPV 18 are directly linked to the development of cervical cancer (PINOTTI et al., 2005). The best treatment for this infection is the one that improves the immune response against the virus. However, current treatments are painful and expensive, affecting significantly women's quality of life (PARADKAR et al., 2014).

Topical application of imiquimod has been shown to reduce the HPV load in patients with external genital warts and therefore it may be an alternative for the treatment of HPV-related diseases (KOUTSKY et al., 1983). While most of the immunomodulatory agents available or in development act as inhibitors of the pathways involved in immune activation, imiquimod is the only one that activates immune function (SAUDER et al., 2000). Imiquimod acts as an agonist of toll-like receptor (TLR) 7, activating both innate (monocytes, macrophages and dendritic cells) and adaptive cellular immunity (Th1) through the induction of pro-inflammatory cytokines, such as interferon alpha (IFN-α), tumor necrosis factor (TNF) and interleukins (IL) 1, 6, 8 and 12. In parallel, this drug induces cell apoptosis and activates B lymphocytes, potentializing the immune response (GUPTA et al., 2002; HENGGE et al., 2004; MEYER et al., 2003) for which high doses of the drug are required.

Drug delivery systems are engineered technologies used as approaches to improve drugs action in the organism. One of these systems named polymeric nanocapsules aim to control the release of the drug in the organism (SOPPIMATH et al., 2001; COUVREUR et al., 2002), to reduce the incidence of adverse effects and target the site action (CONTRI et al., 2014) and to target the drug to the site of action

(BERNARDI et al., 2009; OLIVEIRA et al., 2017). These polymeric nanocapsules carry active molecules to a specific site. This was demonstrated by BERNARDI and co-workers (2009), who observed an increase in the survival rate of rats accompanied by a decrease in glioblastoma size after a treatment with indomethacin-loaded lipid-core nanocapsules. In addition, the polymeric nanocapsules are able to reduce toxicity as demonstrated for Ventirini and co-workers (2015) that observed that imiquimod-loaded polymeric nanocapsules are compatible with non-tumoral keratinocyte cell line (HaCaT) after 12 hours incubation. Moreover, nanoparticle systems are small-sized and, therefore, they easily transpose barriers and penetrate into tissues (TORCHELIN 2000; FRANK et al., 2014). Recently, nanoencapsulation has become a subject of increasing interest in order to boost drug selectivity and efficacy in the treatment of HPV related diseases, including cancer (VIJAYAKUMAR et al., 2012; BLUM et al., 2013; YANG et al., 2014). In vitro studies have demonstrated the potential use of nanostructured systems loaded with doxorubicin (ANTONOW et al., 2017), imiquimode (FRANK et al., 2017) and bromelain (OLIVERIA et al., 2017) as an alternative in the treatment of cancer. Accordingly, encapsulated antitumor drug was able to significantly decrease the tumor cells viability in comparison with their respective control (free drug). This effect was possibly attributed to the fact that polymeric nanocapsules are able to modulate the interaction between drug and cells thus delivering a greater amount of drug in the tumor environment (FRANK et al., 2015). The use of imiquimod as an antitumor drug has already been demonstrated in different cancer cell lines such as prostate cancer (TRAMP-C2 and PC-3) (HAM et al., 2013), basal cell carcinoma (BCC/KMC-1 and A375) (HUANG et al., 2016; WANG et al., 2015; PATEL et al., 2013 and squamous cell carcinoma (SCC12) (SOHN et al., 2014). Indeed, we recently reported that imiquimod-loaded polymeric nanocapsules presented an increased efficiency in inducing cervical cancer cell line death in comparison with the free drug (FRANK et al., 2017). However, the mechanism of action involved on this imiquimod-loaded polymeric nanocapsules effect is still unknown. According to literature, imiquimod per se is able to decrease the viability of cells through induction of apoptosis (HAN et al., 2013; SHON et al., 2014; WANG et al., 2015; HUANG et al., 2016), cell cycle arrest (HAM

et al., 2013) and autophagy (WANG et al., 2015; HUANG et al., 2016). In this work we aim to further elucidate the intracellular mechanism by which the imiquimod-loaded nanocapsules induced cervical cancer cells (SiHa) death, as well as to evaluate its capability to stimulate cancer cells production of proinflammatory interleukin (IL-6) in order to propose its utility as a new agent in HPV-related cancer therapy.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Poly(ε-caprolactone) (PCL) (Mn 80 kg mol^{-1}) and sorbitan monostearate (Span 60®) were purchased from Sigma-Aldrich (Steinheim, Germany). Polysorbate 80 (Tween 80®) was purchased from Henrifarma (São Paulo, Brasil) and copaiba oil was kindly donated by Inovam-Da Lamarta & cia Ltda. Imiquimod (IMIQ) was purchased from Chemical Goods (Guangdong, China). Annexin V, and propidium iodide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Acridine orange (AO) were purchased from Sigma-Aldrich (St. Louis, MO). The cervical carcinoma cell line SiHa was purchased from American Type Culture Collection, Rockville, MD. All solvents and reagents were of analytical or pharmaceutical grade.

5.2.2 Methods

5.2.2.1 *Production of imiquimod-loaded nanocapsules*

The nanocapsules were produced by the interfacial deposition of preformed polymer method (VENTURINI et al., 2015). Briefly, an organic phase consisting of imiquimod (5 mg), PCL (100 mg), copaiba oil (334 µL) and Span 60® (38.4 mg) was dissolved in acetone (27 mL). The solution was maintained under magnetic stirring at 37ºC during 15 minutes. Then, the organic phase was injected into an aqueous phase (53 mL) containing polysorbate 80 (76 mg). After 10 min, the turbid solution was evaporated under reduced pressure in a rotative evaporator at 37ºC (Büchi, Switerland) to approximately 10 mL. The formulation produced was named NC_{imiq}.

5.2.2.2 *Characterization of nanocapsules*

The nanocapsules were characterized in terms of pH, size and zeta potential, immediately after production. The pH analysis was performed by direct measurement using potentiometry (B474 Micronal). The nanocapsules size was measured by different techniques (Laser diffraction: Mastersizer 2000, Nano ZS, Malvern; and Dynamic light scattering: Zetasizer Nano ZS, Malvern) by dilution of nanocapsules in bidestilled water. For determination of the zeta potential, the nanocapsules were diluted in NaCl solution (10 mM) and analyzed by electrophoretic mobility (Zetasizer, Nano ZS, Malvern). Analyses were performed in triplicate.

The drug content (n = 3) was determined after the imiquimod extraction from the nanocapsule aqueous dispersions by High Performance Liquid Chromatography with detection in the ultraviolet (HPLC-UV, Series 200, PerkinElmer, Waltham, MA, USA). The quantification method was adapted (DE PAULA et al., 2008) and validated according to our purposes. A C18 reversed phase column (Merck & Co, Inc, Whitehouse Station, NJ, USA) was used as stationary phase and acetonitrile:acetate buffer (pH4.0; 100mM):diethylamine (30:69:85:0.15 v/v), as mobile phase. An injection volume of 20 µL was used and the drug was detected at 242 nm. Calibration curves (n = 3) were made to determine the drug concentration showing linearity (r = 0.998) in the range of 1 to 25 µg mL^{-1}.

The encapsulation efficiency (EE%) was calculated using Equation 1.

$$EE\% = \frac{C_t - C_{free}}{C_t} \times 100 \quad (1)$$

were C_t is the drug content and C_{free} is the imiquimod concentration determined by HPLC-UV in the ultrafiltrate, which was obtained by using the ultrafiltration/centrifugation technique (Ultrafree-MC 10,000 MW, Millipore, Billerica, USA) at 4,120 ×g for 10 min.

5.2.2.3 *Cell culture*

Cervical carcinoma cell line SiHa containing integrated HPV 16 (American Type Culture Collection, Rockville, MD) was used to verify the antitumor capability of the

formulations tested. Cells were maintained in low glucose DMEM supplemented with 10% FBS and penicillin/streptomycin antibiotics (0.5 U·mL/95% air at 37°C).

5.2.2.4 *Cell treatment*

Cells were seeded and treated after 24 hours with the formulation NC_{imiq}. The free drug (drug solution in DMSO at the same concentration of the nanoformulations) was also used as control. The cultures were exposed to the formulations for 24, 48 and 72 hour with concentrations of 3.0 μmol L^{-1} of nanocapsule aqueous dispersions in culture media. All materials were previously sterilized, and the nanocapsule suspensions were prepared under aseptic conditions.

5.2.2.5 *Cell viability*

Cell lines (40,000 cells/well) were seeded on 24-well plates and 24 hours later they were treated with formulations according to described above. At the end of treatment, medium was removed, cells were washed with 1× PBS, 200 μl of 0.25% trypsin/EDTA was added to detach the cells and 400 μl of DMEM + 10% FBS was added to inactivate trypsin. The viable number of cells was then counted by flow cytometry using FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA). Negative controls were used by treating cells with DMEM supplemented with 10% FBS. Results were expressed in percentage values regarding the control.

5.2.2.6 *Labeling the nucleus of cells with hoechst dye*

Cell nuclei were stained with Hoescht 35565665 (1500 μg/mL) according to the manufacturer's instruction. The dye labeling was done after treatment at the established time intervals (24, 48 and 72h) by fluorescence microscopy.

5.2.2.7 *Annexin v and propidium iodide staining*

Phosphatidylserine externalization was determined by the annexin fluorescence signal of an annexin V–fluorescein isothiocyanate conjugate (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) according to the manufacturer's protocol. Cell

cultures were treated, trypsinized, and centrifuged for 6 min at 1600 rpm, and the supernatant was discarded. The pellet was suspended with 150 µl of annexin binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), incubated with annexin V at 0.75 µl/sample and PI at 15 µl/sample for 15 min at room temperature in the dark, and analyzed in a FACSVerse flow cytometer, using FACSVerse software for analysis (BD Biosciences, San Jose, CA, USA). Cisplatin, 40 µM, was used as positive control for apoptosis, and 0.1% Triton X-100 was used as a positive control for necrosis.

5.2.2.8 *Detection of autophagy using acridine orange (AO)*

The development of acidic vesicular organelles (AVO) was quantified. AVO formation is a typical feature of autophagy, and its development indicates autophagosomes maturation and an efficient autophagic process, since only mature/late autophagosomes are acidic (KLIONSKY et al., 2008; MELLO et al., 2015). For this experiment, cells (20,000 cells/well) were seeded on 24-well multiwell plates, waited to growth for 24 hours and exposed to 3µM NC_{imiq} and IMIQ for 24, 48 and 72 h. Then cells were trypsinized and incubated with AO (2.7 mM) for 15 min at room temperature, and fluorescence emission was analyzed by flow cytometry using a FACSVerse flow cytometer and FACSVerse software. Rapmicyn (200nM) was used in parallel as a positive control of autophagy inducer.

5.2.2.9 *Cell cycle analyses*

After treatment with NC_{imiq} and IMIQ according to described above, cells were detached and centrifuged at 1500 rpm for 5 min. Subsequently, cells were washed 1X with 200 µL of PBS and centrifuged at 1500 rpm for 5 min. Then cells were fixed in 70% ethanol for 2 hours at 4ºC, followed by a new wash with PBS and centrifugation. Finally, propidium iodide (12 µg/ ml), 0.1% triton X-100 and RNAase (50 µg/ml) were added and incubated with the cell suspension for 30 minutes at room temperature, protected from light (FILIPPI-CHIELA et al., 2015). Cells were subsequently analyzed

by FACSVerse flow cytometry. Mitomycin C (5 μg/mL) was used in parallel as positive control of cell cycle arrest.

5.2.2.10*Clonogenic survival assay*

Cells were assayed for the cytotoxic effect of imiquimod after the cell survival according to established methods for clonogenic assay (FRANKEN et al., 2006; MELLO et al., 2015). Subconfluent cultures were exposed to 3 mM of the formulations (NC_{imiq} and IMIQ) for 24, 48, and 72 h. Then the surviving adherent cells were washed with PBS preheated to 37°C, trypsinized, counted, and replated in six-well plates (100 cells/well). After 10 days of incubation in complete culture medium, the colonies, formed from each cell plated, were stained with crystal violet after fixation with methanol and counted manually. In each case results are expressed as survival fraction, which was obtained by dividing the number of colonies that arise after treatment of cells by the number of cells seeded and plate efficiency (PE: number of colonies formed by untreated cells/ number of cells seeded), multiplied by 100.

5.2.2.11*Measurement of IL-6 released by tumor cells*

After treating the cells with the formulations (NC_{imiq} and IMIQ), the culture medium was withdrawn, centrifuged and the resultant supernatant was collected and frozen at -20ºC until analysis. The amount of the IL-6 inflammatory mediator was determined by ELISA kit (boster biological technology, Valley Ave, Pleasanton, CA), according to the manufacturer's protocol. Calibration curves were made to determine the IL-6 concentration in culture medium in the range of 4.69 to 300 pg/ml.

5.2.2.12*Data analysis*

Statistical analyses were performed by means of one-way Analysis of Variance (ANOVA) followed by the post-hoc Tuckey's test for multiple comparison of means ($\alpha = 0.05$). The software SPSS statistics 17.0® was used for the statistical analyses.

5.3 RESULTS AND DISCUSSION

5.3.1 Production of polymeric nanocapsules

Imiquimod-loaded nanocapsule formulation (NC_{imiq}) was produced following Frank and co-workers (2017) procedure. Laser diffraction analysis showed unimodal size distributions for formulation. The volume-weighted mean diameters (D[4,3]) and the diameters at percentiles 10, 50 and 90 under the size distribution curves by volume and by number of particles were plotted in a radar chart (Figure 26). The shape of the curves in the radar chart are the fingerprint characteristic of unimodal particle size distributions, as previously determined for different polymeric nanocapsules (BIANCHIN et al., 2016). All formulations showed d(0.9)v values lower than 500 nm and d(0.5)n values lower than 200 nm, which is in accordance with the findings from Frank and co-workers (2017).

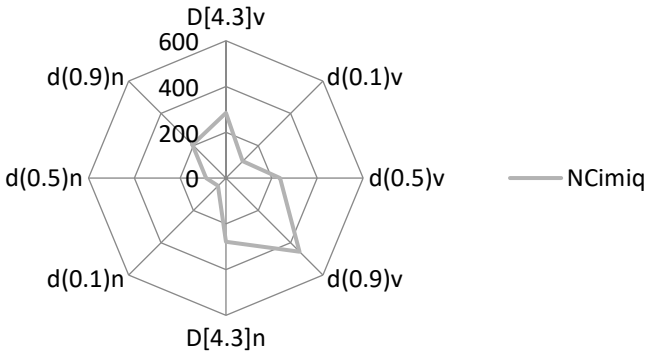

Figura 28 Radar chart presenting the volume-weighted mean diameters (D[4,3]) and the diameters at percentiles 10, 50 and 90 under the size distribution curves by volume and by number of particle.

The dynamic light scattering showed hydrodynamic mean diameters of 242.1±17 nm (NC_{imiq}) with polydispersity indexes of 0.17±0.1. The results demonstrated that nanocapsule aqueous dispersions have narrow unimodal size distributions (data not shown) with high homogeneity of sizes. The zeta potential of NC_{imiq} was -8.9±0.3 mV, which value is in accordance with other formulations prepared with poly(ε-

caprolactone), as wall, and polysorbate 80, as coating material (CATTANI et al., 2010). The pH value for the nanocapsule aqueous dispersions was 6.1±0.07. The drug content showed 0.49±0.05 mg mL^{-1} of imiquimod in NC_{imiq}. Regarding the encapsulation efficiency, NC_{imiq} presented 98±0.2% of imiquimod retained in the dispersed phase.

5.3.2 NC_{imiq} significantly reduces cervical cancer cell viability in a time-dependent manner

According to Figure 27, NC_{imiq} formulation was more effective against the cervical cancer cell line (SiHa), significantly decreasing the cell viability in a time-dependent manner as compared to the free drug in the same concentration. This result is in agreement with our previously published data, which demonstrated that this formulation decreased cervical cancer cell viability at concentrations of 1.5 and 3.0 μmol L^{-1} after 24 hours of treatment (FRANK et al., 2017). Other works have also shown that polymeric nanocapsules loaded with antitumor drugs (i.e. doxorubicin, bromelain) are capable to significantly decrease the viability of the cells (MCF-7) compared to free drug, reinforcing the potential use of nanocapsules to boost traditional antitumor drugs (ANTONOW et al., 2017; OLIVEIRA et al., 2017). Similarly, Chen and co-workers (2014) demonstrated that polymeric nanocapsules containing doxorubicin decrease the viability of MCF-7 at concentrations ranging from 0.2 - 40μM. They also showed that the uptake by cells was greater than the free drug at 1, 8 and 24 hours. Different results were found by Zanotto-Filho and co-workers (2013) regarding the delivery of curcumin into glioma cells (C6). In this case, early evaluated points of time showed higher uptake for the non-encapsulated drug, whereas for late points of time (48 hours or more), the uptake was increased by nanoencapsulation. However, in the *in vivo* experiments they observed better results for the nanoencapsulated formulation.

Here, we show that low concentration of the formulation NC_{imiq} was able to significant decrease SiHa cell line viability in a time-dependent manner, indicating that NC_{imiq} formulation increases the cervical cancer cell death.

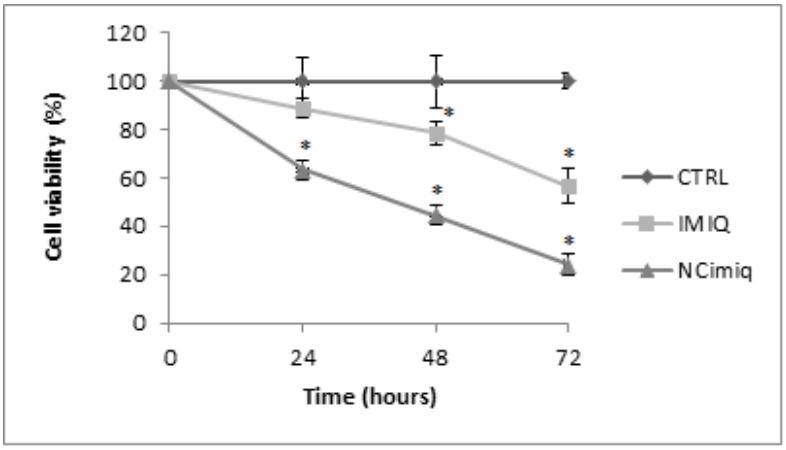

Figura 30 Number of viable cells after 3µM NCimiq and IMIQ after treatment for 24, 48, and 72 h n=3 and *p < 0.05 compared with control (one-way ANOVA, followed by Tukey's test).

5.3.3 NC_{imiq} increases cervical cancer cell shrinkage, membrane blebbing and chromatin condensation

Figure 28 shows that both NC_{imiq} and IMIQ are able to produce cell alterations that resembles apoptotic features such as membrane blebbing, cell shrinkage (upper part) and chromatin condensation (botton part). In turn, NC_{imiq} formulation showed more pronouced effetcs, mainly at 72h after treatment, when compared to IMIQ, suggesting the higher efficacy of the former in inducing cancer cell apoptosis.

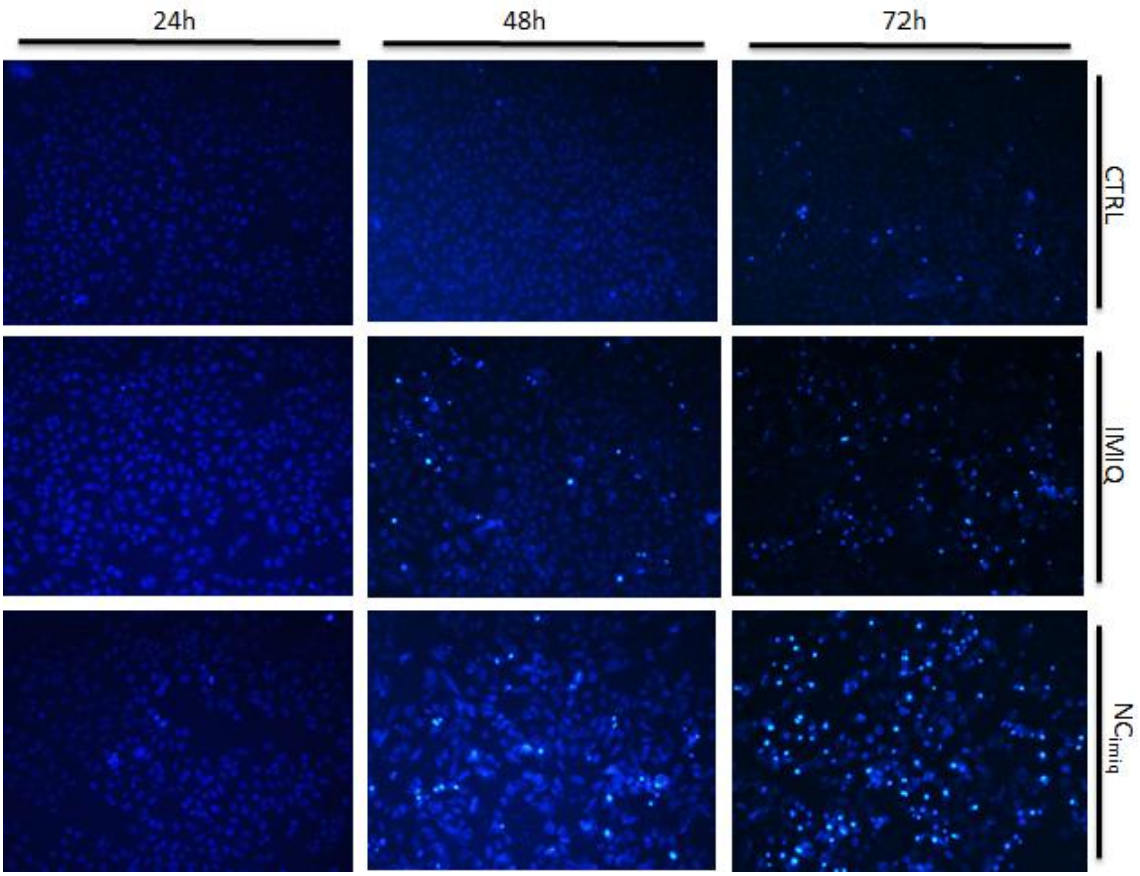

Figura 31 Images of SiHa cell treatment with 3.0µM NCimiq and IMIQ for 24, 48, and 72 h. Cell nuclei was stained with Hoescht 35565665 according to manufacturer's instruction. Note apoptotic features and fragmented nuclei when cells were treated with imiquimod. Scale bars, 20 µm; magnification, 20×.

5.3.4 NC_{imiq}-induced cell death through apoptosis and necrosis

In order to elucidate if NC_{imiq} is able to promote cancer cell death through apoptosis and/or necrosis we evaluated the percentage of annexin V^+/PI^+ cells after exposure to NC_{imiq} or IMIQ for 24, 48 and 72h. According to Figure 29, NC_{imiq} enhanced the number of both PI^+ (necrosis) and annexin V^+/IP^+ (apoptosis) subpopulation in all times tested in relation to the control. Particularly at 72h, IMIQ induced an increase in the number of PI^+ (necrosis) subpopulation only. Those differences may justify the enhanced effect on tumor cell death provoked by the NC_{imiq} formulation.

Imiquimod induced cell death through apoptosis is a longstanding observation and similar mechanism was identified for many types of cell lines such as FL, A375, TRAMP-C2, SCC12, BCC, BLM (KAN et al., 2012; PATEL et al., 2013; HAN et al.,

2013; SOHN et al., 2014; HUANG et al., 2016; EL-KHATTOUTTI et al, 2016). However, in such works, imiquimod induced cell death at higher concentrations (20-150 µg/ml) than those used in this present work (3.75µg/ml). As an example, Ham and co-workers (2013) showed that 20µg/ml of imiquimod was able to induce 8.32% of apoptotic cell death in prostate cancer cells (TRAMPC-2) after 48 hours of treatment. In the same sense, Shon and co-workers (2014) demonstrated that 150µg/ml of imiquimod caused 30% of skin carcinoma cells (SCC12) death through apoptosis after 16 hours of treatment. Similarly, Huang and co-wokers (2016) observed that 50 µg/ml of imiquimod elicited 45% of the skin cancer cells (BCC) death after 48 hours of treatment. The higher percentage of apoptotic cells induced by imiquimod in those settings could be attributed to the higher amount of drug utilized in those works. Here we demonstrated that NC_{imiq} was able to reproduce the imiquimod-related mechanism of cell death, but differently from others the amount of drug used in this formulation was, at least, six times smaller. Moreover, at this lower concentration the free drug was not able to trigger cell apoptosis.

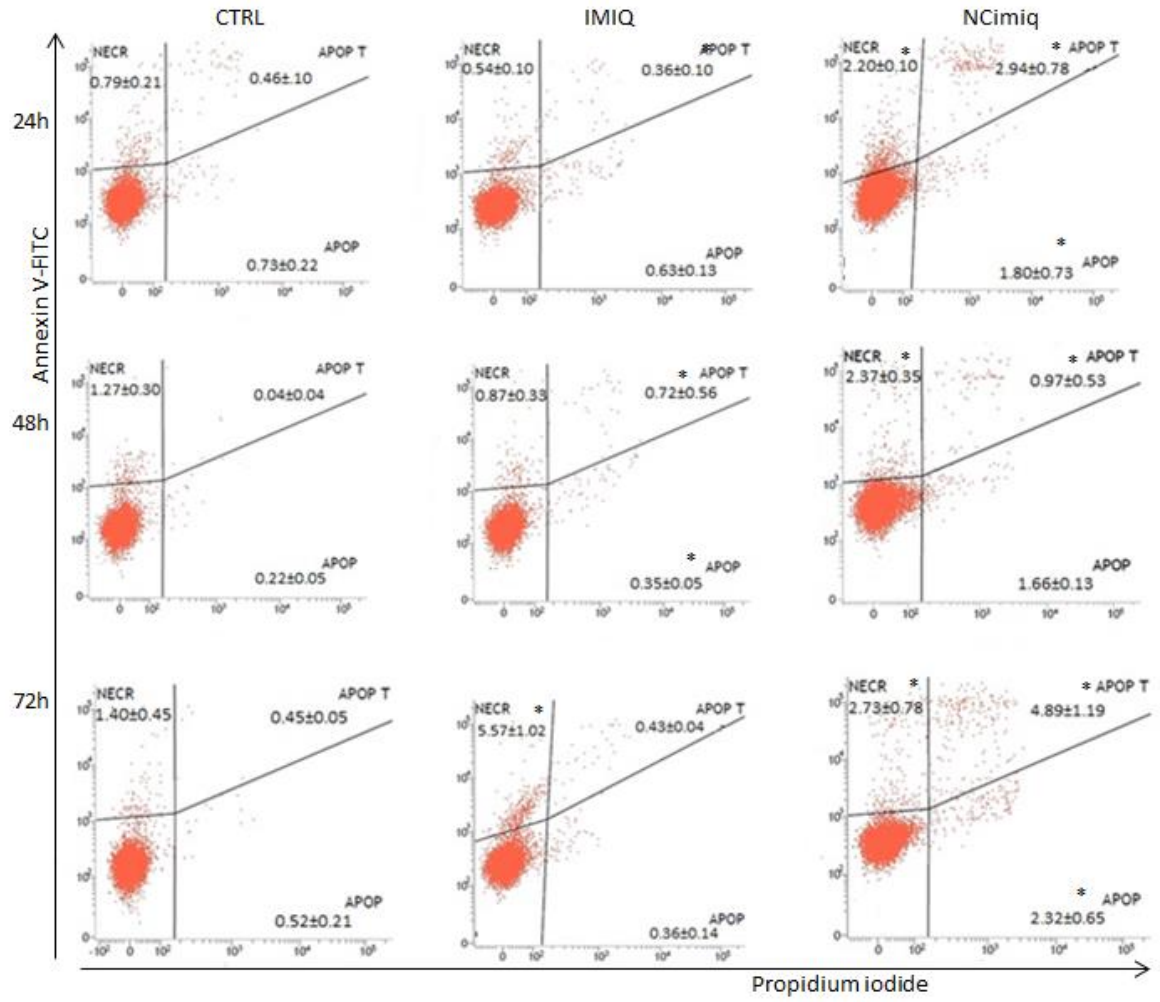

Figura 32 SiHa was exposed or not for 24, 48 and 72 h with 3µm NCimiq and IMIQ. Apoptosis and necrosis were measured according to annexin V/PI binding. n=3 and *p < 0.05 compared with control (one-way ANOVA, followed by Tukey's test). Note: PerCP = annexin V-FITC, FITC-A = propidium iodide.

5.3.5 NC_{imiq} increases the percentage of SubG1subpopulation of SiHa cells after 72h

In order to reinforce the evidences that NC_{imiq} promotes cervical cancer cell death through apoptosis, the content of DNA fragmentation by cell cycle analyses was also evaluated (Figure 30). The eukaryotic cellular cycle is traditionally divided in two main periods: the interphase and the mitosis (M). An entire mammalian cell cycle in culture usually takes 16 hours and is divided in the 3 periods: G1 (growing and preparation for the chromosomes replication), S (DNA synthesis), G2 (preparation for the mitotic

division). However, when cells are undergoing apoptosis and the DNA fragmentation occurs, a subpopulation of cells accumulates in a sub G1 phase. This sub G1 phase is seeing to the left of the G1 peak on the cell cycle graphic. According to Figure 5, after 72 hours of treatment NC_{imiq} showed a significant increase in the percentage of cells at phase <G1 (Sub G1) in relation to the control and IMIQ group, suggesting DNA fragmentation and cell apoptosis.

Differently from the present work, data from literature showed that 50µg/ml of imiquimod induced an increment of subG1 subpopulation in the basocellular carcinoma cells (CCB and A375) (WANG et al., 2015) and 20µg/ml of imiquimod increased the amount of TRAMP-C2 cells in G2/M, while it decreases the percentage of cells at the G1 phase HAM and co-workers (2013). Despite these differences, our result supports the idea that imiquimod induce cancer cell death through apoptosis and the nanoformulation have a better efficiency in producing this effect.

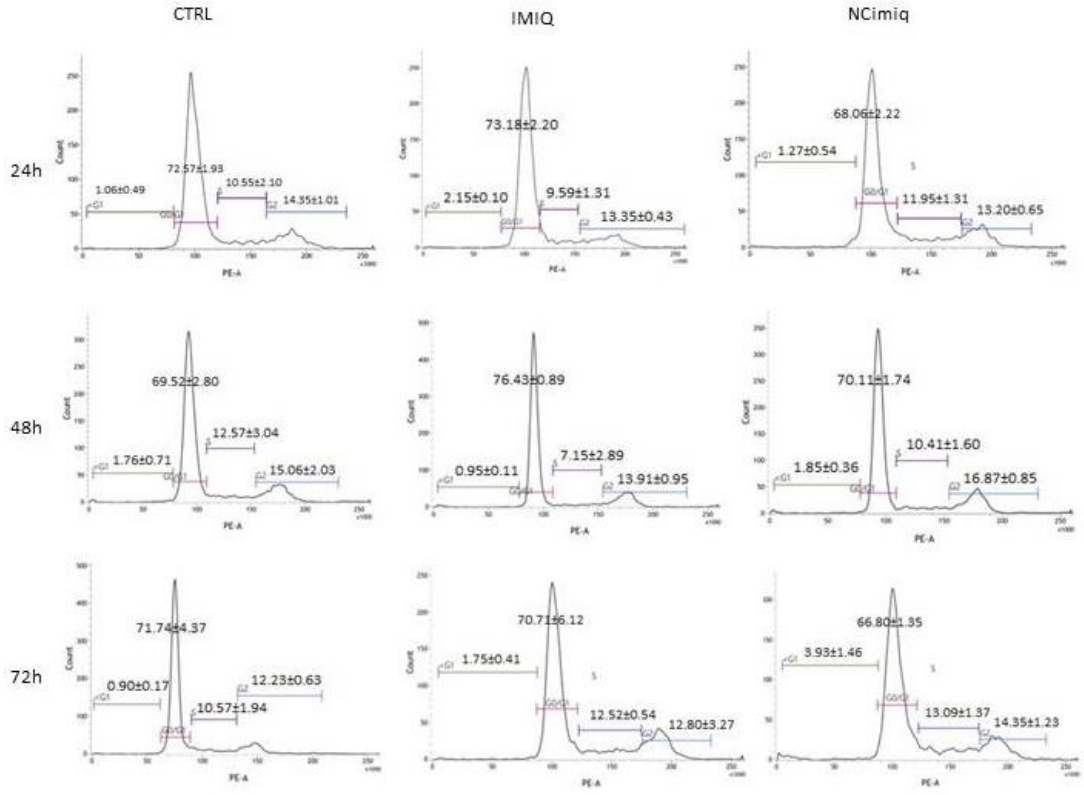

Figura 33 The effect of imiquimod on the cell cycle of SiHa cells determined by DNA content assay.

*p < 0.05 compared with control (one-way ANOVA, followed by Tukey's test).

5.3.6 NC_{imiq} induces increased acridine orange staining in SiHa cells

Increased acridine orange (AO) staining is a suggestive feature of autophagy induction KLIONSKY et al., 2008). Depending on its levels of stimulation, autophagy can contribute to tumor cell death. Therefore, we next evaluated if authophagy is involved with the mechanism of NC_{imiq} induced cervical cancer cell death. The results demonstrated that NC_{imiq} was capable to promote increased amount of AO^+ cells (Figure 31), suggesting the induction of autophagy process. Moreover, this effect was exclusively related to the NC_{imiq} formulation and it occurred in all time point tested. According to the percentage of AO^+ cells we can notice that autophagy is strongly triggered in the first 24h of treatment, despite the number of cells AO^+ are still high after 48h and 72h of treatment. Interestingly, IMIQ was not able to increase the amount of AO^+ cells and presented similar values to those from the control group ($p>0.05$) for the times 48 and 72 hours. This lack of effect could be explained by the fact that IMIQ concentration is very low to trigger such process in to the cells, which is, on the other hand, potentiated by the NC_{imiq} formulation.

Autophagy is a usual process triggered by normal cells exposed to stress condition such as under privation of nutrients, hormones or energy and infection by pathogens (HE et al., 2009). In this context, autophagy prevents the transformation of normal cells to malignant cells by reducing oxygen reactive species, damage to the DNA, protein aggregation and mitochondrial abnormality (ZHONG et al., 2015). It is now understood that, in cancer, autophagy can exert a double role: it can either inhibit tumors formation by avoiding the accumulation of organic substrates and protein aggregates or stimulates tumor growth by serving as a mechanism of cellular survivor (ZHI et al., 2015). Imiquimod-induced autophagy has already been demonstrated by WANG and co-workers (2015) and HUANG and co-workers (2016). According to these studies, high concentration of free imiquimod (50µg/mL) was responsible to trigger cell (BBC) autophagy and contribute to cell death. Here, we demonstrated that low level of imiquimod was also able to promote autophagy, but only after being encapsulated in

nanostructures, reinforcing the beneficial effect of nanocapsules to potentiate drug activity.

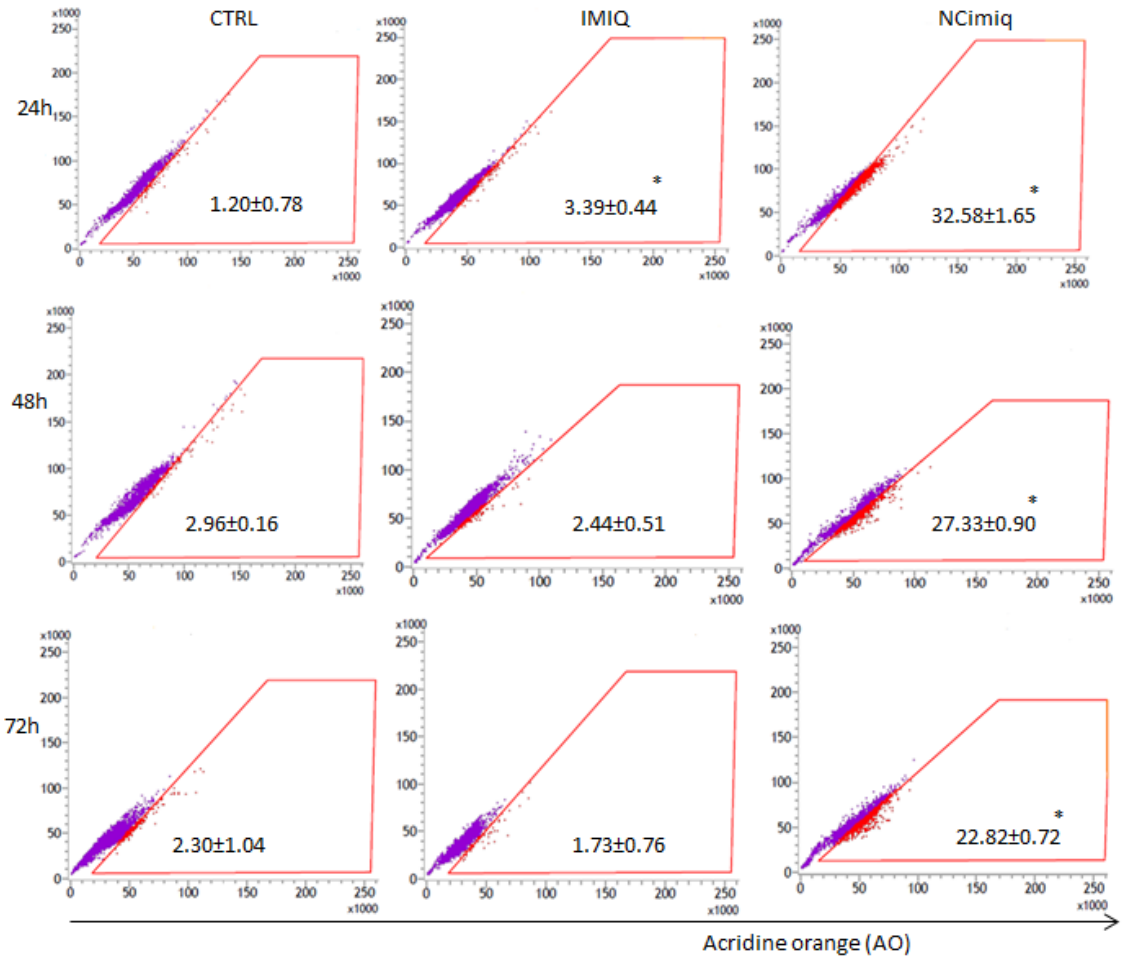

Figura 34 SiHa cells were left untreated or treated with 3µM of NC_{imiq} and IMIQ for 24, 48 and 72 h and autophagy was measured according to the acridine orange (AO) staining. *p < 0.05 compared with control (one-way ANOVA, followed by Tukey's test).

5.3.7 Clonogenic survival assays

Besides acting as a cytotoxic agent, imiquimod also inhibit survival SiHa cells to proliferate and form new colonies. According to the clonogenic assay (Figure 32) single SiHa cell were not capable to grow up in a colony after being exposed to both, formulations NC_{imiq} and IMIQ. Again, this effect was more pronounced when cells were exposed for long period of time (72h) with the NC_{imiq} formulation. To the best of our knowledge, we are the first group to demonstrate this long lasting effect caused by imiquimod per se as well as the potent activity caused by its nanoencapsulation.

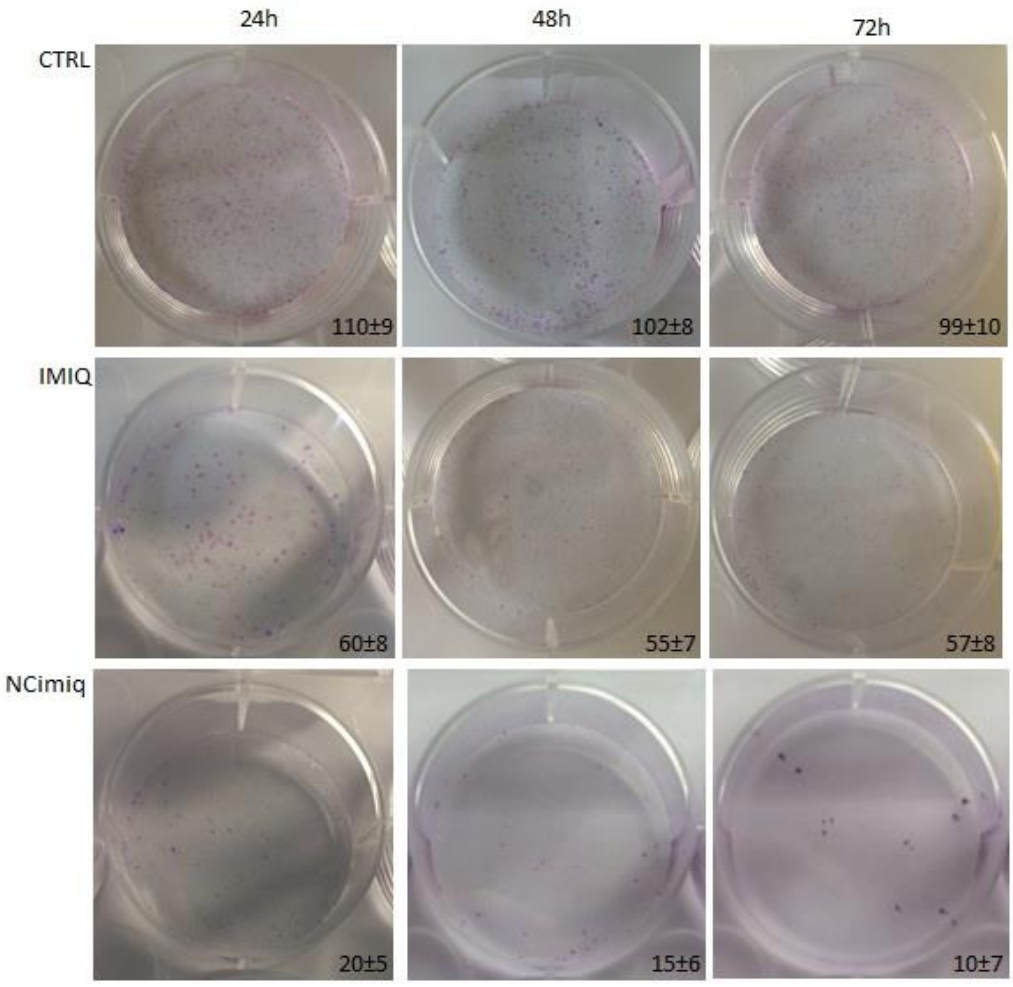

Figura 35 Clonogenic assay: 100 viable cells were seeded in clonogenic assay, and colony formation was evaluated. *p < 0.05 compared with control (one-way ANOVA, followed by Tukey's test).

5.3.8 NC_{imiq} stimulates SiHa cells to release IL-6

Figure 33 depicts the property of both NC_{imiq} and IMIQ formulations in stimulating SiHa cells to release IL-6 in the culture medium. This effect was already expected, since imiquimod is broadly known an immune system activator (SAUDER et al., 2000). Once more, NC_{imiq} formulation was able to boost IL-6 release, triggering the release of higher levels of this cytokine in comparison to IMIQ formulation ($p<0.01$) for all analyzed times. IL-6 is a potent proinflammatory cytokine, which stimulates the maturation and activation of neutrophils and macrophages as well as the differentiation and maintenance of T lymphocytes, modulating both innate and adaptive immune

response (OLIVEIRA et al., 2011; DAHL et al., 2002). Increased circulating levels of IL-6 have already been observed in diverse inflammatory related diseases, including gynecologic cancer (PARADKAR et al., 2014) and this explains the IL-6 values found for the CTRL. In this context, Tjiong and co-workers (1999) showed that IL-6 levels were highly elevated in the blood of patients with cervical cancer (HPV positive) in comparison to the control healthy group (HPV negative), pointing a role for this cytokine in the cervical cancer development. Therefore, NC_{imiq} formulation might be an attractive strategy to treat HPV-related disease such as cervical cancer videlicet its superior capacity to potentiate tumor cell death at the same time that stimulates the immune response by releasing IL-6 in the tumor microenvironment.

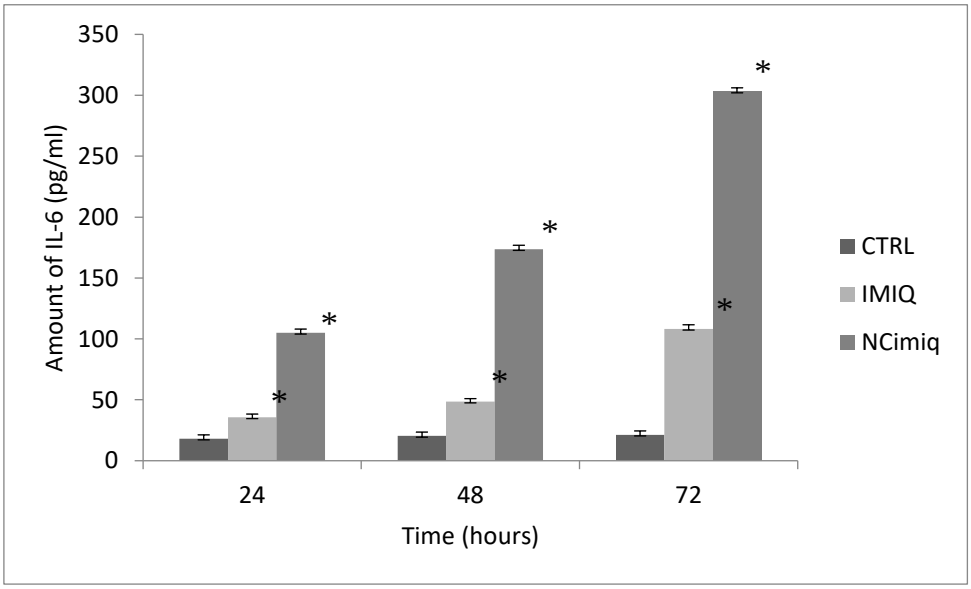

Figura 36 Amount of interleukin 6 after treatment of SiHa cells with the formulations. n=3 and *p < 0.05 compared with control (one-way ANOVA, followed by Tukey's test).

5.4 CONCLUSIONS

In the present study, we successfully demonstrated the efficacy of NC_{imiq} in inducing cervical cancer cell death through multiple of downstream mechanisms, being apoptosis and autophagy the mainstream of those processes. Moreover, our results demonstrated that NC_{imiq} stimulates tumor cells to release the proinflammatory cytokine

IL-6, reinforcing a role for imiquimid in boosting the immune response. These findings open new possibilities for these polymeric nanocapsule aqueous suspensions containing imiquimod as a novel strategy for the treatment of cervical cancer.

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Disclosure of interest

The authors report no conflicts of interest.

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6 CONSIDERAÇÕES FINAIS

6.1 DISCUSSÃO GERAL

A presente tese apresentou diferentes sistemas nanotecnológicos para tratamento de uma enfermidade especifica, o câncer de colo do útero, através do tratamento de cultura de células de câncer cervical (SiHa). A partir disso, a presente tese propôs o desenvolvimento de três diferentes carreadores poliméricos para vetorização do imiquimode. As diferentes estruturas visando aplicação vaginal foram as seguintes: a) nanoemulsão; b) nanocápsulas poliméricas; c) nanocápsulas poliméricas revestidas com quitosana. Tais formulações foram desenvolvidas usando como base pesquisas anteriores desenvolvidas por Bender e colaboradores (2012) e Venturini e colaboradores (2015).

No que se refere às nanoestruturas contendo imiquimode, pode-se destacar que embora essas formulações tenham apresentado menores valores de permeação do imiquimode através da mucosa vaginal em comparação com o fármaco livre, todas apresentaram maiores valores de citotoxicidade em cultura de células de câncer cervical (SiHa). A partir desses achados, foi possível verificar que as nanoestruturas potencializam o efeito citotóxico do imiquimode e também que a presença da parede polimérica nas nanocápsulas não influencia esse efeito, uma vez que após 24 horas de tratamento ambas as formulações (nanocáspulas e nanoemulsões) apresentaram valores semelhantes de diminuição da viabilidade celular (em torno de 40%). Outros trabalhos (FRANK et al., 2015; ANTONOW et al., 2017) têm demonstrado que devido ao tamanho nanométrico que essas estruturas apresentam, elas podem ser internalizadas mais facilmente e isso permite que uma maior quantidade de fármaco seja liberada para as células quando comparado com sua forma livre. Apesar de ambas as formulações apresentarem efeito citotóxico semelhante após 24 horas de tratamento das células, as mesmas apresentaram diferentes efeitos no ensaio clonogênico. Nesse experimento ficou evidenciado que, embora ambas as formulações (nanoemulsões e nanocáspulas) tenham inibido mais do que seu respectivo fármaco livre, a parede polimérica da partícula auxiliou de forma mais efetiva para que menores quantidades de colônias

fossem formadas após as células serem expostas aos respectivos tratamentos. Até onde se tem conhecimento, este é o primeiro estudo na literatura que demonstra que nanoestruturas inibem de forma significativa o aparecimento de novas colônias após tratamento com a formulação em células de câncer cervical. Estudos anteriores não tinham sido capazes de identificar esse tipo de inibição que as nanocápsulas poliméricas apresentam sobre células após seu tratamento, sendo uma novidade dos presentes resultados.

A ação citotóxica do imiquimode sobre diferentes linhagens celulares já é descrita na literatura sobre células de câncer de pele (WANG et al., 2015; HUANG et al., 2016), câncer de próstata (HAM et al., 2013), câncer de ovário (SCHON et al., 2006). Em todos esses trabalhos, as concentrações utilizadas de imiquimode foram muito superiores àquelas utilizadas na presente tese. Isso porque quando o fármaco é encapsulado em nanoestruturas ele apresenta alta performance frente a células (ANTONOW et al., 2017) ou animais (BERNARDI et al., 2009), tornando o fármaco mais apto para ser internalizado (FRANK et al., 2015). O imiquimode na sua forma livre está relacionado a efeitos adversos que incluem eritema, dor, coceira, e ulceração e isso está associado à alta concentração utilizada e ao período prolongado de tratamento, levando os pacientes abandonar o mesmo (GUPTA et al., 2002; WIELAND et al., 2006; KREUTER et al., 2008). A partir desse fato, no presente trabalho foi demonstrado que esses efeitos adversos podem ser em hipótese diminuídos, uma vez que uma dose muito inferior tem efeito significativo em experimentos *in vitro*. Nesse sentido, as formulações desenvolvidas nesta tese têm o potencial de aumentar a adesão do paciente ao tratamento.

Outros sistemas visando aumento da adesão e da permeação a partir de nanocáspulas poliméricas foram desenvolvidos nesta tese. Tratam-se de nanocápsulas poliméricas incoporadas em hidrogéis de hidroxietilcelulose 2% e de quitosana 2.5%. Ambos os sistemas resultaram em formulações nanotecnológicas adesivas inovadoras para um possível tratamento do câncer de colo do útero. Contudo, o hidrogel de quitosana com nanocápsulas poliméricas contendo imiquimode demonstrou ser o mais promissor. Isso porque esse hidrogel apresenta valores de permeação, mucoadesão e

imiquimode retido sobre a mucosa mais promissores. Alguns hidrogéis de diferentes polímeros (celulose, alginato, ácido hialurônico, polissacarídeos, gomas) já foram propostos para entrega de fármacos na via vaginal (Das NEVES 2006; CARAMELLA et al., 2015). Tratam-se de sistemas que visam aumentar o tempo de contato do fármaco com a mucosa para que uma maior quantidade da substância possa interagir com o muco e assim apresentar melhores efeitos. FRANK e colaboradores (2014) propuseram a incorporação de nanocáspulas de EUDRAGIT® RS 100 contendo um marcador de fluorescência (vermelho do nilo) em hidrogéis de quitosana 2.5%. Os autores demonstraram que esse sistema é capaz de aumentar a penetração do ativo encapsulado em mucosa vaginal suína. Neste sentido, o presente trabalho avançou um passo mais, ao demonstrar que nanocápsulas de poli(ε-caprolactona) contendo imiquimode tem efeito sobre células de câncer cervical e também que o sistema desenvolvido (hidrogel de quitosana) quando incorporado com nanocápsulas é efetivo na entrega de fármaco na via vaginal.

Conforme mencionado anteriormente, foram realizados experimentos envolvendo mucosa vaginal suína. Essa mucosa foi utilizada a partir de estudos comparativos entre diferentes mucosas, dentre elas a vaginal e com discos de mucina (Apêndice A). Alguns autores já utilizaram anteriormente diferentes mucosas para elucidar a adesão das formulações, como por exemplo a mucosa sublingual (CHAVES et al., 2017), esofágica (ZATTA et al., 2017), vaginal (SANDRI et al., 2008) e até mesmo diretamente na pele (CONTRI et al., 2014). Porém, outros autores utilizaram discos de mucina para analise de adesividade de suas formulações (LEITNER et al., 2003; BRUSCHI et al., 2007; SRIAMORNSAK et al., 2010; FONSECA et al., 2014). Até o presente trabalho havia uma carência de estudos comparativos que elucidassem a melhor forma de avaliar a mucoadesão das formulações desenvolvidas. Por tanto, este trabalho também avançou nessa frente, sendo que os resultados obtidos demonstraram que discos de mucina apresentam valores mais altos de adesão quando comparados com todas as mucosas utilizados no estudo. Consequentemente, esta tese utilizou somente mucosas suínas para as avaliações de adesividade e permeação.

Para obtenção das nanoestruturas produzidas nesta tese foi utilizado óleo de copaíba. Esta escolha deve-se ao fato de que em estudos preliminares esse óleo foi capaz de solubilizar uma maior quantidade de imiquimode. Já é descrito na literatura que esse composto apresenta ação anti-inflamatória (VEIGA et al., 2007), antitumoral (OHSAKI et al., 1994; LIMA et al., 2003) e antifúngica (SVETLICHNY et al., 2015) além de poder ser utilizado como alternativa para o tratamento de alivio da irritação cutânea e como substituto à corticoides. Visando avaliar esse efeito citotóxico já descrito e a fim de verificar o quanto esse efeito contribuiu para a diminuição da viabilidade das células foi realizado um estudo paralelo abordando o efeito de nanocápsulas poliméricas somente com óleo de copaíba. Os resultados encontrados nesse estudo complementar (Apêndice B) permitiram elucidar que o óleo de copaíba apresenta um ligeiro efeito citotóxico em células de câncer cervical provocando morte por apoptose. A partir desses resultados foi possível demonstrar que a elevada porcentagem de morte que ocorre em células SiHa tratadas com nanocápsulas poliméricas e nanoemulsões (artigo 2 e 3) deve-se ao fato do fármaco imiquimode estar nanoencapsulado, uma vez que seu efeito foi superior as nanocáspulas sem o fármaco bem como superior ao fármaco livre.

6.2 CONCLUSÕES GERAIS

Esta tese defende que formulações nanotecnológicas contendo imiquimode podem ser utilizadas para o tratamento do câncer cervical. Isto se deve às evidências obtidas mediante os estudos desenvolvidos no presente trabalho. As nanopartículas desenvolvidas (nanoemulsões, nanocápsulas poliméricas, nanocápsulas poliméricas revestidas com quitosana), todas contendo imiquimode, apresentaram maior efeito citotóxico em linhagem de câncer do colo do útero (SiHa) e apresentaram mecanismos de morte das células que envolveram apoptose, autofagia e parada de ciclo celular. As nanoemulsões desenvolvidas neste trabalho apresentaram quantidades significativas de imiquimode permeado através da mucosa vaginal suína além de terem produzido uma maior quantidade de morte de células SiHa. Semelhantemente, nanocápsulas poliméricas (revestidas e não revestidas com quitosana) contendo imiquimode

apresentaram permeação do fármaco através da mucosa vaginal, assim como elevado efeito citotóxico do tipo tempo-dependente na mesma linhagem celular. Estas formulações também apresentaram alta adesão quando incorporadas em hidrogéis de hidroxieltilcelulose e de quitosana. Os resultados do trabalho permitiram elucidar a melhor formulação, das avaliadas, para o tratamento de câncer de colo do útero.

Em todos os experimentos, os sistemas nanoestruturados foram comparados com imiquimode na sua forma livre. Em todos esses casos, obtiveram-se melhores resultados quando o imiquimode apresentava-se na forma nanoestruturada quando aplicados em mucosa vaginal e também quando expostos a células SiHa. Além disso, foi possível verificar que com pequenas quantidades de fármaco nanoencapsulado obtém-se um efeito citotóxico significativo em células de câncer de colo do útero. Sendo assim, o presente trabalho desenvolveu formulações nanoestruturadas com potencial para serem futuramente empregadas na terapia do câncer de colo do útero.

6.2.1 Limitações e futuras pesquisas

Esta tese de doutorado apresenta limitações que abrem oportunidades para novas pesquisas. Primeiramente, foi utilizado um modelo vaginal suíno para experimentos envolvendo mucoadesão, permeação e lavabilidade. Embora esse modelo seja o mais utilizado na literatura e o que simula mais adequadamente a via vaginal feminina, outros modelos também podem ser utilizados para avaliação da quantidade de fármaco permeado e aderido sobre a mucosa. Nesse sentido, futuros trabalhos poderiam testar a aplicação de nanocápsulas contendo fármacos para administração vaginal utilizando outros modelos de mucosa animal, como, por exemplo, ovelhas e vacas. Em segundo lugar, outra limitação desta pesquisa é que as formulações de nanocápsulas poliméricas foram incorporadas em somente dois tipos específicos de polímeros para obtenção dos hidrogéis, os quais apresentaram altos valores de adesão sobre a mucosa. Porém, existem trabalhos que demonstraram que outros sistemas poliméricos também podem ser utilizados para entrega de fármacos na via vaginal. Sendo assim, este trabalho comparou especificamente dois sistemas semissólidos para entrega de imiquimode

nanoencapsulado. Novas pesquisas poderiam se dedicar ao estudo comparativo de diferentes hidrogéis adesivos incluindo os sistemas desenvolvidos na presente tese.

Uma terceira limitação do trabalho é que, ao estudar o efeito citotóxico da formulação, foi utilizada uma linhagem de células SiHa. Contudo, existem outros tipos de linhagem celular que poderiam ser utilizados. Portanto, surge-se a possiblidade de futuras pesquisas que comparem o efeito citotóxico da formulação levando-se em consideração as diferenças celulares existentes. Finalmente, a quarta limitação que esta tese apresenta é que as formulações foram aplicadas somente em modelos de estudos *in vitro.* Embora estes resultados tenham demonstrado um panorama promissor para o tratamento do câncer do colo do útero, sabe-se que podem existir diferenças quando o mesmo estudo for transposto para a aplicação de modelos vivos, o qual não foi foco do presente trabalho. Portanto, futuros trabalhos poderiam testar a aplicação *in vivo* das formulações desenvolvidas na presente tese, assim como em pacientes com diferentes graus de avanço da doença abordada nessa tese. Desta maneira, os resultados da presente tese serviriam como uma sólida base de partida para estudos envolvendo modelos vivos.

6.2.2 Implicações práticas

A principal formulação desenvolvida (nanocápsulas poliméricas contendo imiquimode incorporadas em hidrogel de quitosana) neste trabalho apresenta importantes implicações práticas para o tratamento do câncer cervical. Em primeiro lugar, foi demonstrado que esta formulação apresenta uma maior adesividade utilizando dois polímeros específicos. Isto traz importantes benefícios para o tratamento das pacientes com essa enfermidade levando-se em consideração o estilo de vida das mesmas. Sendo assim, a formulação ajuda a garantir um maior tempo de contato do fármaco com a mucosa e garante maiores quantidades de fármaco permeado na mucosa vaginal. Além disso, essa formulação demonstrou ser eficaz mesmo quando se utiliza pequenas quantidades de imiquimode, o que pode melhorar na terapia do câncer de colo do útero, uma vez que a formulação disponível comercialmente é utilizada em altas concentrações e por um longo período de tempo. Além disso, a formulação disponível comercialmente causa efeitos adversos o que acabam levando as pacientes a

abandonarem o tratamento. Nesse sentido, a formulação desenvolvida nesta tese pode contribuir para uma maior aceitação do tratamento devido à melhoria do conforto nas pacientes.

Além disso, é importante destacar que a formulação proposta destina-se ao tratamento da doença já existente. Existem outros tratamentos preventivos contra o vírus HPV, o qual possui subtipos (16 e 18) que estão fortemente associados ao desenvolvimento do câncer de colo do útero. Contudo, a prevalência desta doença é ainda elevada em países em desenvolvimento como o Brasil. Portanto, a formulação desenvolvida neste trabalho pode contribuir para a melhora do tratamento de pessoas já afetadas, o que representa um elemento importante dentro das políticas de saúde pública do país.

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APÊNDICE A

Apêndice (Artigo 5) – Mucoadhesive properties of Eudragit®RS100, Eudragit®S100 and Poly(ε-caprolactone) nanocapsules: influence of the vehicle and the mucosal surface

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Abstract

The use of polymers as mucoadhesive materials has been explored in several drug delivery approaches. However, little attention has been given to their mucoadhesiveness when they are structured in nanocapsules. Mucoadhesion measurements are based on the use of animal mucosa or mucin discs, the glycoprotein responsible for the adhesive characteristic of mucus. Therefore, the objective of this study was to analyze the mucoadhesion of nanocapsules produced with polymers of different ionic properties, Eudragit®RS100, Eudragit®S100 or Poly(ε-caprolactone), when they are incorporated into different vehicles (suspension, hydrogel, and powder) and applied on different mucosal surfaces (mucin, porcine vaginal, and buccal mucosa). Mucoadhesion was measurement by tensile stress tester. Polymeric self-assembling as nanocapsules improved the mucoadhesion of the polymers. The best performance was shown by Eudragit®RS100-nanocapsules. Hydrogels showed higher adhesion when compared to suspensions or powders. Mucin increased mucoadhesiveness of all formulations, but reproduced the difference between them. In conclusion, this study demonstrated that Eudragit®RS100-nanocapsules interacted better with membranes. Furthermore, the vehicle influenced mucoadhesive performance (hydrogel > powders > suspensions). In addition, mucin may be used to compare formulations and in preliminary tests using tensile tester, while the use of porcine mucosa is ideal to mimic adhesion conditions considering *in vivo* experiments.

Keywords: polymeric nanocapsules, mucoadhesion, mucin disc, porcine mucosa, hydrogel, powders.

1. INTRODUCTION

Polymers are the most used materials in the development of mucoadhesive systems due to properties like functional group, pH, charge, and molecular weight, which may influence the form and intensity in which adhesion occurs (ANDREWS *et al.*, 2009). The adhesion of polymers to mucosae has become an important subject in the development of drug delivery systems.

Mucosa is a membrane composed by an epithelial layer covered by a mucus film. Its main function is to protect an organism from the external environment. Formed by lipids, glycoproteins, and inorganic salts suspended in water, the mucus layer has a cohesive gel texture and its thickness varies between 1 and 450 µm (KHUTORYANSKIY, 2011). The glycoprotein mucin is the main component of this structure, and is the main factor responsible for its adhesiveness. Mucin concentration as well as mucus properties vary in function of the mucosal location in the body, which in turn can influence the adhesive performance of the mucosa and consequently the interaction with drug delivery systems (SOSNIK *et al.*, 2014).

Several cavities of the human body are formed by a mucosal surface with adhesiveness characteristics like buccal, esophageal, gastric, intestinal, colonic, rectal, nasal, lung, ocular, and vaginal cavities. These regions have been explored for drug administration aiming towards a more effective local or systemic drug effect (SOSNIK *et al.*, 2014). However, these sites are exposed to a constant flux of biological fluids that can remove part of the applied drug that should be absorbed, influencing drug bioavailability. The mouth, for example, produces 0.5 - 2 L of saliva per day (GOSWAMI *et al.*, 2008), and a woman produces 2 - 3 g of vaginal fluids daily (VALENTA, 2005). In this scenario, drug carriers that strongly interact with the mucosal surface may improve drug absorption, and are amongst the current challenges in the development of polymeric systems for mucosal application.

Previous studies proposed different theories to explain the interaction between polymeric materials and mucosal surfaces (SMART, 2005). Other studies have looked into new ways to promote a stronger interaction between polymeric materials and mucosal surfaces (ANDREWS *et al.*, 2009). One of the proposed alternatives to increase

this interaction is the use of polymers in the form of nanocapsules (FRANK *et al.*, 2014; FONSECA *et al.*, 2014; CHAVES *et al.*, 2017), i.e. structures formed by a polymeric wall stabilized with surfactants around a lipophilic core (JÄGER *et al.,* 2009). It has been demonstrated that the structuration of polymeric materials at the nanoscale increases the surface contact area, facilitating the binding between polymers and mucus (SOSNIK *et al.*, 2014). Moreover, like nanocapsules in general, they are more likely to improve the efficacy of different drugs by controlling the release, improving stability, targeting, improving cellular uptake, modulating permeation, and/or decreasing side effects (POHLMANN *et al.*, 2013; FRANK *et al.*, 2015). Another advantage is that these polymeric nanosystems can be used in different pharmaceutical forms, like suspensions, gels, or powders (FRANK *et al.*, 2015), which facilitates administration because of the flexibility of application while maintaining the original nanostructures and their properties after the drying process (POHLMANN *et al.*, 2002; SCHAFFAZICK *et al.*, 2006; HOFFMEISTER *et al.*, 2012). They are also included in hydrogel formulations (OURIQUE *et al.*, 2011; CONTRI *et al.*, 2010,2014a,2014b; FRANK *et al., 2014*).

Three polymers are often used in the production of polymeric nanocapsules: Eudragit®RS100 (SANTOS *et al.*, 2013; CONTRI *et al.*, 2014a, 2016; KATZER *et al.*, 2014; CHAVES *et al.*, 2017), Eudragit®S100 (SCHAFFAZICK *et al.*, 2005; FRANK *et al.*, 2014, CONTRI *et al.*, 2016), and Poly(ε-caprolactone) (FIGUEIRÓ *et al.*, 2013; DA SILVA *et al.*, 2013; CORADINI *et al.*, 2014; SAVIAN *et al.*, 2015). Eudragit®RS100 [Eudragit RS] is a cationic biocompatible co-polymer of poly(ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) (TRAPANI *et al.*, 2007; ABDALLAH *et al.*, 2012), Eudragit®S100 [Eudragit S100] is a non-toxic anionic co-polymer of poly(methacrylic acid, methyl methacrylate) (ROWE *et al.*, 2009), and Poly(ε-caprolactone) [PCL] is a non-ionic biodegradable and biocompatible polymer (POHLMANN *et al.*, 2013).

Even though the adhesiveness of these three types of polymers is discussed in the literature, little attention has been given specifically to this property when they are structured in nanocapsules. Another important limitation of previous studies is that

mucoadhesion measurements usually comprise the use of animal mucosa, where variations of the biological properties may make result reproducibility more difficult. In addition, the removal of the mucosa may sometimes be difficult, when it is placed in inaccessible locations. In view of this, some authors have used a disc of mucin, which is the glycoprotein responsible for the adhesive characteristic of mucus, instead of mucosal tissue (LEITNER *et al.*, 2003; BRUSCHI *et al.*, 2007; SRIAMORNSAK *et al.*, 2010; FONSECA *et al.*, 2014). Animal mucin shows similar chemical and morphological structure to those of human mucin. Commercially, mucin is extracted from porcine stomach and bovine submaxillary glands (TEUBL *et al.*, 2013).

Summarizing the research problem considered in this paper, the current literature lacks information about the interaction between any of these three different types of polymeric nanocapsules with mucin or animal mucosa models and the resulting mucoadhesiveness of such interaction. Also, there is a lack of information about how the type of vehicle used (e.g. suspension, hydrogel or powder) influences mucoadhesiveness.

In this scenario, the main objective of this paper was to study the effect of the vehicle (suspension, hydrogel, and powder) on the mucoadhesiveness of Eudragit®RS100, Eudragit®S100, or Poly(ε-caprolactone) nanocapsules as well as the effect of different mucosal surfaces (mucin, vaginal mucosa, and buccal mucosa). Moreover, this paper also investigated whether mucin could be an appropriate alternative to the use of fresh animal mucosa in mucoadhesion tests of polymeric nanocapsules using a tensile stress tester.

2. MATERIALS AND METHODS

2.1. Materials

Poly(ε-caprolactone) (Mn 80,000), sorbitan monostearate (Span 60®) and mucin from porcine stomach (type II) were acquired from Sigma-Aldrich (São Paulo, Brazil). Eudragit®RS100 was obtained from Degussa (Darmstadt, Germany), and capric/caprilic triglyceride was obtained from Dellaware (Porto Alegre, Brazil). Eudragit®S100 and hydroxyethyl cellulose were bought from Evonik Industries AG and Embacaps (Porto

Alegre, Brazil), respectively. Polysorbate 80 and acetone were purchased from Vetec (Rio de Janeiro, Brazil). Ethanol and lactose were purchased from Nuclear (São Paulo, Brazil) Dinâmica (São Paulo, Brazil), respectively.

2.2. Preparation and characterization of nanocapsule suspensions

Nanocapsule suspensions were prepared by interfacial deposition according to the preformed polymer method (JÄGER *et al.*, 2009). An organic phase (27 mL) was injected into an aqueous phase (53 mL) formed by 0.077 g of polysorbate 80. The organic phase of particles formed of Eudragit RS (NC-RS) was composed by 0.1g of polymer and 165 µL of capric/caprilic triglyceride dissolved in acetone with magnetic stirring at 40°C. Formulations containing the polymers poly(ε-caprolactone) (NC-PCL) and Eudragit S100 (NC-S100) were produced identically, but the organic phase included 0.0385 g of sorbitan monostearate, and acetone was changed for ethanol in NC-S100. Solvents were removed by reduced pressure (Rotavapor R-114, Büchi, Flawil, Switzerland) and the suspensions were concentrated to the final volume of 10 mL. The formulations (n = 3) were characterized in relation to diameter of particles and polydispersity by laser diffraction (Mastersizer 2000, Malvern Instruments Ltd., UK) inserting the formulations directly in the wet dispersion unit, and by dynamic light scattering (ZetaSizer Nano ZS, Malvern Instruments Ltd., UK) dissolving 20 µL of formulations in 10 mL of ultrapure water. Zeta potential was evaluated by electrophoretic mobility, dissolving 20 µL of formulations in 10 mL of NaCl solution 10 mM (ZetaSizer Nano ZS, Malvern Instruments Ltd., UK), and pH was analyzed using a potentiometer directly in the formulations (VB-10, Denver Instrument, USA).

2.3. Preparation and characterization of hydrogels

Hydrogels were produced by dissolving hydroxyethyl cellulose (2%) into nanocapsule suspensions followed by manual mixing (HG-NC-S100, HG-NC-RS, HG-NC-PCL). The formulations were maintained at 4ºC for 48 h until a hydrogel formed. The pH was determined (n = 3) after the gel was diluted in water (1:10, w/v). The morphology of the hydrogels was analyzed by scanning electron microscopy (SEM; Jeol

Scanning Microscope, JSM-6060, Tokyo, Japan) operating at 10 kV, at the Microscopy Center of the University (Centro de Microscopia Eletrônica - UFRGS, Brazil). For this analysis, the samples were gold-sputtered. The differences in hydrogel morphology was analyzed in order to observe the presence of nanoparticles and to compare the different nanostructures in hydrogels. Moreover, an additional hydrogel was prepared without nanocapsules, as control (HG-HEC).

2.4. Preparation and characterization of spray-dried powders

The powders were prepared according to the spray-dried technique (Mini Spray-Dryer B-290 (Büchi, Flawil, Switzerland) using the following parameters: feed pump rate of 5.0 $ml \cdot min^{-1}$, 100% aspiration, 0.7 mm nozzle, atomization air at 819 $L \cdot h^{-1}$, and an inlet temperature of 120°C with a resulting outlet temperature of approximately 65°C. Lactose was used as drying adjuvant at 10% (w/v). It was added into nanocapsule suspensions (SD-NC-S100, SD-NC-RS, SD-NC-PCL) prior to the drying process and kept under magnetic stirring for 10 min and during the feeding process. The morphology of powders was analyzed by scanning electron microscopy (SEM; Jeol Scanning Microscope, JSM-6060, Tokyo, Japan) operating at 10 kV, at the Microscopy Center of the University (Centro de Microscopia Eletrônica - UFRGS, Brazil). For this analysis, the samples were gold-sputtered. The differences in powder morphology was analyzed in order to observe the presence of nanoparticles in the different powders. An additional powder was produced from an aqueous dispersion of lactose in water (10 % w/v) (SD-Lac), as control.

2.5. Mucoadhesion measurements

A tensile stress tester (TA.XTplus Texture Analyzer; Stable Microsystem, Godalming, UK) was used to analyze mucoadhesion (n = 3) of the polymeric nanocapsules (suspension, hydrogel, and powder). Vaginal and buccal mucosa as well as mucin discs were fixed to the cylindrical probe of the equipment with double-sided adhesive tape. The mucous contacted the nanocapsules samples with a 290-mN preload force for 3 min and, then, they were removed at a constant speed of 0.10 $mm.s^{-1}$ upon

complete detachment. The work (mN.mm) necessary to detach the buccal mucosa, vaginal mucosa, or mucin discs from formulations (NC-S100, NC-RS, NC-PCL, HG-NC-S100, HG-NC-RS, HG-NC-PCL, SD-NC-S100, SD-NC-RS, SD-NC-PCL) was calculated. This calculation is based on the peak of force (mN) and the maximum displacement (mm) upon complete detachment. Solutions of Eudragit S100 (S-S100), Eudragit RS (S-RS), and poly(ε-caprolactone) (S-PCL) were also analyzed for comparison. These solutions were prepared at the same polymeric concentration of the nanocapsule suspensions (0.01 $g.mL^{-1}$). Eudragit S100 was dissolved in ethanol, while Eudragit RS and poly(ε-caprolactone) were dissolved in acetone. Fresh porcine vagina and heads were obtained from Santo Ângelo slaughterhouse (Porto Alegre, Brazil). Vaginal and buccal mucous were excised using a scalpel and were immediately used. Mucin discs were produced by a compression device of the same equipment (TA.XTplus Texture Analyzer; Stable Microsystem, Godalming, UK) using a test speed of 1 $mm.s^{-1}$, post-test speed of 1 $mm.s^{-1}$, and a distance of 4 mm. Mucin discs were hydrated with 20 µL of ultrapure water, and excess water was removed with absorbent paper previously analyzed.

2.6. Statistical analyses

The data collected were analyzed using a full factorial experiment based on the analysis of variance (ANOVA) followed by the Tukey's post-hoc test for multiple comparison of means. These analyses were performed using the SPSS statistics software, version 17.0®. Differences were considered statistically significant for a *p-value* ≤ 0.05.

3. RESULTS

3.1. Physicochemical properties of the nanocapsule suspensions and their respective hydrogels and powders

The mean size of nanocapsules suspensions was determined using the laser diffraction technique. A radar chart containing the mean diameter (d[4,3]), diameter

cumulative of 10 [d(0.1)], 50 [d(0.5)] and 90 [d(0.9)] percent of particles by volume (v) and number (n) distribution is shown in Figure 1.

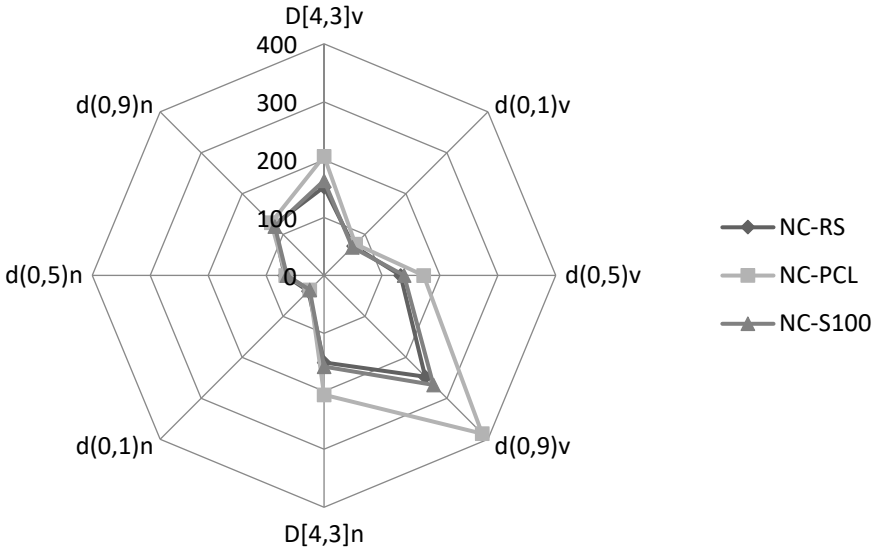

Figure 1 Radar chart of formulations.

All the formulations showed particle size distribution only in the nanoscale range as well as unimodal size distribution (BIANCHIN *et al.*, 2015). The d(0.9)v observed for the three formulations was lower than 400 nm and the d(0.5)n was lower than 100 nm. The volume-weighted mean diameter (d[4,3]v) was 153 ± 25 nm, 164 ± 13 nm, and 206 ± 32 nm for NC-RS, NC-S100, and NC-PCL, respectively. Span values, which represent the polydispersity of the systems, were 1.25 ± 0.35, 1.72 ± 0.08, and 1.44 ± 0.07 for NC-RS, NC-S100, and NC-PCL, respectively. Mean diameter and polydispersity index (PDI) were confirmed by dynamic light scattering analyses (Table 1). Zeta potential was negative for NC-S100 and NC-PCL, though it was positive for NC-RS (Table 1). The pH of all formulations was slightly acid (Table 1).

Table 1: characteristics of formulations in relation to mean diameter and polydispersity index by dynamic light scattering, zeta potential and pH (n = 3, mean ± standard deviation).

	MEAN DIAMETER (nm)	PDI	ZETA POTENTIAL (mV)	pH
NC-S100	154 ± 6	0.141 ± 0.014	-6.76 ± 0.30	5.66 ± 0.12
NC-RS	123 ± 6	0.108 ± 0.013	+6.62 ± 0.52	6.03 ± 0.06
NC-PCL	200 ± 2	0.124 ± 0.017	-5.27 ± 1.38	6.02 ±0.12

The pHs of HG-HEC, HG-NC-S100, HG-NC-RS, and HG-NC-PCL were 6.70 ± 0.06, 6.77 ± 0.06, 6.74 ± 0.03, and 5.86 ± 0.03 respectively. The hydrogel prepared only with hydroxyethylcellulose (Figure 2) had an irregular surface, which was covered with spherical nanoparticles in hydrogels containing the nanocapsule suspensions (Figure 2-B,C,D).

Spray-dried powders were produced using lactose as drying adjuvant. The powder produced only with lactose had irregular structures with smooth surfaces. On the other hand, the powders produced with nanocapsule suspensions showed micro-agglomerates of spherical particles, and surfaces varied according to components of the nanocapsules (Figure 3). Presence of spherical particles at the nanoscale could not be observed in all powders because these particles may be dispersed into the lactose matrix (RIBEIRO *et al.*, 2016)

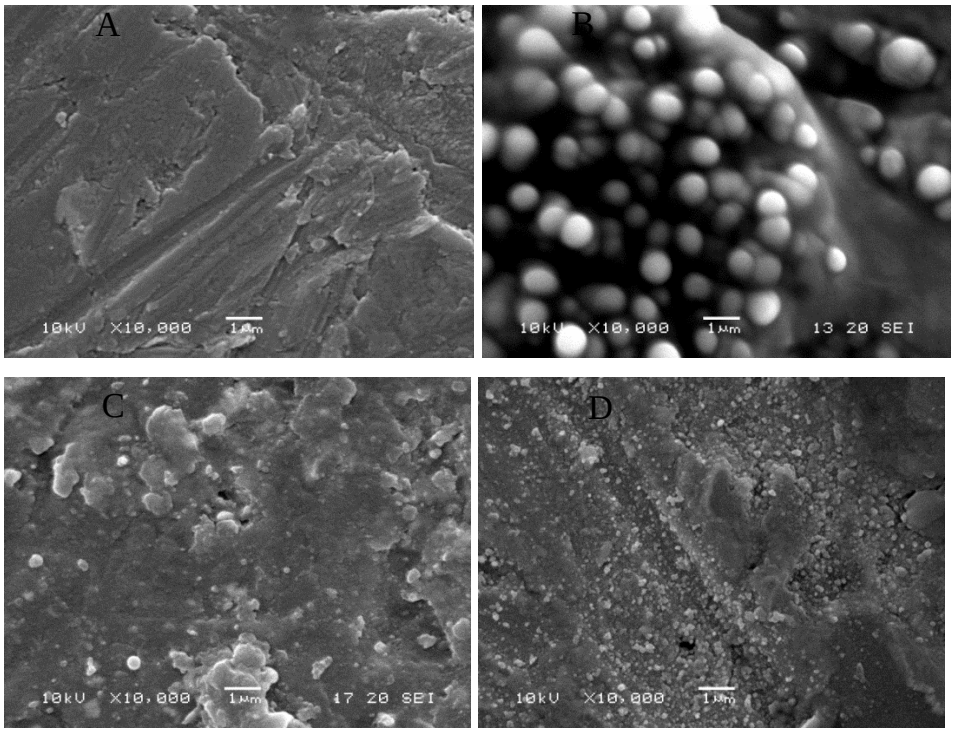

Figure 2 Scanning electron microscopy images of (A) hydrogels of hydroxyethylcellulose (HG-HEC), (B) hydrogels of hydroxyethylcellulose containing nanocapsules of Eudragit®S100 (HG-NC-S100), (C) hydrogels of hydroxyethylcellulose containing nanocapsules of Eudragit®RS100 (HG-NC-RS), (D) hydrogels of hydroxyethylcellulose containing nanocapsules of poly(ε-caprolactone) (GEL-PCL).

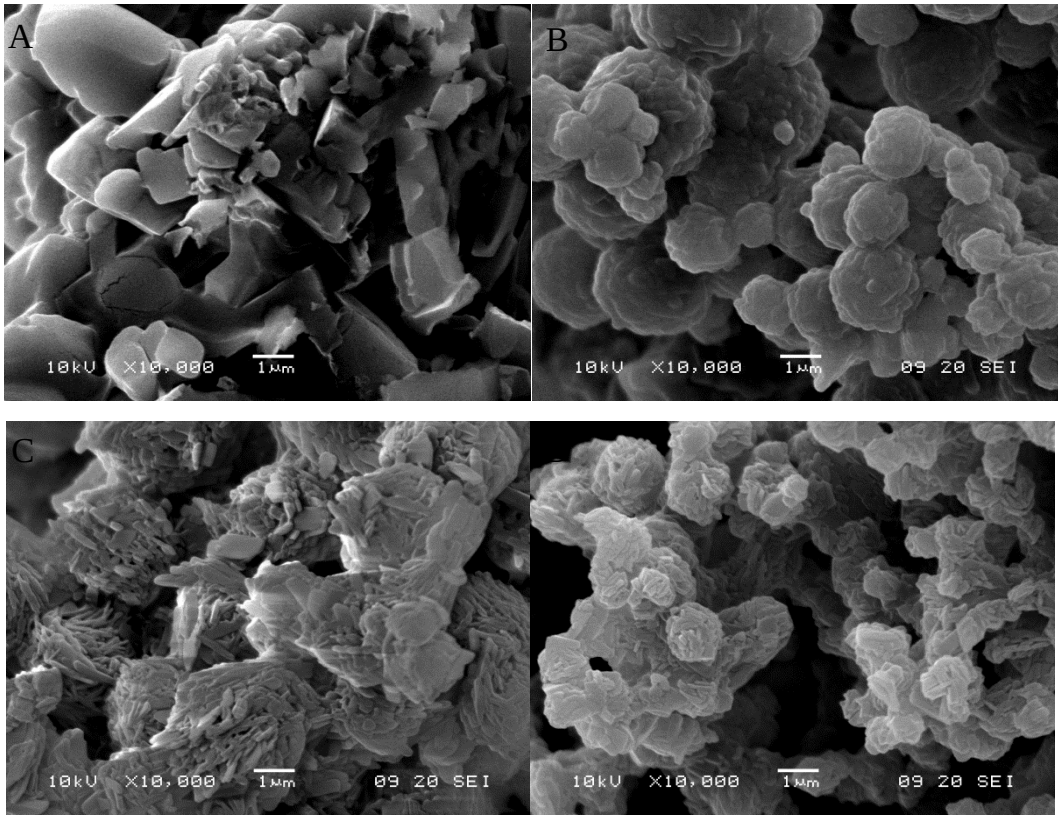

Figure 3 Scanning electron microscopy pictures. (A) lactose powder (SD-Lac), (B) lactose powder containing nanocapsules of Eudragit®S100 (SD-NC-S100), (C) lactose powder containing nanocapsules of Eudragit®RS100 (SD-NC-RS), (D) lactose powder containing nanocapsules of poly(ε-caprolactone) (SD-NC-PCL).

3.2. Mucoadhesion measurements

The first analysis of this study was made to compare the mucoadhesive performance of polymeric nanocapsules in suspension and the solutions of their respective wall-forming polymers. This test was designed to define if it would be necessary to consider both types of polymer structuration in the analysis. The ANOVA results showed that mucoadhesion is much stronger for formulations formed by nanocapsules than for formulations containing dissolved polymer, independently of the type of application surface or polymer used (F-value = 81.38, $p < 0.001$; $\Delta\bar{X} = 84.42$ mN.mm). In view of this, in the second part of analysis, described next, only formulations containing nanocapsules were considered.

In the next step, a three-way ANOVA was performed to study how the combination of the three factors (application surfaces, vehicles, and polymers) can

influence mucoadhesiveness of formulations. Therefore, three types of application surface (mucin, vaginal mucosa, and buccal mucosa), three types of vehicles containing nanocapsules (suspension, hydrogel, and powder) and three types of polymers (Eudragit RS, Eudragit S100, and PCL) were combined. ANOVA results showed that all the main effects (single factor analysis) as well as the second-level interactions (between two factors) were significant ($p < 0.01$). Only the interaction at the third level (all factors at the same time) was not significant in the experiment ($p = 0.276$). Tukey's post-hoc results are shown in Figures 4 and 5.

Regarding the post-hoc results shown in Figure 4, the interaction between the vehicle (suspension, hydrogel, or power) and the type of application surface (mucin, vaginal mucosa, and buccal mucosa) was considered. Regardless of the vehicle type, it is possible to see that the mucin application surface presents the highest mean work at $p < 0.001$: (i) mucin work for suspension = 368.48 mN.mm; (ii) mucin work for hydrogel = 949.94 mN.mm; and (iii) mucin work for powder = 352.91 mN.mm. On the other hand, vaginal mucosa and buccal mucosa did not differ statistically for any of the three different vehicles types: (i) suspension: vaginal work = 88.327 mN.mm and buccal work = 50.88 mN.mm, $p = 0.138$; (ii) hydrogel: vaginal work = 246.678 mN.mm and buccal work = 219.09 mN.mm, $p = 0.27$; (iii) powder: vaginal work = 88.77 mN.mm and buccal work = 69.44 mN.mm, $p = 0.44$). Moreover, for all types of application surfaces, hydrogel was the vehicle that allowed obtaining the highest levels of mucoadhesion, while powder and suspension presented a similar behavior. This could also be corroborated statistically by comparing the main effect of the vehicles types, where the results indicated that hydrogel has a mucoadhesion level (mean work) of 471.9 mN.mm, which is significantly different at $p < 0.001$ from the other two vehicles. On the other hand, suspension (Wmean= 169.23 mN.mm) and powder (Wmean= 170.37 mN.mm) did not differ statistically ($p = 0.937$).

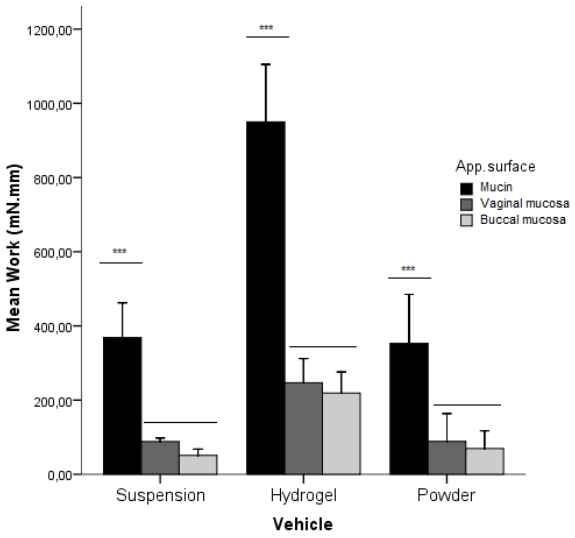

Figure 4 – Mucoadhesiveness of different types of vehicles and application surfaces. *** Application surfaces that differed statistically from the others were highlighted.

Furthermore, the interaction effect between the different types of polymer (Eudragit RS, Eudragit S100, and PCL) and type of application surface (mucin, vaginal mucosa, and buccal mucosa) was analyzed, as shown in Figure 5. Again, the mucin application surface presented the highest levels of mucoadhesion regardless of polymer type at $p < 0.001$: (i) mucin work for Eudragit RS = 735.94 mN.mm; (ii) mucin work for Eudragit S100 = 396.01 mN.mm; and (iii) mucin work for PCL = 539.38 mN.mm. On the other hand, vaginal mucosa and buccal mucosa did not differ statistically concerning each polymer type ($p = 0.075$ for Eudragit RS; $p = 0.107$ for Eudragit S100; and $p = 0.95$ for PCL). Moreover, Figure 2 illustrates the significant differences between all polymers at $p<0.001$ in the following order of mucoadhesion level: (i) Eudragit RS: Wmean = 375.65 mN.mm, (ii) PCL: Wmean = 249.58 mN.mm; and (iii) Eudragit S100: Wmean = 186.27 mN.mm. However, the differences between Eudragit S100 and PCL are prominent because of the mucin effect, while their behavior for vaginal and buccal mucosa seems to be very similar.

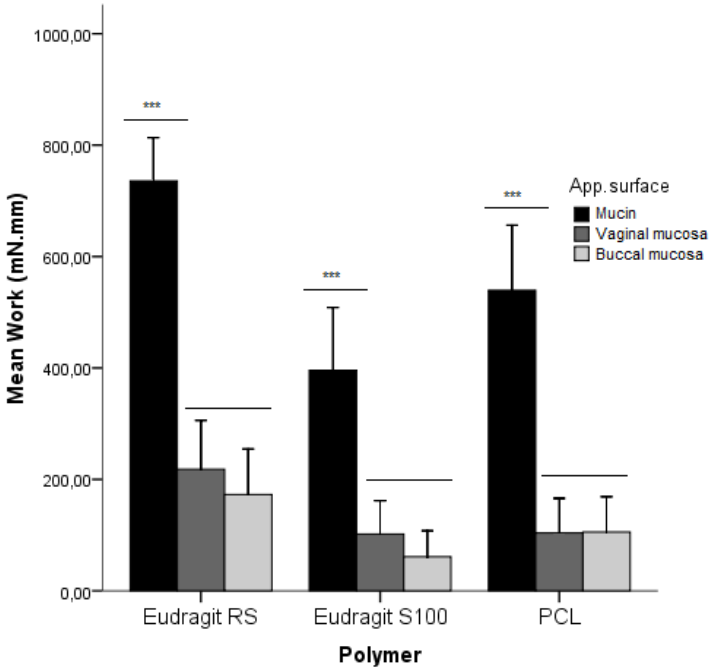

Figure 5 – Mucoadhesiveness of different types of vehicles and application surfaces. *** Application surfaces that differed statistically from the others were highlighted.

Figure 6 summarizes the comparative results of mucoadhesion when the different vehicle types (suspension, hydrogel, and powder) are combined with different types of polymer (Eudragit RS, Eudragit S100, and PCL). As shown in this figure, statistical differences at $p < 0.01$ between all types of polymers in the following order of mucoadhesion levels were observed between hydrogel and powder: (i) hydrogel: HG-NC-RS (606.54 mN.mm); HG-NC-PCL (441.72 mN.mm) and HG-NC-S100 (367.44 mN.mm); and (ii) powder: SD-NC-RS (291.317 mN.mm); SD-NC-PCL (156.12 mN.mm) and SD-NC-S100 (63.69 mN.mm). On the other hand, concerning the suspension vehicle our findings show that NC-RS presented a mean work (mucoadhesion) of 229.09 mN.mm, which differed statistically from the other two polymers at $p < 0.001$, while NC-PCL and NC-S100 did not differ ($p = 0.355$).

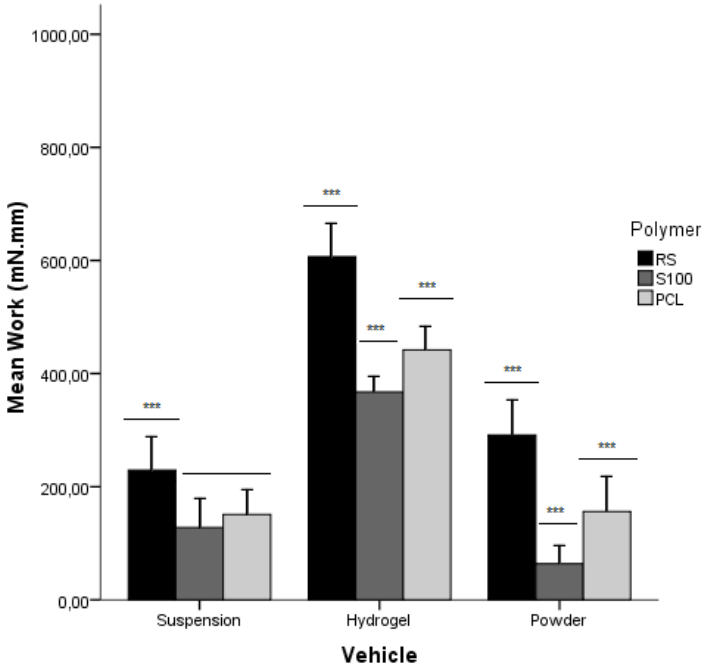

Figure 6- Mucoadhesiveness for different types of vehicles and polymers. *** Polymers that differed statistically from the others were highlighted.

4. DISCUSSION

In order to analyze the nanostructuration effect on mucoadhesiveness of polymers, first it is necessary to be sure of the uniformity of nanocapsules produced. In view of this, nanocapsule suspensions were analyzed regarding their size and polydispersity by laser diffraction and dynamic light scattering. All formulations showed exclusively the presence of nanometric particles with monomodal size distribution, whose values were in agreement with other studies (CONTRI *et al.*, 2014a; CORADINI *et al.*, 2014; FRANK *et al.*, 2014). The zeta potential, which reflects surface charge, varied according to the composition of the system. Eudragit RS nanocapsules showed positive zeta potential, while Eudragit S100 nanocapsules presented negative zeta potential due to cationic and anionic characteristics of the polymers, respectively (FRANK *et al.*, 2014). PCL nanocapsules showed negative zeta potential due to the presence of polysorbate 80 in the interface particle/water, since the polymer is non-ionic (FIEL *et al.*, 2011). The pH values of all formulations were similar, and the influence of this parameter on mucoadhesion could be ignored. Therefore, according to the results

obtained, the developed nanocapsules were uniform enough to afford use in the next steps of this study and in the production of hydrogels and powders. The hydrogels were produced with hydroxyethyl cellulose as thickening agent due to its non-ionic characteristics in water (BRAUN and ROUSEN, 2000), avoiding the effect of any ionic interaction associated with hydrogel composition on mucoadhesion behavior. The semi-solid formulations showed narrow pH, which allowed ruling out its influence in mucoadhesion performance. Microscopy analysis confirmed the maintenance of original nanoparticles in the final pharmaceutical forms produced with nanocapsule suspensions. The spray-dried powders are formed by microagglomerates whose structure is influenced by nanocapsule composing the system.

The results obtained for the mucoadhesion experiments demonstrated that, regardless of the type of polymer used in the preparation of polymeric nanocapsules, they show significantly higher adhesiveness when compared to their respective solution. This may be explained by the increase of their surface area when nanostructured, allowing them to strongly interact with the layers of mucosa (either vaginal or buccal) or with mucin (FRANK *et al.*, 2014, 2017; CONTRI *et al.*, 2014; FONSECA *et al.*, 2015; CHAVES *et al.*, 2017). Moreover, particle size controls the ability of particles to penetrate ("fit") the pores of the mucin mesh (VÀNIC *et al.*, 2013) and the structuration of polymers in nanoparticles may facilitate the "fit" with mucous pores.

The surface properties of the nanocapsules played an important role in their interaction with the mucosa, whereas mucosal surfaces have anionic properties due to the presence of negatively charged mucin molecules (SOSNIK *et al.*, 2014). Eudragit RS nanocapsules had higher adhesiveness when compared to Eudragit S100 or PCL nanocapsules, regardless of mucosal surface. This better performance is observed independently of the vehicle in which nanocapsules were inserted (suspension, powder, or hydrogel). The positive charge of the polymer Eudragit RS may have produced an electrostatic interaction between the polymer and the mucosa, as previously observed by other authors (PIGNATELLO *et al.*, 2002; FRANK *et al.*, 2014; CONTRI *et al.*, 2014a, CHAVES *et al.*, 2017). Therefore, when polymeric nanocapsules are produced with Eudragit RS, they develop positive surface charge, as observed in zeta potential

values, resulting in higher adhesiveness for the application on mucosa (FRANK *et al.*, 2014; CONTRI *et al.*, 2014a; CHAVES *et al.*, 2017). On the other hand, no statistical differences were observed between the adhesiveness values of Eudragit S100 and PCL nanocapsules, regardless of the type of mucosa. A possible explanation for this result may be the fact that the formulations produced with these polymers presented similar negative zeta potential values. When these particles are inserted in hydrogel or powder, a statistical difference could be observed, and formulations containing PCL nanocapsules had higher values of adhesion than formulations containing Eudragit S100 nanocapsules. However, in this case the vehicle may have influenced the interaction with the adhesive surface. PCL has some advantages like, for example, being bioadhesive (SUDHAKAR *et al.*, 2006), biodegradable, and biocompatible (POHLMANN *et al.*, 2013). Therefore, from the practical point of view it is important to assess the viability of the use of each polymer for each specific administration route. In this present work, we took into account only the technical aspect of the adhesiveness of the polymers.

In relation to the vehicle type used, hydrogels showed higher adhesion values in any kind of surface, when compared to suspension or powder vehicles. This was also previously observed by other authors (FRANK *et al.*, 2014; CONTRI *et al.*, 2014a, FRANK *et al.*, 2017). An explanation for this is that an hydrogel has high viscosity and is able to interact longer with the mucosa due to its physicochemical characteristics. Hydrogels have been used in research with the aim of increasing the adhesiveness of drugs on the mucosa (CARAMELLA *et al.*, 2015), and the results obtained demonstrate that it may be a viable option for this purpose. Polymeric nanocapsule suspensions and nanostructured powders presented higher adhesiveness to mucosa when compared to polymeric solutions. They also have other advantages compared to conventional systems, as for instance: (i) controlled drug release; (ii) capacity of drug delivery in targeting sites; (iii) increase in drug photodegradability, among others (BECK *et al.*, 2012; FRANK *et al.*, 2015). Therefore, these systems have been used as vehicles for different drugs such as sprays and tablets or filling of capsules, respectively. Their adhesiveness is related mainly to presence of nanometric particles. Particle sizes

between 200 and 500 nm can substantially influence the diffusion of a drug present in these nanostructures, which can be transported through the mucin mesh that composes the mucosa and fluids (Das NEVES *et al.,* 2015; CARAMELLA *et al.*, 2015).

Regarding the type of application surface for the *in vitro* mucoadhesion measurements, some authors have used fresh animal mucosa and these characteristics were also evaluated by means of different methods (HUSSAIN E RITSCHEL 1989; BONFERONI *et al.,* 2008; SANDRI *et al.,* 2004; FRANK *et al.,* 2014; CHAVES *et al.*, 2017; FRANK *et al.,* 2017). However, given the fact that the biological properties can affect the reproducibility of the results and, depending on the mucosa location, its removal can be a limiting factor, some researchers have proposed the use of mucin discs as an alternative in the determination of the mucoadhesion of formulations (LEITNER *et al.,* 2003; BRUSCHI *et al.,* 2007; SRIAMORNSAK *et al.,* 2010; FONSECA *et al.*, 2014). In this present work, a comparative analysis between both options of surface model – fresh porcine mucosa and mucin discs - was performed to assess adhesiveness. It was observed that the use of mucin discs leads to significantly higher adhesiveness work when compared with fresh mucosa (either vaginal or buccal). When only this substance was considered in the adhesiveness experiments, the resulting values did not reproduce the values of porcine vaginal and buccal mucosa, and mucoadhesiveness was overestimated. This may have happened because the correct mucosa environment was not simulated in this situation. The mucosa has high water contents (~99 %) and around 1% of organic and inorganic materials, including glycoproteins (GOSWAMI *et al.*, 2008). In view of this, a mucin disc has a higher mucin concentration than physiological mucosal surfaces. However, mucin discs were able to reproduce the difference between formulations containing or not nanocapsules as well as between nanocapsules with different ionic characteristics, as observed for porcine vaginal and buccal mucosa. In view of this, for the purpose of comparing formulations, mucin discs may be a substitute model, being useful as adhesive surface for preliminary analyses of mucoadhesiveness. The two porcine mucosae used in the experiment (vaginal and buccal) did differ statistically, and appear to be a more appropriate model for mimicking *in vivo* effects.

5. CONCLUSION

This study demonstrated that structuration of EudragitRS100, EudragitS100, and PCL in nanocapsules improved their interaction with mucosal surface. Due to cationic characteristics, nanocapsules produced with Eudragit®RS100 showed better adhesiveness when compared to anionic nanocapsules formed by Eudragit®S100 and PCL. Incorporation of nanocapsules in hydrogels or the spray-dried process did not alter mucoadhesive profiles. Hydrogels showed higher adhesiveness than powders and suspensions. Work values of the interaction between formulations and mucine were higher than the values observed for the interaction between formulations and porcine vaginal and buccal mucosae. However, mucine was able to reproduce the differences between formulations and vehicles, despite the fact that values were higher. In view of this, mucin discs may be an alternative mucoadhesive surface in preliminary studies. Porcine mucosa is ideal to mimic *in vivo* adhesion conditions; therefore, its use is more appropriate for studies about *in vivo* effects.

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Disclosure of interest

The authors report no conflicts of interest.

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APÊNDICE B

Apêndice B (Artigo 6) - Copaiba oil-loaded polymeric nanocapsules – modern nanostructures for the encapsulation of other lipophilic cytotoxic agents in order to fight cervical cancer

SHORT COMMUNICATION

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Abstract

Copaiba oil is broadly known in popular medicine by its anti- inflammatory action. Moreover, several studies have indicated its use in the treatment of skin cancer due to its direct anti-tumoral activity. Therefore, developing new formulations that explore copaiba oil anti-tumoral effect associated to other cytotoxic drugs can be a successful strategy to fight cancer. We produced polymeric nanocapsules containing copaiba oil as an alternative system to solubilize other lipophilic cytotoxic agents and therefore boost the anti-cancer treatments. As expected, copaiba oil-loaded polymeric nanocapsules were able to slightly decrease cervical cancer cell viability *per se*, triggering cell death through apoptosis. Thus, copaiba oil-loaded polymeric nanocapsules appears to be a versatile nanostructured system to improve cancer treatment not only for their direct anti-tumoral effect but also for their ability to incorporate other lipophilic cytotoxic agents. We propose the application of this formulation in association with different active drugs as a possible modern approach to boost other anti-cancer agents against cervical cancer.

Keywords: polymeric nanocapsules; copaiba oil; SiHa; cervical cancer.

1. INTRODUCTION

The non-selective cytotoxic action of many drugs constitutes an important limiting step in the treatment of cervical cancer, often resulting in tumor recurrence and patient death (THULER et al., 2008). Therefore, there is an urgent need for new formulations with specific therapeutic action on the tumor cells. Nanostructured systems have been used to improve drug performance and therapeutic index, by specifically delivery drugs to the selected sites (FRANK et al., 2015). In this sense, the encapsulation of multiple cytotoxic drugs in solid lipid nanoparticles may be a promising strategy to potentiate tumor cell death and therefore improve cervical cancer outcomes.

Copaiba oil - extracted from the stem of Copaifera trees from the tropical region of Latin America - has gained increasing attention in the pharmaceutical industry due to its wide pharmacological application, especially as anti-inflammatory (VEIGA et al., 2007), antifungal (SVETLICHNY et al., 2015) and antineoplastic agent. Yet, many studies have supported its use as an adjuvant agent in the treatment of melanoma (LIMA et al., 2003), invasive micropapillary carcinoma (OHSAKI et al., 1994) and vaginal and cervical cancer (BRITO et al., 2000; BRITO et al., 2010).

Therefore, incorporating copaiba oil into the polymeric nanocapsules could be an innovative approach to boost anti-cancer therapy not only for its intrinsic anti-tumoral activity but also for its ability to solubilize other lipophilic cytotoxic agents. In this work we intent to develop polymeric nanocapsules containing copaiba oil in their nucleus as well as evaluate their cytotoxic effect in cultures of cervical cancer cells to further associate them with different anti-tumoral drugs and potentially improve cervical cancer treatment.

2. MATERIALS AND METHODS

2.1. Materials

Annexin V and propidium iodide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Acridine orange (AO) was purchased from Sigma-Aldrich (St. Louis, MO). Poly(ε-caprolactone) (PCL) (Mn 80 kg mol^{-1}) and sorbitan monostearate (Span 60®) were purchased from Sigma-Aldrich (Steinheim, Germany). Polysorbate 80

(Tween 80®) was purchased from Henrifarma (São Paulo, Brasil) and Copaiba oil was kindly donated by Inovam-Da Lamarta & cia Ltda. Imiquimod (IMIQ) was purchased from Chemical Goods (Guangdong, China). The cervical carcinoma cell line SiHa was purchased from American Type Culture Collection, Rockville, MD. All solvents and reagents were of analytical or pharmaceutical grade.

2.2. Methods

2.2.1. Production of oil-loaded nanocapsules

Polymeric nanocapsules containing copaiba oil in their core were produced by the nanoprecipitation method, with the same concentrations of materials used by Frank and co-workers (2017). The formulation produced was named NC_{cop}.

2.2.2. Characterization of nanocapsules

The NC_{cop} was characterized by two complementary methods: laser diffraction (LD) (Mastersizer 2000, Malvern Instruments Ltd., UK) and dynamic light scattering (DLS) (ZetaSizer Nano ZS, Malvern Instruments Ltd., UK). The morphology of NC_{cop} was analyzed by transmission electron microscopy (TEM, Jeol JEM 1200-ExII, 100 mV, Tokyo, Japan) at the Microscopy Center of the University (Centro de Microscopia Eletrônica - UFRGS, Brazil). The samples were diluted in (1:10 v/v) ultrapure water deposited on specimen grid (Formvar-Carbon support film, Electron Microscopy Sciences) and negatively stained with uranyl acetate solution (2%, w/v).

2.2.3. Cell culture

For the experiments involving analysis of the cytotoxicity of the formulations, the cervical carcinoma cell line SiHa was used (American Type Culture Collection, Rockville, MD). Cells were maintained in low glucose DMEM supplemented with 10% FBS and penicillin/streptomycin antibiotics (0.5 U·mL/95% air at 37°C). All materials were previously sterilized, and the formulations were prepared under aseptic conditions.

2.2.4. Cell viability

Cell lines (40,000 cells/well) were seeded on 24-well and treated with 3.0µM of NC_{cop} in culture media for 24 and 48 hours. Then the number of viable cell was

determined by flow cytometry using FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA).

2.2.5. Cell death mechanism assay

Tests were performed to verify the type of cell death mechanism involved after cells being treated with NC_{cop}. The experiments were: a) annexin V and propidium iodide staining for verification of possible death by apoptosis and necrosis; b) labeling the nucleus of cells with hoechst dye; to detect fragmented nuclei c) detection of autophagy using acridine orange (AO); d) cell cycle analyses. For all experiments, the methodology followed was that described by Frank and co-workers (2017).

2.2.6. Data analysis

Statistical analyses were performed by means of Analysis of Variance (ANOVA) followed by the post-hoc Tuckey's test for multiple comparison of means ($\alpha = 0.05$), using SPSS statistics 17.0®.

3. RESULTS AND DISCUSSION

3.1. Production of nanocapsules loaded with copaiba oil

Figure 1 shows the nanocapsule sizes measured by different techniques. Figure 1A represents the volume-weighted mean diameters. The shape of the curves in this figure are fingerprint characteristic of unimodal size distributions. In this figure, it is possible to observe that 50% of the particles d(0.5)n presented diameters below 200 nm. The values found was SPAN<2.0 indicating homogeneity of the particles. Figure 1B represented the morfhology of formulation NC_{cop}. This figure evidences homogeneous particles with nanometric size (all less than 500 nm). The dynamic light scattering showed particle sizes of 190.50±8.20 and PDI<0.1. These results are in agreement with those presented in Figure 1A. Thus, it was possible to verify the presence of an adequate nanotechnological system.

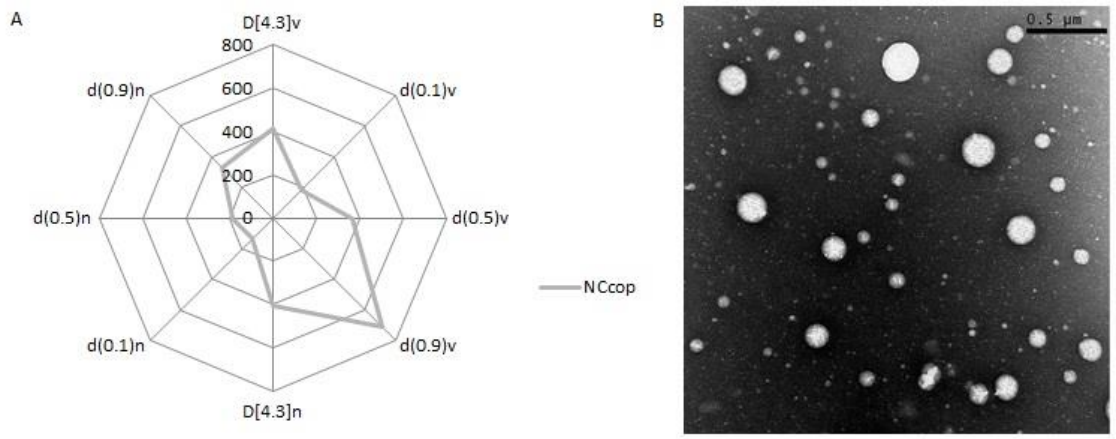

Figure 1: Particle size distribution. A- Radar chart presenting the volume-weighted mean diameters (D[4,3]) and the diameters at percentiles 10, 50 and 90 under the size distribution curves by volume and by number of particle. B- Transmission electron microscopy (TEM) micrographs: (50000x)

3.2. NCcop slightly decreases SiHa cell viability

According to figure 2, polymeric nanocapsules containing copaiba oil at a very low concentration (0.125µL copaiba oil) led to a slight but significant decrease on SiHa cell viability after 24h (85.57%±8.82) and 48h (80.94%±5.06) of treatment when compared to control. However this NC_{co} effect wasn't time-dependent since no significant difference on cell viability was found over the time ($p > 0.05$).

An effect for copaiba oil on uterine cervix epithelium of oophorectomized rats was previously described by Brito and coworkers (2000). According to this study, topical application 0.3 mL of copaiba oil on the vaginal mucosa triggered morphological and morphometric changes in uterine cervix resulting in thickening of the epithelium. The reasons attributed to this result are still not completely understood, but the authors suggest a possible pseudo-hormonal effect or a topical irritation caused by the oil. In a different approach BRITO and coworkers (BRITO et al., 2010) also verified the effect of topical application of copaiba oil on the vagina and uterine cervix of Walker 256 tumor-bearing rats. Animals inoculated with Walker 256 carcinoma cells into vagina and uterine cervix were treated with 4.8 ml / kg of copaiba oil or with water (control group) and the effect on tumor growth was evaluated. Surprisingly copaiba oil showed a stimulatory effect over cancer cells boosting tumor growth. This tumor promoting effect was attributed to its immunosuppressive activity – described in high doses -

therefore contributing to tumor immune escape. In contrast, Lima and co-workers (2003) reported an anti-tumor effect for copaiba oil in a mouse model of melanoma. Accordingly, copaiba oil was able to decrease 58% of tumor growth when used at concentration of 2 g/kg. At the same time, it reduced melanoma cell line (B16F10) viability *in vitro* in a concentration and time-dependent manner. Of note, this latter result was collected after 1 and 3 hours of experiment and the copaiba oil concentration used was 0.5 and 1 mg/ml. Despite its contradictory effects, copaiba oil final activity either pro- or anti-tumoral appears to vary according to the concentration, formulation, administration route and time of exposure. To the best of our knowledge we are the first group to test copaiba oil-loaded polymeric nanocapsules effect on cervical cancer cell lines.

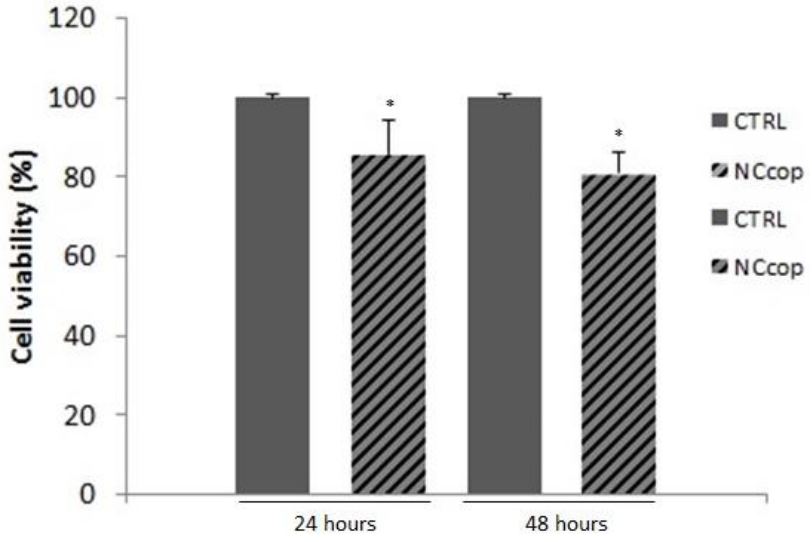

Figure 2 – - Number of viable cells after 3µM NCcop treatment for 24h and 48 hours *p < 0.05 compared with control (one-way ANOVA, followed by Tukey's test).

3.3. NCcop triggers cervical cancer cell death through apoptosis

Considering the slight reduction in cell viability triggered by NC_{cop} we futher investigated the mechanism enrolled in this process. Figure 3 shows that NC_{cop} is capable to produce modest cellular changes similar to apoptotic features such as cell shrinkage, membrane blebbing, chromating condensation and increased annexin V

stainig at both 24h and 48h post treatment. Moreover, an increase annexin V/IP double staining can be noticed, which is representative of late apoptosis. So far no studies have elucidated the mechanism involved in the cell death triggered by copaiba oil. We are the first group to support an apoptosis related cell death trigged by this compound.

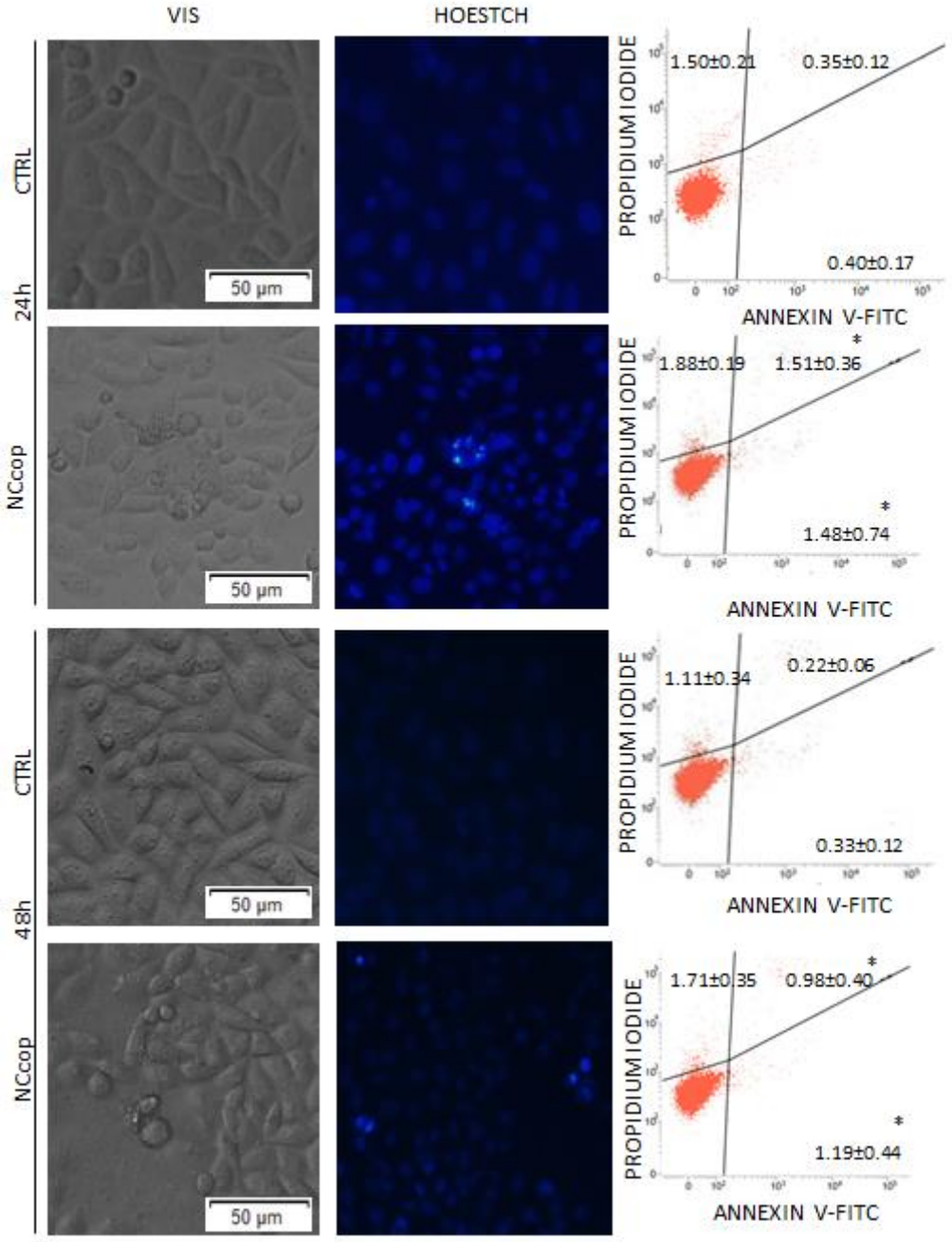

Figure 3 –SiHa cells were treated with 3.0µM of NCcop for 24 h and 48 hours. Then pictures were taken before (left) and after (middle) cell nuclei stained with Hoescht. Note apoptotic features such as cell shrinkage, membrane blebbing and fragmented nuclei when cells were treated with NC_{cop}. Scale bars, 50 µm; magnification, 20×. Right: Apoptosis and necrosis measurement according to annexin V/PI binding. n=3 and *p < 0.05 compared with control (one-way ANOVA, followed by Tukey's test).

3.4. NCcop lead cells to accumulate at the SubG1phase of the Cell cycle

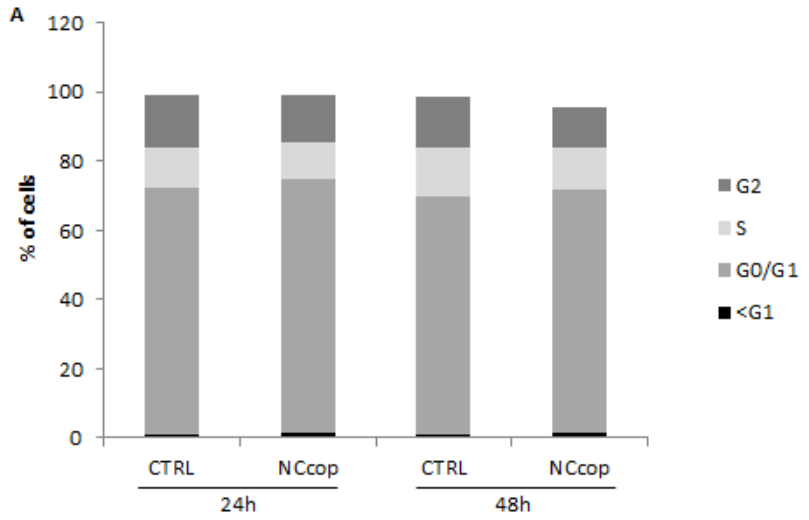

Figure 4 demonstrated that NC_{cop} treatment for 24 and 48 hours lead cells to accumulate at the SubG1 phase of the cell cycle - suggestive of apoptosis induction. At the same time, a reduction in the percentage of G2/S subpopulation was observed. In agreement with the data showed at the Figure 3 this result reinforce the fact that apoptosis is the main mechanisms of cell death triggered by NC_{cop} in SiHa cells.

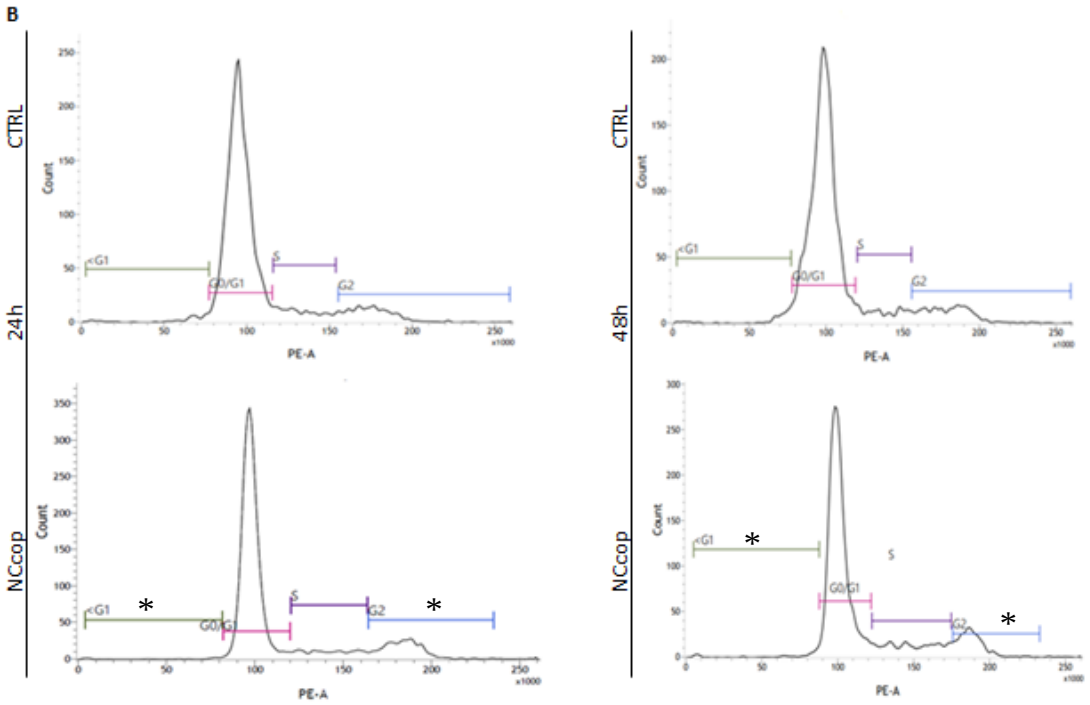

Figure 4- Cell cycle distribution after 24 h and 48 h of treatment of SiHa cells with 3µm of NC_{cop}. Results are mean values±SD (n=3). *p < 0.05 compared with control (one-way ANOVA, followed by Tukey's test). NOTE: Cout = number of cells; PE-A = fluorescence intensity.

3.5. Autophagy is not related to NCcop mechanism of cell death induction

NC_{cop} ability to induce autophagy - as an auxiliary mechanism of cell death - was also investigated. According to Figure 5, there was no increase into the number of AO^+ cells despite NC_{cop} exposure for 24h and 48h, suggesting that NC_{cop} triggers cell death independently of autophagy process. Despite the literature has supported a cytotoxic effect for copaiba oil either *in vivo* or *in vitro* (LIMA et al., 2003; BRITO et al., 2000; BRITO et al., 2010), a role for autophagy in this process has never been reported. Regardless, in our experimental settings, autophagy does not appear to contribute with NC_{cop} ability to induce cell death.

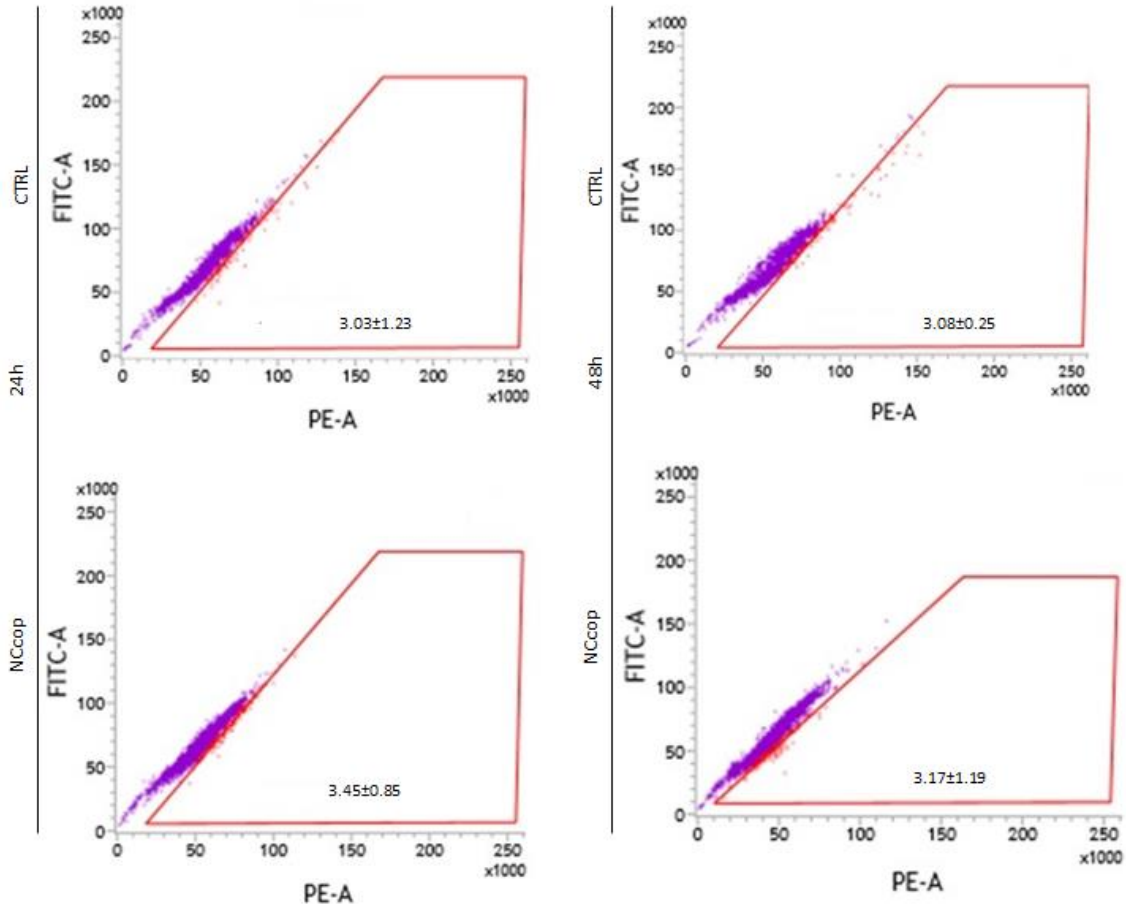

Figure 5 – SiHa was left untreated or treated with 3µm of NC_{cop} for 24h and 48h. Autophagy were measured according to the acridine orange (AO) staining. Note: PE-A: positive AO staining.

4. CONCLUSIONS

Polymeric nanocapsules with copaiba oil in their nucleus were produced with nanometric size. Considering its slight ability to induce cervical cancer cell line death through apoptosis, NC_{cop} could be used as a versatile system designed to solubilize other lipophilic cytotoxic agents in order to improve their tumor killing activity. Therefore we propose the application of this formulation in association with different active drugs as a possible modern approach to boost other anti-cancer agents against cervical cancer.

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Disclosure of interest

The authors report no conflicts of interest.

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