



UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMÁCIA

Izi Vieira Nunes Cunha

**DESENVOLVIMENTO DE INVASOMAS CONTENDO APIGENINA
PARA O TRATAMENTO DO CÂNCER DE PELE**

Florianópolis

2023

Izi Vieira Nunes Cunha

**DESENVOLVIMENTO DE INVASOMAS CONTENDO APIGENINA PARA
O TRATAMENTO DO CÂNCER DE PELE**

Dissertação submetida ao Programa de Pós-Graduação em Farmácia da Universidade Federal de Santa Catarina para a obtenção do título de Mestra em Farmácia.

Orientador: Prof. Thiago Caon, Dr

Coorientadora: Angela Machado de Campos, Dra.

Florianópolis-SC

2023

Cunha, Izi Vieira Nunes

DESENVOLVIMENTO DE INVASOMAS CONTENDO APIGENINA PARA O TRATAMENTO DO CÂNCER DE PELE / Izi Vieira Nunes Cunha ; orientador, Thiago Caon, coorientadora, Angela Machado Campos, 2023.

160 p.

Dissertação (mestrado) - Universidade Federal de Santa Catarina, Centro de Ciências da Saúde, Programa de Pós-Graduação em Farmácia, Florianópolis, 2023.

Inclui referências.

1. Farmácia. 2. apigenina; invasomas; melanoma; terapia antitumoral. I. Caon, Thiago. II. Campos, Angela Machado. III. Universidade Federal de Santa Catarina. Programa de Pós-Graduação em Farmácia. IV. Título.

Izi Vieira Nunes Cunha

Título: Desenvolvimento de invasomas contendo apigenina para o tratamento do câncer de pele

O presente trabalho em nível de Mestrado foi avaliado e aprovado, em 29 de setembro de 2023,
pela banca examinadora composta pelos seguintes membros:

Prof. Prof. Luis Felipe Costa Silva, Dr.
Instituição Universidade Federal de Santa Catarina

Profa. Vânia Rodrigues de Lima, Dra.
Instituição Universidade Federal do Rio Grande

Profa. Mariele Paludetto Sanches, Dra.
Instituição Universidade Federal de Santa Catarina

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THIAGO CAON
Data: 21/12/2023 09:14:08-0300
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Prof. Thiago Caon, Dr. Orientador

Florianópolis, 2023

AGRADECIMENTOS

À minha mãe, Vania, por me mostrar sua visão sensível sobre o mundo, pela sabedoria e por ser uma inspiração.

Ao meu pai, Amilton, que mesmo em memória sempre esteve presente em meu coração, me lembrando de ser forte e ter coragem.

Aos meus irmãos, Plínio e Kim, por estarem sempre ao meu lado.

À minha família em geral, que sempre esteve presente.

Ao meu orientador, Prof. Dr. Thiago Caon, por me desafiar e acreditar no meu potencial, por toda sua dedicação, ensinamentos e paciência.

Aos meus colegas de mestrado, Júlia e Douglas por toda a ajuda no desenvolvimento experimental deste trabalho, mas acima de tudo por compartilharem sonhos, risadas e momentos de leveza.

À colega Ingrid Vicente Farias, pelo apoio e auxílio experimental.

À minha coorientadora, Prof^a. Dr^a. Ângela Machado de Campos, por toda sua sabedoria e por tornar a jornada mais leve.

À Prof^a. Dr^a. Adriana Passarella Gerola, por todo auxílio, dedicação e paciência para ensinar.

À Active Farmacêutica pela doação da substância ativa utilizada para a pesquisa.

A todos os colegas do Laboratório de Farmacotécnica pelo convívio e apoio.

À banca examinadora pela disponibilidade e contribuições.

À CAPES e CNPQ pela bolsa e apoio financeiro deste projeto.

À UFSC e ao PGFar por disponibilizarem toda a infraestrutura necessária para a execução deste projeto.

À todos que direta ou indiretamente colaboraram na execução deste trabalho e na minha formação acadêmica e que não foram aqui mencionados.

RESUMO

Terapias anti-melanoma têm sido acompanhadas de vários efeitos adversos indesejáveis, recidivas e resistência a fármacos, o que tem motivado a busca por novas alternativas terapêuticas. Estudos têm demonstrado que a apigenina (APG), um flavonoide obtido de plantas, atua de maneira “seletiva” na modulação de várias vias de sinalização que controlam o desenvolvimento, crescimento e metástases de células tumorais. Apesar da alta eficácia, a APG é caracterizada por baixa solubilidade aquosa, alta metabolização hepática, forte ligação a proteínas plasmáticas e lenta absorção oral. A fim de superar estas limitações, duas estratégias foram propostas neste estudo: a substituição da rota oral pela percutânea e a incorporação da APG em invasomas, um tipo especial de lipossomas que contém baixas concentrações de etanol e um ou mais terpenos. A administração percutânea da APG permite encurtar o caminho até os tecidos-alvo enquanto invasomas trazem como principal vantagem o aumento da solubilidade e permeação cutânea. Por se tratar de um sistema pouco explorado, primeiramente avaliou-se como seus componentes (colesterol, limoneno e etanol) afetam as propriedades biofísicas (rigidez de membrana e estabilidade) e interações com a pele utilizando-se técnicas espectroscópicas e de espalhamento de luz. Um aumento proporcional na rigidez das membranas foi observado para concentrações de colesterol acima de 10%, além de maior grau de desordem do estrato córneo. Por outro lado, este agente não resultou em melhora da estabilidade das vesículas. O limoneno tornou as vesículas de fosfatidilcolina mais estáveis, flexíveis, monodispersas e potencializou interações com a pele. Finalmente, baixas concentrações de etanol (3% p/v) resultaram em um aumento do tamanho das vesículas e diminuição da rigidez de membrana mesmo na presença de colesterol. Para a próxima etapa do estudo, que incluiu a incorporação da APG nestes sistemas inovadores, lipossomas e invasomas com e sem colesterol foram preparados, caracterizados e então avaliou-se a distribuição do flavonoide nas diferentes camadas da pele utilizando-se o modelo de difusão das câmaras de Franz. A APG foi eficientemente incorporada em invasomas (>98%), além de torná-los mais estáveis. O limoneno contribuiu para um incremento significativo na permeação/retenção da APG na pele humana, o que foi ainda maior na presença do colesterol. Estes achados podem ser associados ao efeito de desestruturação/fluidificação de domínios lipídicos do estrato córneo (identificado por análises de ATR-FTIR). Como consequência, maior quantidade de APG alcança a região da lâmina basal, que é onde ficam os melanócitos (células envolvidas na patogênese do melanoma). Em resumo, invasomas mostraram-se mais promissores que lipossomas convencionais para a incorporação da APG tendo em vista a maior estabilidade, deformabilidade/flexibilidade das vesículas e interação com a pele, encorajando a realização de ensaios de atividade antitumoral e/ou incorporação de outros agentes antitumorais que possuem problemas de toxicidade. Estudos avaliando um provável efeito anti-melanoma sinérgico de APG e limoneno também devem ser realizados, tanto na forma livre quanto invasomal, sempre com a perspectiva de se alcançar terapias mais seguras e efetivas.

Palavras-chave: apigenina; invasomas; melanoma; terapia antitumoral.

ABSTRACT

In anti-melanoma therapies, several undesirable adverse effects, disease recurrences and drug resistance have been common, which has motivated the search for new therapeutic alternatives. Literature studies have shown that apigenin (APG), a flavonoid obtained from plants, acts in a “selective” way in the modulation of several signaling pathways that control the development, growth and metastasis of tumor cells. Despite its high efficacy, APG is characterized by low aqueous solubility, high hepatic metabolism and binding to plasma proteins as well as slow oral absorption. Two approaches were proposed in this study aiming to overcome these limitations: the change of its oral route for the percutaneous route and the incorporation of APG in invasomes, a particular type of liposomes that contain low concentrations of ethanol and one or more terpenes. The percutaneous administration of APG makes it possible to shorten the pathway/route to the target tissues while invasomes have the main advantage of increasing both solubility and cutaneous permeation. Given that invasomes are underexplored systems, the effect of vesicle composition (cholesterol, limonene and ethanol) on the biophysical properties (membrane rigidity and stability) and interactions with the skin was firstly studied. Spectroscopic and light scattering techniques were selected for this step. A proportional increase in membrane rigidity was found for cholesterol concentrations above 10%, besides a greater disruption of stratum corneum. On the other hand, this agent did not improve vesicle stability. Limonene, in turn, resulted in vesicles more stable, flexible, monodisperse and presenting enhanced interactions with the skin. Finally, low concentrations of ethanol (3% w/v) resulted in an increase in vesicle size and a decrease in membrane rigidity even in the presence of cholesterol. The next step of this study included the incorporation of APG in these innovative systems. Liposomes and invasomes with and without cholesterol were prepared, characterized and then the distribution of the flavonoid in the different layers of the skin was evaluated through the Franz-type diffusion cell model. APG was efficiently incorporated into invasomes (>98%), besides making them more stable. Limonene contributed to a significant increase in APG permeation/retention in human skin, which was even greater in the presence of cholesterol. These findings may be associated with the disruption/fluidification effect of stratum corneum lipid domains (identified by ATR-FTIR analyses). As a consequence, a greater amount of APG reaches the region of the basal lamina, which is where melanocytes (cells involved in the pathogenesis of melanoma) are located. In summary, invasomes proved to be more promising than conventional liposomes for the incorporation of APG in view of the greater stability, deformability/flexibility of vesicles and interaction with the skin, encouraging the evaluation of antitumor activity of these systems and/or incorporation of other antitumor drugs that present toxicity problems. Studies evaluating a probable synergistic anti-melanoma effect of APG and limonene should also be performed to ensure that safer and more effective therapies are found.

Keywords: apigenin; invasomes; melanoma; antitumoral therapy.

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1 INTRODUÇÃO

O câncer de pele afeta aproximadamente 1,2 milhões de indivíduos anualmente (WHO, 2022), sendo o tipo de carcinoma mais comum no mundo (*Skin Cancer Foundation*, 2022). No Brasil, representa 33% de todos os diagnósticos de câncer no país (INCA, 2020). Tumores de diferentes linhagens podem ser encontrados e, desta forma, podem ser classificados de acordo com a célula de origem e seu comportamento. Embora o melanoma seja o tipo de câncer de pele mais raro, também é o mais agressivo e letal. A taxa de sobrevida depende do estágio em que o tumor se encontra. Em seu estágio mais avançado, estima-se uma taxa de sobrevida de apenas 31,9% (NCI, 2022). O melanoma cutâneo é uma neoplasia relacionada com a proliferação de melanócitos atípicos na camada basal da epiderme. Esta patologia possui origem multifatorial resultante da interação entre exposição ambiental e susceptibilidade genética. A exposição à radiação UV está entre os principais fatores de risco (SAGINALA et al., 2021).

A primeira opção terapêutica para melanomas diagnosticados em estágios iniciais é a excisão cirúrgica, sendo efetiva na maioria dos casos. Por outro lado, alguns pacientes apresentam recidiva do melanoma de forma disseminada ou são diagnosticados de forma tardia. Nestas situações, a excisão cirúrgica pode não ser suficiente (LUKE et al., 2017). Ainda que nos últimos anos tenha ocorrido uma revolução em termos de tratamento do melanoma, com a aprovação de fármacos que compõem as terapias direcionadas (inibidores MAPK) e imunoterápicos (anticorpos monoclonais), observa-se que estes medicamentos ainda possuem limitações em termos de resposta, com recidivas frequentes. Outra problemática envolvendo estes medicamentos é a capacidade dos tumores em desenvolver resistência a estas substâncias e/ou ativarem vias de proliferação alternativas (DE LUCA et al., 2017), além de estimularem excessivamente o sistema imune e serem específicos para melanomas que apresentam determinadas mutações, sendo inefetivos para os demais (HONG; AHN, 2017). Sendo assim, mesmo com os recentes avanços, ainda há interesse em novos compostos que auxiliem no tratamento desta patologia e que atuem por mecanismos distintos dos disponíveis (LIU et al., 2019).

Compostos naturais tais como os polifenóis passaram a ter relevância clínica devido às propriedades antimutagênicas e, desta forma, têm sido alvo de vários estudos (KATİYAR, 2011; NICHOLS; KATİYAR, 2010; SOUTO; SAMPAIO; CAMPOS, 2019). A apigenina (APG), flavonoide foco dessa dissertação, tem sido considerada uma opção promissora devido a sua alta seletividade por células tumorais comparativamente

a saudáveis e ação multidirecionada, ou seja, envolve vários alvos de ação (CAO et al., 2016a; STEVENS; REVEL; MAIER, 2017; ZHAO et al., 2017).

É categorizada como classe II no Sistema de Classificação Biofarmacêutico, tendo a solubilidade como o principal desafio a ser superado. Em frutas e vegetais, geralmente é encontrada na sua forma glicosilada, o que torna a sua absorção oral um tanto desafiante. Assim, sua conversão para a forma aglicona se mostra necessária para garantir uma absorção mais efetiva (PÁPAY et al., 2015; STEVENS; REVEL; MAIER, 2017; WANG et al., 2019a).

Após a administração oral, a APG sofre uma série de reações de deglicosilação, conversão para a forma glicosilada novamente, recirculação entero-hepática e ligação à proteínas plasmáticas, o que traz uma série de desafios farmacocinéticos a serem superados até o alcance do sítio-alvo (STEVENS; MAIER, 2016).

Apesar destas limitações, vários estudos apontam que a APG é uma molécula promissora para terapias antineoplásicas. De fato, este flavonoide atua em diferentes vias de sinalização relacionadas ao crescimento, desenvolvimento e metástase de células tumorais. Além disto, possui ação seletiva frente as células cancerígenas (MENG et al., 2017; SHI et al., 2015; TSENG et al., 2016; ZHANG et al., 2015), o que não ocorre com a maioria dos antitumorais atualmente disponíveis comercialmente.

A fim de superar as limitações biofarmacêuticas e farmacocinéticas da APG e possibilitar sua utilização de forma efetiva na terapia do melanoma, duas estratégias são propostas neste trabalho. A primeira consiste na administração percutânea deste ativo. Com isto, evita-se o efeito de primeira passagem, ligação a proteínas plasmáticas bem como problemas de absorção decorrentes da não conversão da forma glicosilada em aglicona (HALDER et al., 2022). Além disso, esta rota de administração encurta o caminho que o composto ativo precisa fazer até alcançar o sítio de ação.

Outra estratégia considerada foi a incorporação da APG em sistemas nanoestruturados, os quais trazem uma série de vantagens, a considerar: permitem uma liberação sustentada e direcionada (ou sítio-específica) do ativo, aumento da solubilidade aquosa, redução de efeitos adversos bem como proteção frente a degradação motivada por fatores ambientais e/ou fisiológicos (DESAI, 2012; JEEVANANDAM; CHAN; DANQUAH, 2016; GAGLIARDI *et al.*, 2021; KASHYAP *et al.*, 2021).

Os lipossomas têm sido extensivamente estudados como alternativa para terapias antitumorais. Devido a composição ser muito parecida com a de membranas biológicas (BANGHAM, 1977), proporcionam alta biocompatibilidade e biodegradabilidade (EL-

TOKHY et al., 2021). Como a APG precisa alcançar as camadas mais profundas da pele para exercer sua ação, a seleção de lipossomas contendo promotores de permeação pode ser de particular interesse. Invasomas foram selecionados para esta pesquisa uma vez que ainda não tinham sido investigados para a incorporação da APG. Estes sistemas possuem pequenas quantidades de etanol e terpenos na sua composição, contribuindo para a obtenção de vesículas menos rígidas e com efeito promotor de absorção. Estes agentes aumentam a fluidização e/ou extração de lipídeos do estrato córneo, principal barreira da pele, garantindo uma entrega de fármacos ainda mais efetiva que sistemas convencionais (DRAGICEVIC-CURIC *et al.*, 2008; BABAIE *et al.*, 2020).

Outra vantagem para o uso de invasomas é que muitos terpenos utilizados na sua composição já tem mostrado atividade antiproliferativa frente a melanoma (ALIPANAH et al., 2021; MOHAMMED; BABEANU; RADU, 2022). Assim, estes compostos podem agir sinergicamente com a APG, resultando em maior eficácia ou redução de efeitos adversos. Ainda, combinações de ativos tem se mostrado mais eficientes nos casos de resistência tumoral, particularmente quando atuam por mecanismos distintos. (FERRARI, 2005; YU et al., 2022). Até o momento, poucas terapias tópicas têm sido consideradas para o tratamento do melanoma. Ainda, a pele apresenta uma estrutura complexa que acaba limitando o número de moléculas que podem ser administradas por esta via (devem apresentar baixo peso molecular, lipofilicidade intermediária, reduzido potencial irritante, dentre outros aspectos) (HALDER et al., 2022).

A primeira etapa desta dissertação incluiu uma revisão da literatura para identificar os principais sistemas nanoestruturados contendo APG para o tratamento do câncer. Aspectos tecnológicos bem como vantagens e desvantagens de cada sistema foram discutidos em detalhes. Na segunda etapa, lipossomas e invasomas, ainda sem APG, foram desenvolvidos e caracterizados quanto ao tamanho, índice de polidispersão e carga de superfície. O efeito do colesterol, limoneno e etanol na estabilidade da vesícula e rigidez de membrana foi investigado. Ainda, avaliou-se como o colesterol e o limoneno afetam a interação com domínios proteicos e lipídicos da pele. Por fim, incorporou-se a APG nos sistemas otimizados, seguido de nova caracterização e ensaios de permeação cutânea para avaliar o impacto de diferentes veículos na distribuição da APG nas camadas da pele (derme e epiderme).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Desenvolver e caracterizar invasomas contendo o monoterpene limoneno e o flavonoide apigenina para o tratamento local do câncer de pele como uma estratégia para aumentar eficácia e segurança.

2.2 OBJETIVOS ESPECÍFICOS

- Avaliar a literatura científica de modo a identificar as principais nanopartículas contendo apigenina utilizadas no tratamento do câncer de pele;
- Preparar invasomas através do método clássico de hidratação do filme lipídico seguido de tratamento ultrassônico;
- Analisar o tamanho e índice de polidispersão das vesículas por espalhamento de luz dinâmico (DLS) e a carga de superfície por mobilidade eletroforética;
- Avaliar o efeito do etanol, limoneno e colesterol na rigidez de membranas de fosfatidilcolina por deslocamento de sonda fluorescente;
- Avaliar o efeito destes mesmos agentes na estabilidade das formulações por métodos espectroscópicos e analisador do tipo LUMiSizer®;
- Avaliar o efeito de diferentes concentrações de colesterol e limoneno em domínios lipídicos e proteicos da pele por técnicas espectroscópicas;
- Desenvolver método de cromatografia líquida de alta eficiência (CLAE) para a quantificação da apigenina;
- Avaliar a eficiência de encapsulação (E.E.) da apigenina nas vesículas através do método de ultrafiltração em membranas do tipo Amicon®;
- Avaliar tamanho, índice de polidispersão, carga superficial, estabilidade e rigidez das vesículas após a incorporação da APG pelas técnicas já mencionadas;
- Avaliar a taxa de permeação e retenção da APG incorporada em invasomas através do modelo de difusão em câmara de Franz considerando pele humana como modelo de membrana.

3 CAPÍTULO I - REVISÃO BIBLIOGRÁFICA

3.1 CÂNCER DE PELE E MELANOMA

3.1.1 Epidemiologia

Dados recentes disponibilizados pela Organização Mundial de Saúde (OMS) relatam o aumento na incidência dos casos de câncer de pele, o qual afeta aproximadamente 1,2 milhões de indivíduos a cada ano (WHO, 2022). No Brasil, esta doença representa 33% de todos os diagnósticos de câncer no país. O Instituto Nacional do Câncer (INCA) registra, anualmente, cerca de 185 mil novos casos. O câncer de pele do tipo não melanoma é o mais comum no Brasil, acometendo cerca de 177 mil indivíduos por ano. A doença é provocada pelo crescimento anormal das células que compõem as camadas da pele. Podem ser classificados em carcinomas baso- e espinocelulares (INCA, 2020). Os carcinomas basocelulares são caracterizados por um crescimento lento e capacidade de invasão mínima. Os carcinomas espinocelulares, por sua vez, possuem crescimento rápido, podendo evoluir e formar tumores invasivos, o que pode levar ao aparecimento de metástases (GLOSTER & NEAL, 2006).

O melanoma é o tipo de câncer de pele mais agressivo, registrando cerca de 8,4 mil casos anuais no Brasil (INCA, 2020). Em países como a Austrália, cuja incidência é a maior do mundo, a chance de um indivíduo de 85 anos ser diagnosticado com melanoma é de 1 em 17 (5,9%) (*Australian Government*, 2022). Nos Estados Unidos, entre 1975 e 2018, a incidência de melanoma aumentou 320% (CDC, 2019; KALYAN *et al.*, 2021).

Apesar de menos frequente, o melanoma possui elevada letalidade, respondendo por 80% das mortes relacionadas a câncer de pele (LUAN *et al.*, 2022). De acordo com a Agência Internacional de Pesquisa em Câncer GLOBOCAN, 57 mil indivíduos morreram em decorrência do melanoma em 2020 (IARC, 2022). Em sua atualização mais recente (2012-2018), o Instituto Nacional do Câncer norte-americano estimou uma sobrevida de 99,5% quando diagnosticado em estágios primários (I-II), diminuindo para 70,6% se o diagnóstico ocorrer no estágio III e 31,9% no estágio IV (NCI, 2022).

Contrariamente ao que ocorre com tumores sólidos, que tem adultos de idade mais avançada como principal alvo, casos de melanoma não são raros em jovens de meia-idade. Estudos epidemiológicos têm mostrado que indivíduos do sexo masculino possuem 1,5 vezes mais chance de desenvolver melanoma do que as mulheres. O local

de ocorrência dos tumores também parece ter uma relação com o sexo. Em homens, as costas representam o local mais comum, enquanto que braços e pernas são os locais do corpo mais afetados. Caucasianos possuem 10 vezes mais risco de desenvolver melanoma cutâneo do que negros, asiáticos ou hispânicos (provavelmente relacionado com a maior sensibilidade à exposição solar). Por outro lado, tanto as populações caucasianas quanto as afrodescendentes possuem risco semelhante para o desenvolvimento de melanoma plantar. Os melanomas não cutâneos (por exemplo melanomas de mucosa) são mais comuns em populações não-caucasianas (MARKOVIC et al., 2007).

3.1.2 Fisiopatologia do melanoma cutâneo

O melanoma cutâneo é uma neoplasia cuja origem está relacionada com a proliferação de melanócitos atípicos na camada basal da epiderme. Em condições fisiológicas, os melanócitos produzem e transferem a melanina para os queratinócitos mitoticamente ativos. A melanina transferida concentra-se principalmente no espaço perinuclear dos queratinócitos e protege o núcleo dos danos da radiação ultravioleta (UV) (TUONG; CHENG; ARMSTRONG, 2012).

O melanoma é uma doença multifatorial que tem relação tanto com a exposição ambiental quanto susceptibilidade genética. A exposição à radiação UV está entre os principais fatores de risco para o desenvolvimento da doença (SAGINALA et al., 2021). Esta exposição faz com que os queratinócitos produzam o hormônio estimulante dos melanócitos (MSH), que se liga aos receptores de melanocortina-1 (MC1R) presentes na superfície destas células. Essa interação resulta na produção e acúmulo de melanina nos melanossomos, os quais são transportados para os queratinócitos e conferem proteção frente a radiação UV. Essa proteção ocorre devido à capacidade da melanina em bloquear um amplo espectro de radiação UV. Ela confere proteção ao núcleo celular, evitando alterações no DNA. A predisposição genética à doença aliada a uma exposição solar excessiva pode levar a transformação dos melanócitos em células tumorais, contribuindo para o aparecimento do melanoma (LEONARDI et al., 2018).

O melanoma, assim como outros tipos de câncer, possui mecanismos complexos envolvendo múltiplas vias e o sistema imunológico. Danos no DNA podem resultar em perda do controle da proliferação celular, resultando em lesões hiperplásicas. Na maioria dos casos, estas lesões não progridem; entretanto, algumas células podem se manter viáveis e dar origem ao melanoma. Esse processo é chamado de tumorigênese do

melanoma, o qual é caracterizado pela proliferação anormal de melanócitos atípicos na epiderme, podendo se espalhar para a derme e tecido subcutâneo. Ao alcançar os tecidos mais profundos, pode confundir o sistema imunológico, alcançando a via linfática ou sanguínea e então outros órgãos, caracterizando a metástase (MOREIRA et al., 2020).

O tumor pode surgir na pele sem lesões prévias ou com lesões pigmentadas como lentigo e nevo melanocítico. Estas lesões iniciais podem evoluir e se transformarem em nevos melanocíticos com hiperplasia, atipias nucleares e/ou displasia, espalhando-se superficialmente e caracterizando a fase de crescimento radial, onde as lesões ainda permanecem restritas a epiderme e com baixo potencial invasivo (melanoma *in situ*, estágio 0). Com o passar do tempo e a persistência do estímulo carcinogênico, estas células podem adquirir a capacidade de invadir a derme, caracterizando o crescimento vertical (estágios I e II, lesões de 1-2 mm e 2-4 mm, respectivamente, sem comprometimento de linfonodos), podendo alcançar os linfonodos (estágio III) e, finalmente, outros órgãos distantes (estágio IV, tumor com capacidade de realizar metástase) (NCCN, 2019; TUONG; CHENG; ARMSTRONG, 2012).

Diversos estudos têm demonstrado que a disseminação do melanoma é resultado de mutações e alterações no microambiente tumoral decorrentes da superexpressão de proteínas que favorecem a invasão e infiltração circundante (MORO; MAUCH; ZIGRINO, 2014; CHIRIBOGA *et al.*, 2016; FALZONE *et al.*, 2016; SANDRI *et al.*, 2016). A superexpressão de metaloproteinases de matriz (MMPs), particularmente MMP-9 e MMP-2, que degradam componentes extracelulares, favorece a infiltração de células tumorais (MORO; MAUCH; ZIGRINO, 2014; FALZONE *et al.*, 2016; SANDRI *et al.*, 2016). A superexpressão dessas proteínas e as alterações observadas no microambiente tumoral são decorrentes das alterações genéticas e desregulação da via NF- κ B (LEONARDI et al., 2018). A via de sinalização do fator transcrricional NF- κ B está envolvida na sobrevivência e proliferação celular. Trata-se de um heterotrímero formado pela ligação das proteínas p50, p65 e I κ B α que, em seu estado inativo, permanece no citoplasma. Quando TNF- α , FasL e TRAIL ligam-se a receptores de membrana, ocorre a ativação do complexo IKK, que é responsável pela fosforilação, ubiquitinação e degradação de I κ B α . Em seguida, o dímero p50/p65 é translocado para o núcleo, onde se liga a sequências específicas de DNA, promovendo a transcrição de proteínas tais como a MMP-9, BCL-2, BCL-XL, survivina e XIAP e ciclina D1 (proteína relacionada ao ciclo celular) (HOFFMANN, 2006). Interessantemente, a radiação UV parece ativar a via de sinalização NF- κ B de forma independente, sem a necessidade de ativação do complexo

IKK, promovendo a degradação de I κ B α e consequente liberação do dímero p50/p65. O mecanismo pelo qual a radiação UV ativa esta via de sinalização permanece incerto. Sugere-se que a radiação UV seja capaz de regular negativamente a quantidade de I κ B α através da redução na tradução dos mRNAs que contém as informações para síntese dessa proteína (WU *et al.*, 2004; LEWIS & SPANDAU, 2007; O'DEA; KEARNS; HOFFMANN, 2008) (Fig. 1).

Além das alterações no microambiente tumoral, o melanoma possui uma das maiores cargas de alterações genéticas somáticas de todos os tumores humanos (VOGELSTEIN *et al.*, 2013; AKBANI *et al.*, 2015). As mutações somáticas mais frequentes nos melanomas cutâneos afetam genes que controlam a proliferação (BRAF, NRAS e NF1), crescimento e metabolismo (PTEN), ciclo celular (CDKN2A) e tempo de vida replicativo (TERT) (HODIS *et al.*, 2012; KRAUTHAMMER *et al.*, 2012). Estas alterações genômicas geralmente levam à ativação exacerbada de duas vias de sinalização importantes: a via das proteínas ativadas por mitógeno (MAPK) e a da fosfatidilinositol-3-quinase (PI3K/AKT), as quais são responsáveis pela proliferação celular, diferenciação, sobrevivência e homeostase. Mais de 90% dos melanomas exibem ativação exacerbada da via MAPK, tornando-a um alvo terapêutico interessante. A exposição frequente aos raios UV leva a mutações no gene BRAF, afetando a via MAPK. Entre 37 e 50% dos melanomas apresentam mutações neste gene (CURTIN *et al.*, 2005). Mutações nos genes NRAS (presente em 15 a 30% dos melanomas) e NF1 (presente em 10 a 15% dos melanomas) também contribuem para a ativação das vias MAPK e PI3K/AKT (CURTIN *et al.*, 2005; FALZONE *et al.*, 2016). Embora a mutação destes genes tenha papel determinante para o desenvolvimento do melanoma, vários outros genes podem estar envolvidos e são importantes para caracterizar o genótipo invasivo e metastático (KRAUTHAMMER *et al.*, 2012; GIBNEY & SMALLEY, 2013; MAERTENS *et al.*, 2013; WHITTAKER *et al.*, 2013; NISSAN *et al.*, 2014).

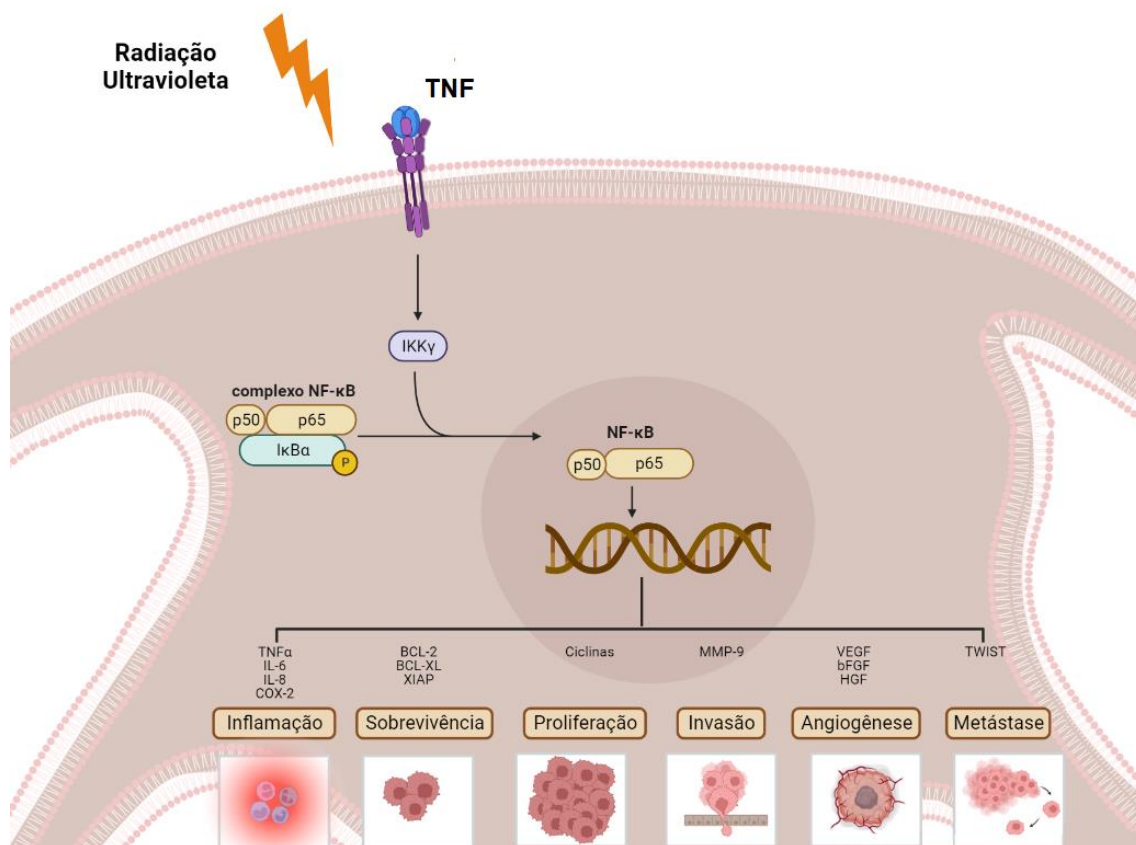


Fig. 1. Via de sinalização do fator de transcrição NF-κB. A exposição à radiação UV promove alterações no microambiente tumoral através da ativação de NF-κB e, consequente, transcrição de proteínas relacionadas à inflamação, sobrevivência, proliferação, invasão, angiogênese e metástase.

3.1.3 Opções terapêuticas

A excisão cirúrgica é o tratamento de primeira escolha, sendo efetivo na maior parte dos casos (ROSS; GERSHENWALD, 2011). Por outro lado, alguns pacientes apresentam reincidência do melanoma de forma disseminada ou são diagnosticados com melanoma em um estágio avançado (cerca de 10%). Nestes casos, apenas a excisão cirúrgica do tumor não tem sido suficiente. Aproximadamente 1/3 dos melanomas em estágio IV apresentam metástases cerebrais e viscerais, o que indica grave prognóstico e menor probabilidade de ter uma resposta efetiva ao tratamento (LUKE et al., 2017).

O tratamento para melanomas em estágio avançado sofreu uma revolução desde 2011 com a aprovação de diversos agentes terapêuticos. Estes agentes possuem diferentes alvos e dividem as terapias disponíveis em dois grandes grupos, sendo eles:

- (1) Terapias direcionadas: inibidores da MAPK (inibem o oncogene BRAF e via de sinalização MAPK), os quais são administrados por via oral.
- (2) Imunoterapias: inibidores de *checkpoints* imunológicos [Ex.: anticorpos dirigidos ao antígeno-4 de linfócitos T citotóxicos (anti-CTLA-4) e anticorpos contra a proteína de morte celular programada 1 (anti-PD1)], os quais administrados por via intravenosa.

Uma vez que 37-50% dos pacientes com melanoma possuem mutações no gene BRAF (mutação BRAF V600), a seleção de inibidores BRAF (p.ex.: vemurafenibe e dabrafenibe) pode ser efetiva nestas situações. Em estudo clínico realizado por Flaherty *et al.* (2010), a utilização destes agentes terapêuticos demonstrou uma resposta inicial em 70-80% dos pacientes, entretanto, observou-se recidiva da doença em todos os pacientes dentro do período de 9 meses. Isto ressalta a necessidade de combinar inibidores BRAF com outros fármacos para alta sobrevida (FLAHERTY, 2010; FLAHERTY *et al.*, 2010).

Outros fármacos que atuam como inibidores da via de sinalização MAPK tais como o trametinibe e cobimetinibe proporcionam um benefício clínico inicial, porém, não são capazes de impedir a mortalidade do paciente já que o tumor torna-se resistente a estas moléculas de maneira muito rápida ou ocorre ativação de outras vias (DE LUCA *et al.*, 2017).

Ainda que as terapias alvo-específicas e imunoterapia possam trazer maior seletividade, desenvolvimento de resistência tumoral e estímulo excessivo do sistema imunológico podem ocorrer (SPAIN; JULVE; LARKIN, 2016; BERNING *et al.*, 2019). Ainda, as terapias direcionadas/alvo-específicas se aplicam apenas para pacientes cujo melanoma apresenta mutação BRAF V600E, não abrangendo outros tipos de mutações (HONG; AHN, 2017). Sendo assim, mesmo com os recentes avanços, ainda há interesse na investigação de novos compostos que auxiliem no tratamento do melanoma (LIU *et al.*, 2019) ou estudos com combinações de diferentes fármacos.

3.1.4 Compostos naturais como alternativa terapêutica

Vários estudos demonstram que polifenóis podem agir como moduladores de vias de sinalização dos processos de crescimento e proliferação celular bem como no combate de radicais livres de oxigênio (ROS) (ASENSI *et al.*, 2011; BRIGUGLIO *et al.*, 2020; KATIIYAR, 2011; NICHOLS; KATIIYAR, 2010). Estes compostos podem atuar no

reparo do DNA e possuem capacidade de absorver radiação UV (SOUTO; SAMPAIO; CAMPOS, 2019). Dentre os polifenóis, os flavonoides se destacam por possuírem uma estrutura benzo- γ -pirona característica que tem sido associada as mais diversas atividades biológicas que variam com o padrão de hidroxilação, substituições e conjugações na estrutura principal (DURAZZO et al., 2020). Em um estudo que avaliou o efeito de flavonoides polihidroxilados no crescimento de células de melanoma B16F10, sugeriu-se que a presença de uma ligação dupla C2–C3 e três grupos hidroxila adjacentes nos anéis A ou B proporciona maior atividade antiproliferativa (MARTÍNEZ et al., 2003). Casagrande e Darbon (2001), por sua vez, investigaram o efeito de uma série de flavonoides na proliferação e ciclo celular em células de melanoma humano (OCM-1). Interessantemente, a presença de um grupo hidroxila na posição 3' do anel B na quercetina e luteolina foi correlacionada a uma parada do ciclo celular G1 enquanto sua ausência no kaempferol e apigenina foi correlacionada a um bloqueio de G2. A presença de unidades isoprenóides em flavonoides também parece ter um efeito positivo na destruição de células de melanoma (ARUNG et al., 2010). Até o momento, fisetina, apigenina, diosmina, luteolina e quercetina tem sido os flavonoides mais estudados para o tratamento do melanoma (JÚNIOR et al., 2017).

3.2 APIGENINA

3.2.1 Características físico-químicas

A apigenina (APG), quimicamente conhecida como 4,5,7'-trihidroxi-flavona é um composto bioativo pertencente ao grupo dos flavonoides (Fig. 2). Em sua forma nativa, a APG pode ser encontrada em vegetais e frutas tais como salsa, cebola, laranja, camomila e outras especiarias (JEETAH; BHAW-LUXIMON; JHURRY, 2014; PÁPAY et al., 2015).

É categorizada como classe II no Sistema de Classificação Biofarmacêutico, possui baixa massa molar (MM=270,24 g/mol) e temperatura de fusão de 347,5°C. (PATEL; SHUKLA; GUPTA, 2007). Ainda, apresenta um log P de 2,87, com solubilidade em água de 1,35 $\mu\text{g/mL}$ a 25°C. Em pH intestinal, sua solubilidade tem um pequeno aumento (2,16 $\mu\text{g/mL}$) (PÁPAY et al., 2015). A APG pura é considerada instável para o armazenamento em longo prazo a temperatura ambiente e, desta forma, condições

especiais devem ser consideradas (WANG et al., 2019b). Em plasma de ratos, manteve-se estável por 24 h a temperatura ambiente e por pelo menos 4 semanas a -20°C (DUAN et al., 2011; SHI et al., 2011). A decomposição dos flavonoides depende dos substituintes ligados à molécula. Grupos hidroxilas favorecem a degradação, enquanto que, açúcares e grupos metoxilas tem um efeito contrário. Assim, é possível afirmar que glicosídeos são quimicamente mais estáveis que as agliconas, além de apresentarem uma maior solubilidade devido a presença do açúcar (WANG et al., 2019b).

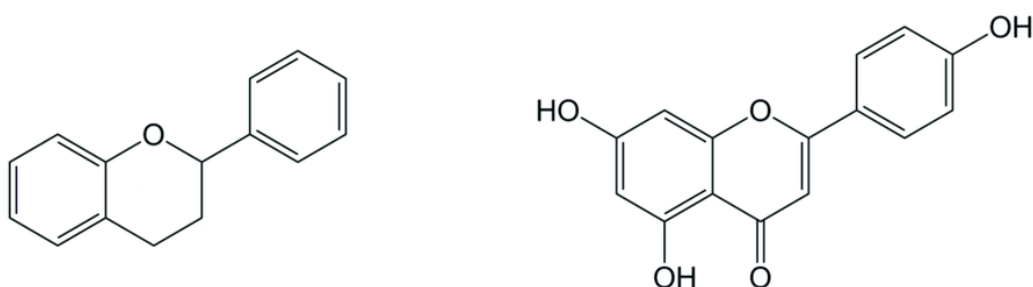


Fig. 2. Estrutura química básica dos flavonoides (à esquerda); estrutura química da apigenina (à direita).

Nas plantas, flavonoides como APG são tipicamente encontrados na sua forma glicosilada, com um ou mais açúcares ligados à aglicona (C-glicosídeos ou O-glicosídeos), o que implica em uma absorção intestinal incompleta devido a maior massa molar. Assim, a conversão para sua forma aglicona é crucial para uma efetiva absorção oral (PÁPAY et al., 2015; STEVENS; REVEL; MAIER, 2017; WANG et al., 2019a).

Após a ingestão, a deglicosilação da APG é mediada por β -glicosidases sintetizadas pelo fígado, intestino e/ou microbiota intestinal. Esta reação representa uma etapa crítica para a absorção e metabolização da APG por via oral (DAY et al., 1998; NÉMETH et al., 2003). Na sua forma aglicona, a APG pode se difundir livremente através do epitélio (difusão passiva), alcançando a circulação sanguínea. Ao alcançar a circulação entero-hepática, pode ser revertida na forma O-glicosilada original através da ação de UDP-glicuroniltransferases, ou ainda, sofrer a ação de sulfonil-transferases (STEVENS; MAIER, 2016; VAZHAPPILLY et al., 2021).

Antes de ser absorvida, a APG pode ser metabolizada no lúmen intestinal. Estes metabólitos e agliconas são transportados para o fígado via sistema porta-hepático, onde novas reações de metabolização podem ocorrer. Os metabólitos produzidos no fígado podem atingir a circulação sistêmica, de onde são distribuídos a tecidos e células-alvo; podem ser excretados para a bile e sofrer recirculação entero-hepática, ou ainda, serem

eliminados nas fezes/urina (THILAKARATHNA; VASANTHA RUPASINGHE, 2013). Alta taxa de ligação a proteínas plasmáticas é relatada para a APG, explicando uma alta variação farmacocinética (CHEN; LIN; HU, 2003; PÁPAY et al., 2015).

Estudos farmacocinéticos em humanos geralmente são realizados com a ingestão de salsa (*Petroselinum crispum*) por se tratar de um vegetal rico em APG e pobre em outros flavonoides (NIELSEN et al., 1999; PATEL; SHUKLA; GUPTA, 2007). Em seus estudos, Meyer *et al.* (2006) relataram uma concentração plasmática máxima de APG de 33,46 nmol/L, a qual foi alcançada após 10 h da ingestão de dose única de 2 g de salsa por quilograma (65,8 µmol de APG). Após 28 h de ingestão, a concentração plasmática reduziu para 2,3 nmol/L (MEYER et al., 2006). Em geral, baixas concentrações deste composto são encontradas na urina. Meyer *et al.* (2016), por exemplo, encontraram apenas 0,22% da dose total de APG ingerida em amostras de urina após 24 h. Nielsen et al. (1999), por sua vez, encontraram 0,58% da dose total de APG ingerida neste mesmo fluido (NIELSEN et al., 1999).

Um estudo farmacocinético completo, avaliando a absorção, metabolismo, distribuição e excreção foi realizado em modelo murino. Após uma única dose oral, a APG ligada ao marcador radioativo foi detectada no plasma a partir de 9 h, alcançando a concentração plasmática máxima 24 h após a ingestão. Quanto a sua eliminação, 51% do composto radioativo foi recuperado na urina, 12% nas fezes, 1,2% no sangue, 0,4% nos rins, 9,4% no intestino, 1,2% no fígado e 24,8% no restante dos compartimentos corporais, durante 10 dias. Um longo tempo de eliminação foi encontrado (91,8 h) com *clearance* plasmático de 1,95 mL/h. O volume de distribuição estimado foi 25 vezes maior que o volume teórico de sangue, sugerindo uma acumulação da APG nos tecidos. A partir deste estudo, sugeriu-se que a APG apresenta lenta absorção, metabolismo e eliminação (GRADOLATTO et al., 2005).

3.2.2 Atividade antitumoral

Nos últimos anos, a APG ganhou destaque como composto natural promissor para o tratamento de diversos tipos de câncer, o que tem relação com sua atuação em várias vias de sinalização. Exerce sua atividade antitumoral através da modulação dos processos de apoptose celular (induzindo a morte das células cancerígenas de forma seletiva) (BUDHRAJA et al., 2012; LIN et al., 2012; SHI et al., 2015; MAEDA et al., 2018; SHI et al., 2015; ZHAO et al., 2011), além de modular o ciclo celular (induzindo a

parada e supressão de células cancerígenas) (MENG et al., 2017; SHI et al., 2015; TSENG et al., 2016; ZHANG et al., 2015) e vias de sinalização relacionadas à progressão, proliferação e metástase das células tumorais como via PI3K/AKT, mTOR, MAPK/ERK, NF- κ B, Wnt/ β -catenina e JAK/STAT (CAO et al., 2016b; LIN et al., 2017; LIU et al., 2015; RUELA-DE-SOUSA et al., 2010; SEO et al., 2015a; XU et al., 2016).

A atividade antiproliferativa da APG foi estudada por Woo *et al.* (2020) em células com baixa (A375P) e média (A375M) capacidade invasiva e metastática. A APG demonstrou inibir o crescimento celular devido a ativação de vias apoptóticas (indução das proteínas BAX, p53, caspase-9, caspase-3 e PARP e inibição das proteínas anti-apoptóticas como BCL-2) (WOO et al., 2020).

Outros estudos relatam a capacidade da APG em induzir a citotoxicidade dose e tempo-dependente nas linhagens celulares de melanoma A375 e A2058, acompanhado pela redução no número de células e indução de mudanças morfológicas compatível com o processo de “anoikis” (processo de apoptose desencadeado pela perda da adesão celular). Ainda, o tratamento de linhagens celulares de melanoma WM1361B e WM983A com APG aumentou a expressão da proteína p27, resultando em diminuição de ciclina-D, ciclina-E e nas proteínas CDK2/4/6, culminando na parada do ciclo celular na fase G1/G0 (HASNAT et al., 2015). Em células A375 e C8161, observou-se que a APG (até 280 μ M) é capaz de induzir a parada do ciclo celular na etapa G2/M e induzir a apoptose com o aumento da expressão de caspase-3 (ZHAO et al., 2017).

Além das vias apoptóticas e *checkpoints* no ciclo celular, a APG também mostrou inibir a fosforilação da p-FAK e ERK, levando a uma diminuição na migração celular e, consequentemente, redução de metáteses (HASNAT et al., 2015).

3.3 ESTRUTURA DA PELE E ADMINISTRAÇÃO CUTÂNEA

A pele é uma complexa estrutura multicamada que, em adultos, possui uma área de aproximadamente 1,5 a 2 m². Em termos anatômicos a pele pode ser dividida em epiderme, derme e hipoderme (Fig. 4). Além de evitar a perda de água do organismo e da ação termorreguladora, a principal função deste órgão é atuar como uma barreira física (ex.: proteção a radiação UV), química, mecânica e imunológica (EYERICH et al., 2018).

A derme, camada intermediária da pele, apresenta espessura variável conforme a região do corpo e idade do indivíduo. É formada por fibras de colágeno, elastina e matriz extracelular, cuja função é garantir a elasticidade e resistência da pele. Por ser uma região

ricamente vascularizada, é responsável pela nutrição e oxigenação da epiderme. Como os vasos sanguíneos encontram-se na derme, preparações farmacêuticas “transdérmicas” necessariamente devem garantir que o princípio ativo penetre o estrato córneo, permeie a epiderme viável e, finalmente, atinja a derme (ABDO; SOPKO; MILNER, 2020).

A epiderme, camada mais externa da pele, pode ser dividida em diferentes camadas, que correspondem a células em diferentes estágio de maturação, a considerar: camada basal, estrato espinhoso, estrato granuloso e estrato córneo (EC). Estas camadas são constantemente renovadas à medida que as células das camadas basais passam por processos de diferenciação e migram para a superfície da pele. Em seu último estágio, estas células advindas outrora da camada basal (queratinócitos) se diferenciam em corneócitos, onde perdem seus núcleos, as principais organelas citoplasmáticas e os lipídios são liberados no espaço intercelular. Células desvitalizadas ricas em queratina (corneócitos) envoltas por uma mistura complexa de ceramidas, colesterol e ácidos graxos livres formam a camada mais externa da pele conhecida como estrato córneo (EC) (RAUBER; ARGENTA; CAON, 2020).

O estrato córneo representa a principal barreira que limita o transporte de moléculas através da pele. O epitélio escamoso estratificado cobre os últimos 50-120 µm da epiderme. O mecanismo principal pelo qual a maior parte das moléculas é internalizada é a difusão passiva (HALDER et al., 2022). Neste tipo de transporte, regido pela Lei de Fick, as moléculas se movem aleatoriamente de uma região para outra, através de uma membrana, de acordo com o gradiente de concentração. O movimento ocorre sempre na direção da região de menor concentração (AULTON; TAYLOR, 2016). A difusão passiva pode ser trans- ou intercelular quando ocorre através ou entre as células, respectivamente. Moléculas de natureza mais lipofílica tem maior afinidade por estas rotas tendo em vista a composição lipídica das membranas celulares e o conteúdo lipídio presente nos espaços intercelulares (YU et al., 2021). Apêndices tais como folículos pilosos e glândulas sebáceas, por sua vez, são vias que podem ser utilizadas para o transporte de moléculas com maior massa molar e de natureza mais hidrofílica ou ainda sistemas vesiculares (RUELA et al., 2016).

Ainda que os compostos ativos ultrapassem o EC, existem outros obstáculos a serem superados tais como as junções estreitas entre as células do estrato granuloso (principalmente em regiões com lesão tecidual) ou ainda a presença de células de Langerhans que compõe o sistema imune e são responsáveis por englobar bactérias e outros materiais estranhos ao organismo (BRANDNER et al., 2015).

A fim de superar as barreiras da pele, promotores químicos, físicos ou sistemas vesiculares podem ser considerados. Promotores físicos tais como a iontoforese ou ultrassom permitem o transporte de moléculas mediante fenômenos eletrostáticos e de cavitação, respectivamente (MITRAGOTRI, 2000; WANG et al., 2005).

Os promotores químicos, por sua vez, podem interagir com constituintes do EC e causar alterações em sítios lipídicos ou proteicos da pele, facilitando o transporte de moléculas (MARTINS; VEIGA, 2002). Podem se intercalar nas lamelas lipídicas do EC, promovendo o afrouxamento e/ou fluidificando as cadeias lipídicas ou então modificar a conformação da queratina, causando intumescimento e aumento da hidratação ou atuam sob os corneodesmossomos, afetando a coesão entre os corneócitos. Os promotores que possuem um efeito indireto atuam por modificação da atividade termodinâmica ou solubilidade do fármaco. Tensoativos, por exemplo, podem aumentar a fração solúvel de um fármaco pouco solúvel no veículo e assim favorecer sua permeação (KOVÁČIK; KOPEČNÁ; VÁVROVÁ, 2020). Muitos promotores de absorção atuam por mais de um dos mecanismos citados. Acredita-se que os promotores de absorção mais potentes atuem nos lipídios intercelulares do EC já que estes representam a principal barreira para a permeação de compostos na pele (WILLIAMS; BARRY, 2004).

O primeiro composto considerado promotor de absorção químico foi o Azone[®] (*N*-dodecil-2-caprolactama), o qual foi patenteado em 1976. As propriedades anfífilas observadas neste composto levaram ao aparecimento de outros promotores contendo cadeias alquílicas de 10 a 12 carbonos (WILLIA; BARRY, 1991).

As propriedades necessárias para um promotor de absorção ideal compreendem sua capacidade em ser específico (os promotores devem aumentar a permeação das substâncias sem exercerem atividade farmacológica), não tóxicos, não-irritantes ou alergênicos, apresentar ação rápida, previsível e reprodutível. Ainda, propriedades de barreira devem ser restabelecidas após a remoção dos mesmos e devem ser compatíveis com o fármaco e excipientes que compõe a formulação aplicada (KOVÁČIK; KOPEČNÁ; VÁVROVÁ, 2020).

Sistemas vesiculares tais como lipossomas e suas modificações representam uma estratégia aprimorada para a entrega de fármacos através da pele. Por serem constituídos primordialmente por fosfolipídios, os quais possuem caráter anfílico, estes sistemas são capazes de alterar as propriedades de barreira do EC. De forma geral, processos de fusão das vesículas na pele levam à miscibilidade dos fosfolipídios lipossomais com os lipídios do EC, afrouxando as lamelas lipídicas do EC, permitindo então a passagem de fármacos

através desta barreira. Ainda, por ação oclusiva, este sistema é capaz de promover a hidratação das camadas de queratina, o que também aumenta a difusão de fármacos pela pele (SINICO; FADDA, 2009).

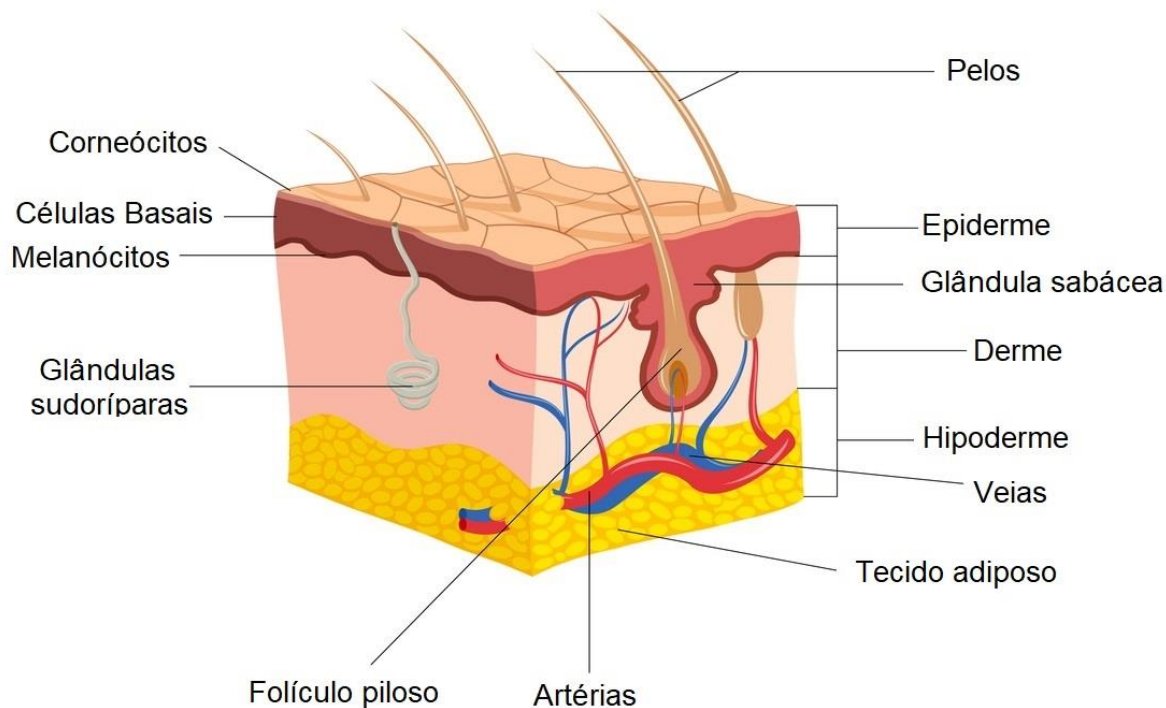


Fig. 4. Estrutura anômica da pele. Fonte: Traduzido de *Kona Dermatology* (2023).

3.4 NANOTECNOLOGIA

Desvantagens dos tratamentos convencionais para terapia antitumoral, como baixa biodisponibilidade, administração de altas doses, efeitos adversos, resistência a múltiplos fármacos e falta de seletividade podem ser superadas com a nanotecnologia (DESAI, 2012; GESSNER et al., 2000; KASHYAP et al., 2021; MOGHIMI; SZEBENI, 2003).

Os sistemas nanoestruturados são promissores como veículos para agentes quimioterápicos pois apresentam diversas vantagens em relação às formulações convencionais. Estas vantagens incluem a redução de efeitos colaterais, aumento do índice terapêutico, administração mais conveniente e maior adesão do paciente ao tratamento. Os nanocarreadores permitem a entrega da SA diretamente no local do tumor, reduzindo a toxicidade e a interação com tecidos saudáveis. A utilização de nanocarreadores pode ainda promover a redução da depuração renal e hepática e diminuir

o reconhecimento pelo sistema imunológico, alterando as propriedades farmacocinéticas e a biodistribuição da SA. Melhoria da estabilidade ou solubilidade também podem ser alcançados, garantindo um rápido desenvolvimento de novos medicamentos (YU et al., 2021).

Dentre os sistemas nanoestruturados para incorporação de SA destacam-se os lipossomas que, por apresentarem uma composição parecida a de membranas biológicas, demonstram alta biocompatibilidade (EL-TOKHY et al., 2021). Os lipossomas são vesículas esféricas compostas por bicamadas fosfolipídicas que, em solução aquosa, se auto-organizam de forma concêntrica. Estas vesículas são constituídas basicamente por fosfolipídeos, esteróis e antioxidantes (materiais lipídeos podem oxidar facilmente na presença de oxigênio). As fosfatidilcolinas são os fosfolipídeos mais empregados em estudos de formulação já que apresentam alta estabilidade química frente a variações de pH ou da concentração de sal no meio (BATISTA; DE CARVALHO; MAGALHÃES, 2007). Devido à sua estrutura, os lipossomas possuem a capacidade de incorporar tanto fármacos hidrofílicos (no interior da vesícula) quanto fármacos hidrofóbicos (em meio as cadeias carbônicas dos fosfolipídios). Apesar da versatilidade quanto à encapsulação de ativos, a fusão prematura dos lipossomas convencionais no estrato córneo podem dificultar a chegada do ativo até as camadas mais profundas da pele ou corrente sanguínea (EL MAGHRABY; BARRY; WILLIAMS, 2008). Isto é particularmente relevante para a patologia selecionada nesta dissertação, já que muitos tumores de pele podem chegar até a derme ou ainda levar a metástases. Assim, outros constituintes podem ser adicionados aos lipossomas tradicionais, tais como tensoativos, terpenos e etanol, de modo que as vesículas se tornem mais flexíveis, ou ainda, permaneçam intactas até alcançar as camadas mais profundas. Niosomas, transferosomas, etosomas e invasomas são alguns exemplos destes lipossomas (JAIN; TRIPATHI; TRIPATHI, 2021).

3.4.1 Invasomas

Os invasomas são vesículas compostas por fosfolipídios, etanol e um ou mais terpenos. A combinação destes agentes resulta em vesículas mais flexíveis e com maior potencial de permeação na pele comparativamente aos lipossomas convencionais (EL-TOKHY et al., 2021) (Fig 5).

O etanol interage com a porção polar dos fosfolipídios do EC, contribuindo para que as membranas fiquem mais fluidas. Devido ao baixo peso molecular, o etanol é

também capaz de penetrar o interior de corneócitos, que são preenchidos por fibras de queratina aglutinadas, promovendo a desnaturação e afrouxamento destas estruturas fibrilares. Baixas concentrações de etanol são suficientes para perturbar as estruturas lamelares do EC. Ainda, a presença do etanol torna as vesículas mais flexíveis, permitindo a difusão entre os corneócitos (BABAIE *et al.*, 2020; DRAGICEVIC-CURIC *et al.*, 2008; HORITA *et al.*, 2015).

A capacidade dos terpenos em melhorar a penetração percutânea depende de fatores como lipofilicidade, tamanho, quiralidade, entalpia de vaporização, ponto de ebulição e grau de insaturação (KUMAR *et al.*, 2022). De forma geral, os terpenos atuam como promotores de absorção através das interações com a porção apolar dos lipídios intercelulares bem como grupamentos hidrofílicos da cabeça polar, afetando tanto a via inter- quanto transcelular (CHEN *et al.*, 2016). Os terpenos podem extrair ou fluidificar os lipídios do EC, desestruturando a barreira da pele (AHAD *et al.*, 2011a, 2011b; WANG *et al.*, 2017). Além disso, podem romper ligações de hidrogênio entre as moléculas de ceramida, estabelecendo novas interações com estas estruturas fisiológicas. As ceramidas são proteínas do EC que se encontram fortemente organizadas por ligações de hidrogênio, resultando em uma rede que aumenta as propriedades de barreira (WANG *et al.*, 2017). Os terpenos dissolvidos no domínio lipídico intercelular também podem melhorar o coeficiente de partição das substâncias ativas no EC (CHANTASART *et al.*, 2009).

Embora vários mecanismos de interação vesícula-pele tenham sido descritos na literatura, dois deles são mais aceitos pela comunidade científica e podem ocorrer simultaneamente. A primeira teoria é de que as vesículas se desintegram na superfície da pele e os materiais que a compõe (p. ex.: terpenos e etanol) desorganizam e/ou rompem lamelas lipídicas intercelulares do estrato córneo, formando canais que permitem o transporte de moléculas (VADDI *et al.*, 2002; OTA *et al.*, 2003; DRAGICEVIC-CURIC *et al.*, 2008; CHEN; LIU; FAHR, 2011).

A segunda hipótese sugere que pequenas vesículas intactas penetrem através das células que compõe o EC sob a influência do gradiente osmótico transepidérmico, atuando como sistemas carreadores. Isto seria observado apenas para vesículas altamente flexíveis (CEVC; BLUME, 1992; CEVC; SCHÄTZLEIN; RICHARDSEN, 2002; HONEYWELL-NGUYEN; GOORIS; BOUWSTRA, 2004).

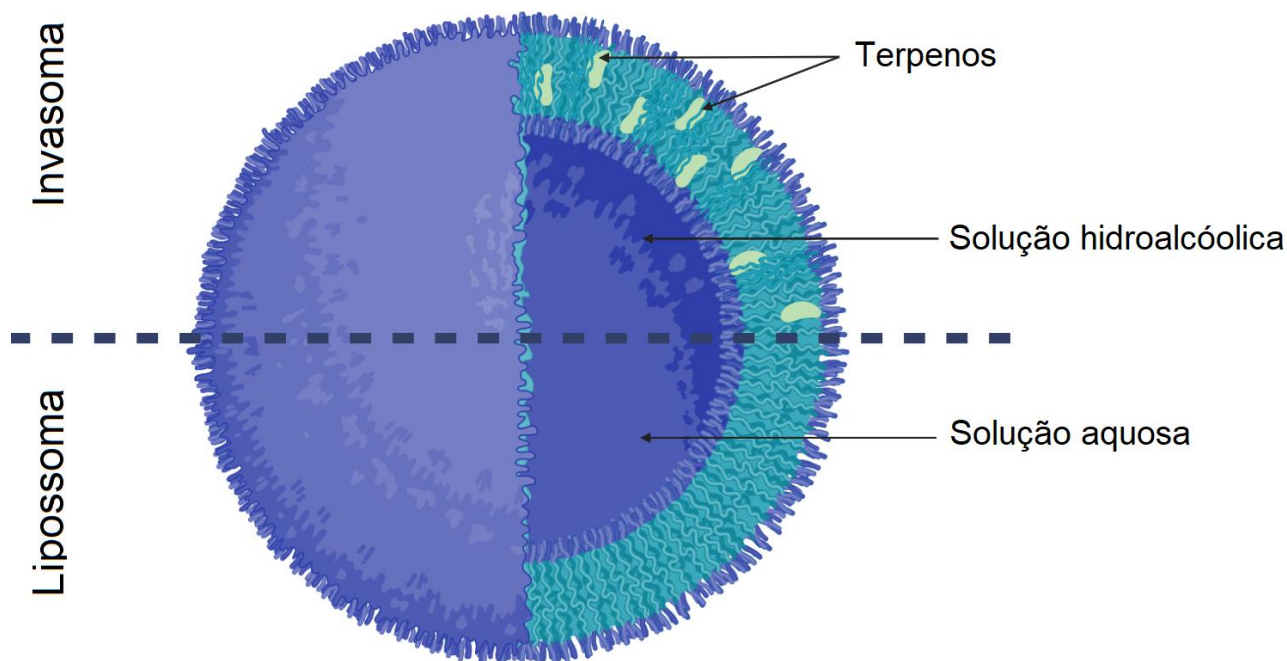


Fig. 5. Estrutura de invasomas *versus* lipossomas.

3.4.1.1. Métodos de preparo dos invasomas

3.4.1.1.1. Hidratação do filme lipídico

Nesta técnica, que tem sido a mais amplamente utilizada, primeiramente solubilizam-se fosfolipídios e componentes hidrofóbicos em solvente adequado de modo a obter uma solução homogênea. O solvente orgânico é então eliminado em evaporador rotatório, formando um filme lipídico na parede do balão. Por fim, é realizada a etapa de hidratação através da adição de solução hidroalcoólica. Ao entrar em contato com a água, os fosfolipídios se auto-organizam para um estado de menor energia, onde as porções hidrofóbicas interagem entre si a fim de diminuir a superfície de contato com água, formando as vesículas lipossomais. O sistema é submetido à agitação para o desprendimento total do material lipídico da parede do balão. Como vesículas polidispersas e com os mais diversos graus de lamelridade são obtidas, metodologias acessórias devem ser consideradas a fim de reduzir e padronizar o tamanho das vesículas (CHEN; LIU; FAHR, 2011; NAYAK; TIPPAVAJHALA, 2021). Nesta dissertação, considerou-se um tratamento em ultrassom de ponteira.

3.4.1.2. Caracterização físico-química

Os invasomas devem, idealmente, apresentar um tamanho de partícula entre 100-300 nm para a aplicação na pele, serem flexíveis e elásticos, apresentar estabilidade físico-química e alto grau de interação com a pele. Testes de caracterização são cruciais para averiguar as propriedades mecânicas, estabilidade e interações destas vesículas com o EC (BABAIE et al., 2020).

3.4.1.2.1. Tamanho, índice de polidispersão e potencial zeta

A análise do tamanho das vesículas é crucial quando a administração percutânea é abordada, isto porque o tamanho afeta a capacidade das vesículas penetrarem o EC. Vesículas com tamanho bem reduzido (60-180 nm) podem atravessar o EC sem alterações na estrutura, alcançando as camadas mais profundas da pele (CEVC; SCHÄTZLEIN; RICHARDSEN, 2002; DRAGICEVIC-CURIC *et al.*, 2008b).

O diâmetro médio e índice de polidispersão podem ser determinados pela técnica de espalhamento de luz dinâmico. Nesta técnica, uma fonte de luz (laser) é emitida sob a amostra e o movimento browniano das partículas em suspensão faz com que a luz seja espalhada (ou difratada) com diferentes intensidades. A observação da luz espalhada é geralmente realizada em um ângulo de 90°. A análise dessas flutuações de intensidade resulta na velocidade do movimento browniano e assim, o tamanho de partícula pode ser obtido através da relação Stokes-Einstein (NOMURA et al., 2014).

O índice de polidispersão (PDI) das formulações revela a homogeneidade de tamanho na população de vesículas formadas. É importante que as vesículas possuam tamanhos semelhantes para que o comportamento de entrega dos ativos seja sempre o mesmo. Além disto, este parâmetro é um indicativo de estabilidade ao longo do tempo (DRAGICEVIC-CURIC et al., 2009). Outro parâmetro indicativo de estabilidade ao longo do tempo é a carga superficial das vesículas. Altos valores de potencial zeta contribuem para maior repulsão eletrostática entre as partículas, o que evita fenômenos de instabilidade tal como a coalescência, por exemplo (SMITH et al., 2017).

A carga superficial (ou potencial zeta) pode ser determinada por anemometria laser Doppler. Nesta técnica, as partículas com carga migram para o eletrodo de carga oposta com uma velocidade proporcional a carga superficial. Essa velocidade da partícula

em um campo elétrico é denominada de mobilidade eletroforética, a qual depende da potência do campo elétrico, da constante dielétrica do meio bem como viscosidade do meio. Através da aplicação da lei de Henry, que relaciona a mobilidade eletroforética com o potencial zeta, é possível obter um valor que representa a magnitude da repulsão ou da atração eletrostática entre as partículas (NETO, 2015).

3.4.1.2.2. *Rigidez de membrana*

A flexibilidade de membrana é uma das características mais marcantes dos invasomas já que pode ter relação com a taxa de liberação do fármaco, eficiência de encapsulação, grau de interação com as células e estabilidade (CARRERAS et al., 2020; COLDREN et al., 2006; ROMERO; MORILLA, 2015). A medida que a deformabilidade das vesículas aumenta, a estabilidade diminui. Apesar de contribuir para a entrega de fármacos em camadas mais profundas da pele, a flexibilidade das membranas pode levar a processos de agregação e formação de estruturas multilamelares ao longo da estocagem. Além disso, vesículas flexíveis são mais suscetíveis a degradação térmica, apresentando estabilidade reduzida (HUSSAIN et al., 2017). Por este motivo, é necessário que haja um equilíbrio entre flexibilidade e rigidez da membrana.

Chwastek *et al.* (2020) apresenta um método em que uma sonda fluorescente sensível à viscosidade (DCVJ) é incorporada às vesículas para avaliação deste parâmetro, a qual foi considerada nesta dissertação. As propriedades fotofísicas da sonda são sensíveis à viscosidade do meio e, portanto, podem ser usadas para estimar a viscosidade de membranas lipídicas. Em seu estado singleto excitado, a molécula de DCVJ sofre rápida rotação em torno da ligação simples que conecta as subunidade julolideno e dicianovinil, atuando como um rotor molecular. Dado que a rotação interna e a emissão de fluorescência são processos competitivos, em solventes não viscosos e no estado singleto excitado, a rotação interna é preferida e a emissão de fluorescência é reduzida. Portanto, o DCVJ é fracamente fluorescente em soluções fluidas enquanto que, o aumento da viscosidade do meio dificulta a rotação interna, favorecendo a desativação do estado excitado por emissão de fluorescência (JEE; BAE; LEE, 2009). Para a avaliação da viscosidade da membrana (isto é, rigidez) de lipossomas e invasomas, a intensidade de emissão de DCVJ incorporada nestas vesículas foi comparada à de um solvente de viscosidade conhecida (metanol).

3.4.1.2.3. Estabilidade

No que diz respeito à estabilidade, sistemas baseados em fosfolipídios são susceptíveis a oxidação e ainda podem apresentar, ao longo do tempo de armazenagem, coalescência, sedimentação, bem como outros fenômenos. Técnicas espectroscópicas são úteis na investigação de fenômenos de instabilidade. A espectroscopia no UV-visível mede as mudanças na luz transmitida devido ao espalhamento provocado por partículas em suspensão (turbidimetria) ou absorção da luz (absorbância). A turbidimetria é um método bem estabelecido para avaliar eventos de agregação coloidal. Nesta técnica, as mudanças de sinal são medidas ao longo do tempo e indicam mudanças no tamanho das partículas, o que se traduz em eventos de instabilidade como agregação ou sedimentação (RAY et al., 2015). Para avaliar fenômenos de separação de fases e sedimentação, o equipamento LUMiSizer[®] pode ser considerado. A análise com LUMiSizer[®] é baseada na sedimentação centrífuga das amostras, que permite uma rápida classificação da estabilidade e estimativa do prazo de validade de dispersões não diluídas em sua concentração original. Os perfis de transmissão das amostras permitem traçar o comportamento da mistura e estabilidade. As dispersões coloidais estáveis mostram a formação de um leito plano sob um campo centrífugo enquanto que as partículas agregadas podem mostrar um perfil escalonado (a aceleração centrífuga causa diferentes velocidades de sedimentação de partículas que diferem em tamanho) (CADDEO et al., 2013).

3.4.1.2.4. Interações com estrato córneo

Análises espectroscópicas têm sido úteis para a investigação do grau de interação das vesículas com a pele. Informações a respeito do grau de desordem de domínios proteicos e lipídicos podem ser extraídas com o auxílio do FT-IR. Vibrações das ligações CH simétricas e assimétricas de hidrocarbonetos dos lipídeos do EC aparecem em 2,920 e 2,850 cm^{-1} , respectivamente. Mudanças na fluidização ou extração lipídica promovidas por promotores de absorção contribuem para o alargamento e redução da intensidade (área) destas bandas espectrais (LEVANG; ZHAO; SINGH, 1999).

Vibrações de estiramento das amidas I e amidas II de proteínas do EC aparecem em 1650 e 1550 cm^{-1} , respectivamente. A banda observada para amidas I ocorre devido à vibração de estiramento C=O enquanto a banda observada para amidas II ocorre devido

ao estiramento C-N e vibração de flexão N-H. Estas duas bandas, principalmente a banda de amidas I, são sensíveis e podem ser deslocadas para frequências mais altas ou mais baixas quando ocorrem alterações na conformação de proteínas (VADDI et al., 2002).

3.4.1.2.5. *Permeação cutânea ex vivo*

A permeação cutânea *ex vivo*, ou seja, a quantidade de fármaco capaz de permear a pele pode ser avaliada utilizando células de difusão de Franz. Este aparato é composto por dois compartimentos (doador e receptor), uma cânula de amostragem e um sistema de aquecimento para manter a temperatura a 37°C (Fig. 6). A membrana, que neste caso é a pele, é disposta na interface entre os compartimentos doador e receptor. Idealmente, peles de tecido “humano” devem ser priorizadas neste tipo de ensaio, as quais são frequentemente obtidas de cirurgias plásticas e de cadáveres, com a devida aprovação ética de uso. No compartimento superior ou doador, é inserida a solução do SA ou formulação-teste. Quantidade finitas ou infinitas de fármaco podem ser consideradas. Em geral, doses infinitas são utilizadas para a composição de sistemas transdérmicos. No compartimento inferior, encontra-se o fluido receptor, o qual deve mimetizar o comportamento fisiológico do sangue, manter a integridade do tecido e uma completa solubilização do fármaco (o volume do meio de dissolução deve ser suficiente para que o soluto se encontre em uma concentração cerca de 10 vezes inferior à sua concentração de saturação). A presença de um agitador magnético visa garantir uma distribuição uniforme do fármaco na fase receptora, após sua passagem pela pele, impedindo o acúmulo na região abaixo da membrana. Além disto, há a presença de uma jaqueta de água externa para garantir um controle da temperatura do experimento, que deve ser de 37 ou 32°C (a depender da literatura consultada). As amostras para análise são retiradas deste último compartimento em intervalos pré-definidos. Por fim, o fármaco é quantificado a fim de se conhecer a quantidade permeada (AULTON; TAYLOR, 2016; FINNIN; WALTERS; FRANZ, 2012).

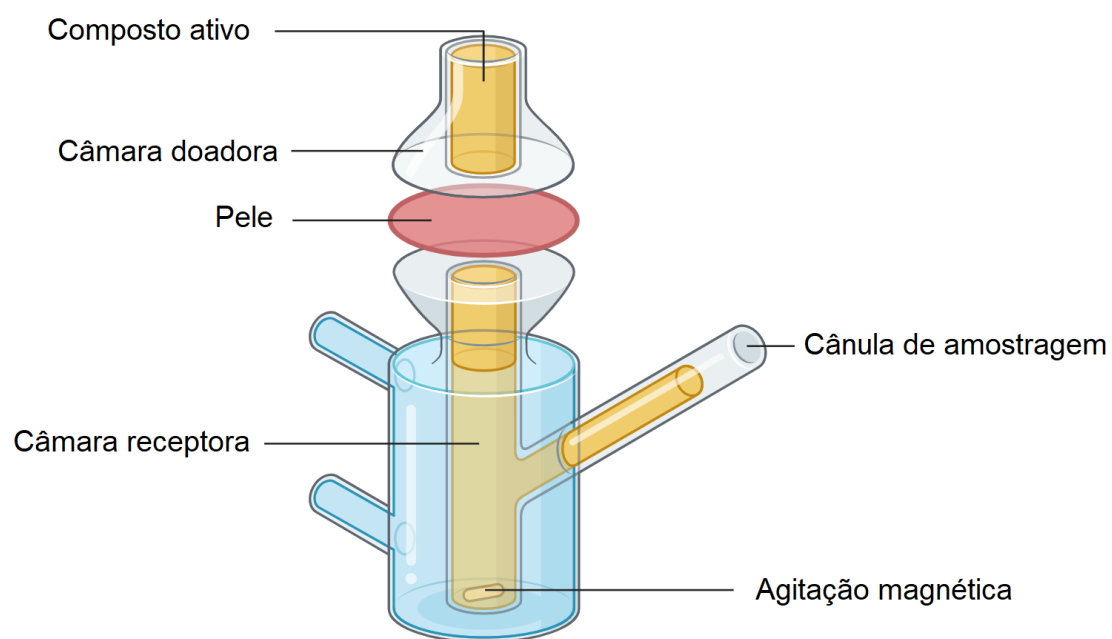


Figura 5. Câmara de difusão de Franz.

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4 CAPÍTULO II - NANOCARREADORES DE APIGENINA PARA APLICAÇÕES EM TERAPIAS ANTINEOPLÁSICAS

Este capítulo foi estruturado na forma de um artigo de revisão cujo objetivo foi levantar dados da literatura referentes aos principais sistemas nanoestruturados utilizados para incorporação da APG no contexto das terapias antineoplásicas. A parte inicial deste trabalho de revisão dedicou-se a apresentar limitações farmacocinéticas/biofarmacêuticas ou relativas a estabilidade da APG que justificam o desenvolvimento de sistemas nanoestruturados, além dos diferentes mecanismos de ação antitumoral já descritos para este flavonoide. Na sequência, detalharam-se estudos de atividade antitumoral realizados com diferentes tipos de nanopartículas de APG. Lipossomas e nanopartículas poliméricas foram identificados como os sistemas nanoestruturados mais estudados. Os primeiros trazem como principal vantagem a biocompatibilidade enquanto que as nanopartículas poliméricas um perfil de liberação sustentado. O produto deste capítulo é um artigo científico, que se encontra sob revisão no periódico *Journal of Drug Delivery Science and Technology*.

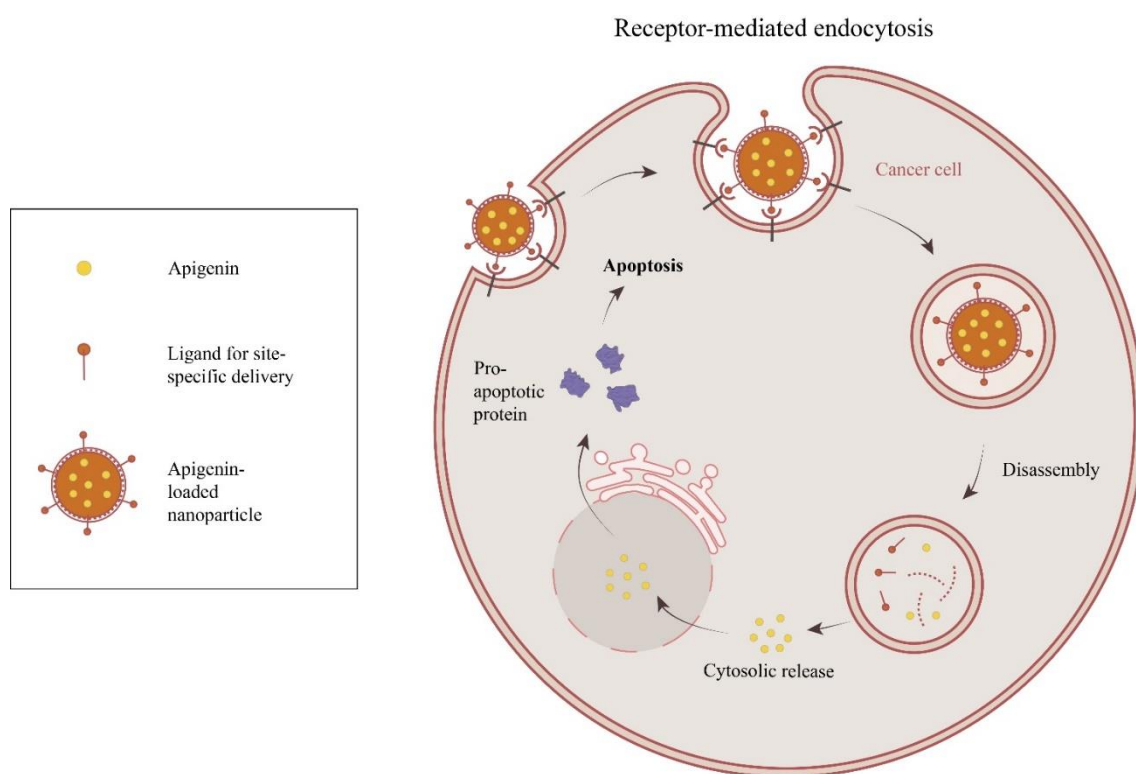
**NANO-BASED APIGENIN DELIVERY SYSTEMS FOR CANCER
APPLICATIONS**

Izi Vieira Nunes Cunha, Angela Machado Campos, Thiago Caon[#]

Postgraduate Program in Pharmacy, Federal University of Santa Catarina, Campus
Reitor João David Ferreira Lima, s/n - Trindade, Florianópolis, SC 88040-900, Brazil.

[#]Corresponding author: caon.thiago@ufsc.br; thiagocaon@gmail.com.

Graphical abstract



Abstract

Apigenin (APG) has been considered in treatment of various types of cancer due to its capacity of modulating cell proliferation, apoptosis, cell cycle arrest, invasion, metastasis and autophagy by regulating different cell signaling pathways. Moreover, this molecule exhibits high selectivity to cancer cells compared to healthy cells, a key aspect in relation to other anticancer therapies. On the other hand, APG is characterized by low aqueous solubility, slow oral absorption, high metabolization and association rate with plasma proteins. As a consequence, low plasma concentrations are often found after oral administration. Nanotechnology emerges as an alternative to overcome these pharmacokinetic or physicochemical limitations, providing a targeted and controlled drug delivery, increased oral bioavailability and aqueous solubility as well as improved side effect profile. In this review, studies addressing the anticancer properties of APG nanoparticles were analyzed in detail. The synergism of action between drugs already used clinically and APG nanoparticles allows to reduce the dose of the drug administrated, which would minimize adverse effects. *In vivo* studies, in turn, showed its potential in reducing tumor volume, encouraging trials with humans.

Keywords: apigenin; cancer; drug delivery; nanoparticles.

1. Introduction

Apigenin (APG), chemically known as 4,5,7,-trihydroxyflavone, is a bioactive compound belonging to the flavonoid group. Flavonoids are secondary metabolites of plants that play a crucial role in their physiology and protection against pathogens and insects [1]. In its native form, APG can be found in vegetables and fruits such as parsley, onions, oranges, chamomile tea, wheat sprouts and other spices [2,3].

Solvent extraction has been the most traditional method to obtain this flavonoid. Methanol results in a higher extraction efficiency than boiling water, ethanol and propanol [4]; however, it is a more toxic solvent. As an alternative, ultrasonic and microwave-assisted extraction techniques [5] or even supercritical fluid [6] have been purposed to reduce energy or solvent consumption as well as providing shorter processing times and higher extraction yields.

Recently, APG has attracted interest due to its remarkable therapeutic properties such as anti-inflammatory, antioxidant, anti-proliferation activity, changes in cellular signaling and apoptosis induction, which suggest a potential activity against cancer [1,7]. Many studies have reported that APG can inhibit the proliferation of cancer cells and tumor suppressor activity against various types of cancer, including skin, colon, prostate, oral, breast, lung, pancreatic, colorectal and hepatic cancer [8–11].

Although several therapies against cancer have been developed in the last decade, including chemotherapies, radiotherapy, immunotherapy, phototherapy and surgery; they have not been effective for all types of cancer [7]. Low action selectivity is also conventionally found, which requires high doses, and then adverse effects such as diarrhea, stomatitis, cognitive dysfunction, fatigue, bowel and bladder dysfunction as well as mental health issues are often observed [12–14].

In this context, APG emerges as an anticancer candidate due to its low intrinsic toxicity and selective action against cancer cells. It is considered safe, even at high doses, and no toxicity has been reported [15]. This flavonoid is widely distributed in several dietary sources and the daily intake is relatively high. Even so, low plasma concentrations are found after an oral administration [16]. Its low aqueous solubility could be the reason for the poor intestinal absorption [1].

Advances in the nanotechnology field have provided the opportunity to improve the bioavailability of poorly water-soluble drugs, enable a site-specific drug delivery and provide a controlled drug release. The development of nanoparticles is also able to protect

APG from biodegradation and excretion, affecting its pharmacokinetics [14,16], and further improve its anti-cancer activity.

Polymer and lipid-based delivery systems as well as metallic nanoparticles have been the main nanocarriers exploited for the incorporation of apigenin for antitumoral therapies. This review presents a comprehensive discussion on biopharmaceutical or pharmacokinetic limitations of this active compound, its action in multiple targets related to the cancer and finally the nanostructured systems developed aiming to obtain safer and/or more effective antitumor therapies.

2. Physicochemical properties and oral bioavailability

APG is categorized as a class II compound in Biopharmaceutical Classification System, which indicates high intestinal permeability and low aqueous solubility. APG has a low molar mass (MM=270.24 g/mol), a melting point of 347.5°C and the raw material structurally appears as yellow needles [17]. Its log P is 2.87, presenting a solubility in water of 1.35 µg/mL at 25°C. Under specific conditions such as pH = 7.5, APG has a slightly higher solubility value (2.16 µg/mL), but this parameter still limits effective intestinal absorption [1].

In plants, flavonoids such as APG usually remain in a glycosylated form with one or more sugar components linked to the aglycon by O- or C-glycosidic bonds, leading to incomplete absorption. Therefore, APG should be converted into an aglycone form to be effectively absorbed [1,18,19].

APG deglycosylation is mediated by epithelial β-glucosidases (lactase-phlorizin hydrolase and cytosolic β-glucosidase), which are enzymes synthesized by the liver, intestine and/or gut microbiota. This process represents a critical step in the absorption and metabolism of this compound in humans [20,21]. Once in its aglycon form, APG can passively diffuse through the epithelium, achieving the bloodstream. When these flavonoids reach the liver, they can be converted back to O-glycoside by the UDP-glucuronyltransferase or sulfonated by sulfonyl-transferases [18,22].

Different authors have performed pharmacokinetic studies on APG in humans [23–25]. The oral ingestion of parsley (*Petroselinum crispum*) is typically considered in these pharmacokinetic studies as it presents a large amount of APG and a low concentration of other flavonoids [17,26]. Meyer et al. (2006), for example, found a maximum APG plasma concentration of 33.46 nmol/L, which was achieved 10 h after a single oral dose of 2 g blanched parsley (65.8 µmol APG) per kilogram. Plasma

concentrations fell under the detection limit (2.3 nmol/L) within 28 h [25]. In a randomized cross-over study with oral administration of a similar amount of dried parsley (5 g/84 mg APG) for 7 days, APG was not detected in plasma. The authors attributed this result to the low limit of the quantification method [27]. Low content of APG was also found in urine. Meyer et al. (2006) found 0.22% of the ingested total dose in 24-h urine samples. Nielsen et al. (1999) found a similar urinary excretion value, which was 0.58% of ingested total APG.

A complete pharmacokinetic study evaluating absorption, distribution, excretion and metabolism of radiolabeled APG was also performed in a rat model. The APG started to be detected in plasma 9 h after a single oral administration, reaching a maximum concentration at 24 h post-ingestion time. On the APG elimination, 51% the radioactivity was recovered in urine, 12% in feces, 1.2% in the blood, 0.4% in the kidneys, 9.4% in the intestine, 1.2% in the liver, and 24.8% in the rest of the body within 10 days. A high elimination half-time (91.8 h) and a plasmatic clearance of 1.95 mL/h were found. The estimated distribution volume of radioactivity was 25 times higher than the theoretical blood volume, suggesting an accumulation of APG in the tissues. Taken together, these results suggest a slow absorption, metabolism and elimination of this flavonoid [28].

Before absorption, APG may present extensive phase I and phase II metabolism in the intestinal lumen. These metabolites and aglycone forms are then transported to the liver via the hepatic portal vein, where may undergo further metabolism. Liver metabolites can be released into the bloodstream to reach target cells and tissues, excreted to bile and undergo enterohepatic re-circulation, or eliminated in urine and/or feces (Fig. 1) [29].

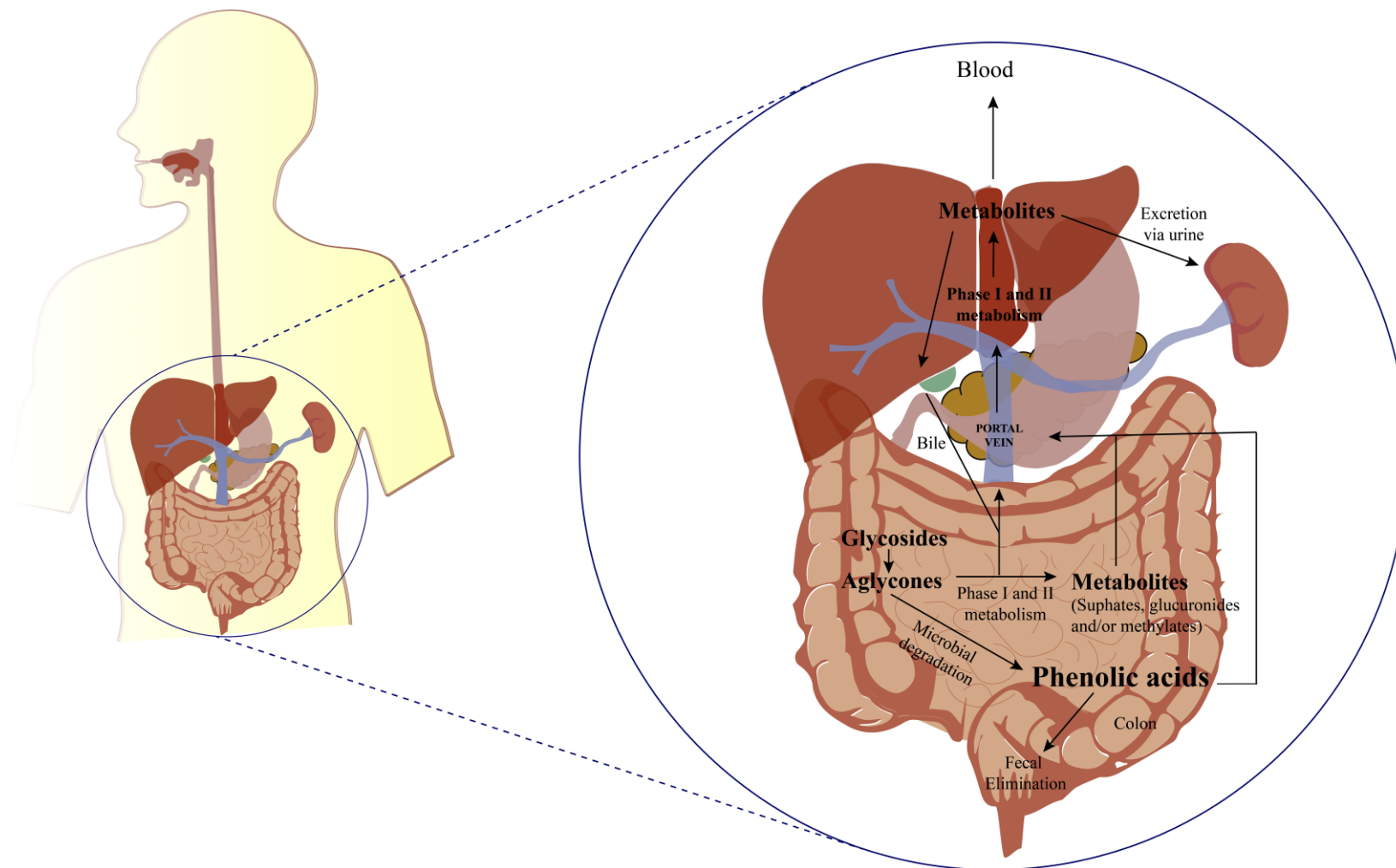


Figure 1. A scheme representing the APG metabolism. After ingestion, APG may go through phase I and phase II intestinal metabolism. Metabolites and aglycones are transported to the liver via hepatic portal vein and undergo further metabolism. Liver metabolites can be released into bloodstream, excreted to bile and undergo enterohepatic re-circulation or even eliminated in urine and/or feces.

The high level of protein binding and poor release from the protein-bound form represents another critical aspect related to bioavailability of APG [30]. These data added to the metabolism in the small intestine and liver in combination to enterohepatic recycling may play an important role in the poor systemic bioavailability [1,31].

3. Mechanisms of anti-cancer activity

APG has been considered for different types of cancer due to its multitargeted capacity of modulating cell proliferation, apoptosis, cell cycle arrest, invasion, metastasis and autophagy through the regulation of various cell signaling pathways [32–38], which will be discussed in detail.

3.1 Effect of apigenin on apoptosis

Apoptosis is a form of programmed cell death resulting in morphological change and DNA fragmentation. It is crucial for maintaining an equilibrium between cell growth and death. Disturbs in this balance can lead to tumor proliferation, invasiveness and even the development of drug resistance in cancer [39].

APG induces apoptosis through the activation of both intrinsic and extrinsic pathways as represented in Figure 2 [35,40]. The intrinsic apoptotic pathway is activated in response to intracellular stressors, with mitochondrial outer membrane permeabilization (MOMP). MOMP contributes to the release of cytochrome c from the mitochondria, which binds to the apoptotic protease activating factor (APAF-1) for apoptosome formation. Procaspase-9 binds to the apoptosome, gets cleaved and activated, and then activates caspase-3 to induce cell death [41,42]. The intrinsic apoptotic pathway is positively regulated by the proapoptotic BCL-2 family members such as BAX, BAK, BIM, BID, PUMA (BAX and BAK are central effectors of apoptosis as they lead to the formation of macropores in the mitochondria outer membrane) and negatively regulated by the anti-apoptotic BCL-2, BCL-XL, BCL-W and MCL1 family members. Preclinical studies evaluating different cancer cell lines and animal models have shown that APG increases the expression of proapoptotic proteins while it reduces the expression of antiapoptotic proteins [35,43–45].

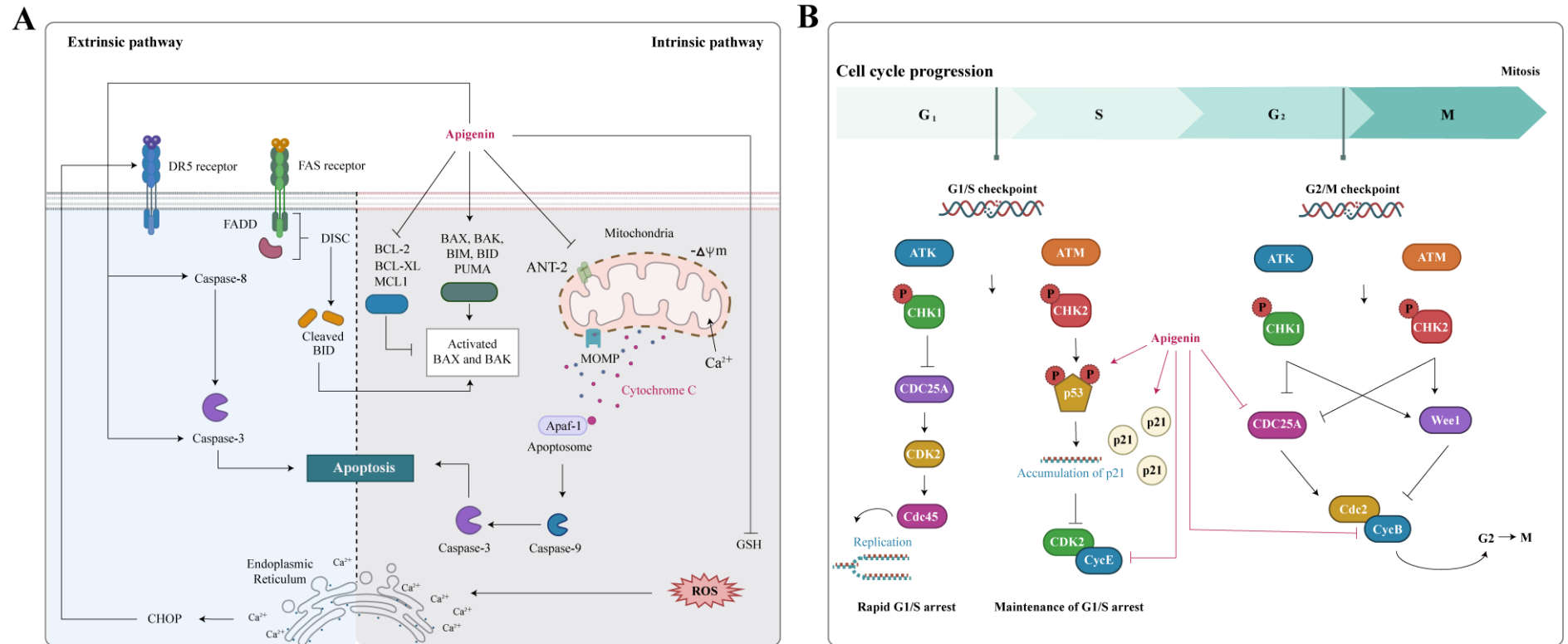


Figure 2. Intrinsic and extrinsic pathways modulated by APG (A); cell cycle modulated by APG (B).

The extrinsic apoptotic pathway (known as the death receptor pathway) may be also modulated by APG. The apoptosis by extrinsic pathway occurs through the binding of death-inducing ligands to cell surface receptors [42]. The apoptosis process initiates by the binding of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), TNF- α and/or Fas ligand (FasL) to death receptors such as TRAIL, TNFR-1, Fas receptors and pro-apoptotic death receptor 5 (DR5). Death ligand stimulation results in oligomerization of the receptors and recruitment of the adaptor proteins [*i.e.*, Fas-associated protein with death domain (FADD); TNF receptor-associated protein with death domain, (TRADD)], which lead to activation of cell death effectors (caspase 8 and caspase 10). Then, the interaction between adaptor proteins and caspases results in the death-inducing signaling complex (DISC), which initiates an apoptotic signaling cascade. Large amounts of caspase 8 contribute to the activation of other effector caspases (*e.g.*, caspase 3, caspase 6, and caspase 7) while the cleavage of the proapoptotic BH3-only protein Bid depends on small amounts of this caspase [46]. Both extrinsic and intrinsic apoptosis pathways converge on caspase-3 and subsequently on other proteases and nucleases that drive the terminal events of programmed cell death [47].

APG induces activation of the extrinsic pathway in cancer cells by upregulating the mRNA expression of caspase-3, caspase-8, TNF- α and overexpression and cleavage of poly ADP-ribose polymerase (PARP), which is a known cellular substrate of caspases and, thus it is considered a hallmark of apoptosis [48–50]. Oishi et al. (2013) also reported that APG can inhibit adenine nucleotide translocase type 2 (ANT-2) by direct binding. ANT-2 has anti-apoptotic properties and is overexpressed in many malignant tumor cells. This inhibition increases the expression of death receptor (DR5), leading to an increase in sensitivity to TRAIL and then the apoptosis is enhanced [51].

Another apoptotic pathway that is modulated by APG involves the endoplasmic reticulum stress (ER stress), inducing reactive oxygen species (ROS) generation and Ca^{2+} release, contributing to damage to the mitochondrial membrane and activation of caspase 3 [35,40,52,53].

Studies performed with different cancer cells have suggested that APG increases ROS levels within cells, which leads to the continuous release of Ca^{2+} from the ER lumen to the cytosol, causing ER stress. Cytosol Ca^{2+} overload increases the ion influx into the mitochondria, reducing mitochondrial membrane potential ($\Delta\psi\text{m}$). Therefore, the opening of the mitochondrial permeability transition pore leads to apoptosis via the intrinsic pathway [35,40,52,53].

In an attempt to overcome ER stress, the endoplasmic reticulum upregulates the expression of a sensor protein, the C/EBP homologous protein (CHOP). CHOP upregulates the expression of pro-apoptotic proteins (e.g., BAX and BAK) and downregulates the anti-apoptotic proteins (e.g., BCL-2 and BCL-XL), inducing the apoptotic intrinsic pathway. CHOP also upregulates the expression of DR5. DR5 overexpression increases sensitivity to death-inducing ligands and leads to cleavage of caspase-8, which cleaves the Bid and thus triggering apoptosis through extrinsic and intrinsic pathways [52,54,55].

Interestingly, APG has greater selectivity for cancer cells compared to healthy cells, which could be related to $\Delta\psi_m$ differences. The cancer cells have a higher $\Delta\psi_m$ (~ -210 mV) compared to healthy cells (~ -140 mV), which may be associated with changes in mitochondrial membrane composition such as electron transport chain, ATP synthase, uncoupling proteins (UCPs) and adenine nucleotide translocases (ANTs). Tumor cells down-regulate the expression of ANT type 1 (ANT1), which is responsible to transport ATP from the mitochondrial matrix to the cytosol. The ANT1 down-regulation also stimulates glycolysis via AMP-activated protein kinase (AMPK). Unlike ANT-1, ANT-2 is overexpressed in cancer cells. This enzyme is responsible to export ADP from the matrix to the cytosol by the change of an ATP equivalent, increasing ATP levels in the matrix. Consequently, ATP synthase switches to the reverse mode, creating a state of hyperpolarization (high $\Delta\psi_m$). In addition to glycolysis stimulation, the cancer cells benefit from high $\Delta\psi_m$ given that these conditions inhibit the release of proapoptotic factors [56].

3.2 Effect of apigenin on cell cycle

The cell cycle refers to a series of events that leads to DNA duplication, organelle and cytoplasm division, to generate two new daughter cells. This cycle is divided into the G0/G1, S and G2/M phase. Uncontrolled and/or irregular cell division represents an important hallmark of cancer. Checkpoints at different stages of the cell cycle can prevent cells with damaged DNA from duplicating [57]. Cell cycle progression involves the sequential activation of cyclin-dependent kinases (CDKs). Therefore, eukaryotic cells have developed effective and well-regulated mechanisms to control cell cycle progression [35,58]. Several *in vitro* and *in vivo* studies have reported that APG can modulate the cell cycle, inducing the arrest and suppression of cancer cells [35,57,59,60].

In the G1 phase, the major checkpoint is the ATM(ATR)/CHK2(CHK1)–p53/MDM2-p21 pathway, which can induce sustained or even permanent G1 arrest, preventing the cell from proceeding to phase S. When DNA damage is identified, the activation of protein kinases such as ATM and ATR is initiated, which represents the first step for cell cycle inhibition. ATM/ATR can directly phosphorylate the p53 transcription factor (controls genes involved in cell death and DNA repair mechanisms) or indirectly by effector kinases CHK1 and CHK2. These phosphorylation events result in the activation of two signal transduction pathways, one to “initiate” and one to “maintain” the G1/S arrest. The initial arrest is mainly modulated by ATR/CHK1. The increased level of CHK1 downregulates the CDC25A phosphatase, resulting in the accumulation of the phosphorylated (inactive) form of CDK2. Consequently, CDC45 cannot be phosphorylated to initiate the replication, leading to a delay of a few hours in the G1/S transition [61].

The maintenance of G1/S arrest is mediated by p53, where both ATM and ATR can phosphorylate Ser15 of p53 directly and Ser20 through the CHK1/CHK2 expression. The phosphorylation of p53 increases p21^{WAF1/CIP1} expression, which inhibits the S-phase-promoting CDK2-cyclin E complex and thus maintains the G1/S arrest. In addition, p21^{WAF1/CIP1} also inhibits the CDK4-cyclin D complex, preventing phosphorylation of the retinoblastoma protein (Rb). Therefore, the release of transcription factor E2F, which is crucial for the transcription of S-phase genes, is avoided (SANCAR; LINDSEY-BOLTZ, 2004). In summary, the G1 checkpoint is crucial to preventing the critical tumor suppressor pathways governed by p53 and Rb, which are often dysregulated in human cancers [62].

Another checkpoint modulated by APG occurs in the G2/M stage, which prevents cells from undergoing mitosis in the presence of DNA damage. Depending on the agent type causing DNA damage (e.g., UV light, ionizing radiation), the ATM-CHK2-CDC25 pathway can initiate the cell cycle arrest and ATR-CHK1-CDC25 pathway can maintain it or vice versa. In any situation, CHK1/CHK2 inhibits the entry into mitosis by downregulating the activity of CDC25A phosphatase and upregulating the WEE1 kinase. The reduction in CDC25A phosphatase levels as well as the increase in WEE1 expression control the CDC2/Cyclin B activity, delaying the G2/M transition [61]. The maintenance of phase G2 is mainly regulated by p53 and BRCA1, which leads to the upregulation of cell cycle inhibitors such as p21^{WAF1/CIP1} [61].

APG has been reported as a modulator of the cancer cell cycle, affecting both G0/G1 and G2/M phases in a selective manner. It is able to inactivate CDK1, CDK2, CDC2, reduce expression of cyclin A, B, D1, D2 and E, downregulate CDC25 proteins, increase p53 protein levels and stability, and increase the p21^{WAF1/CIP1} expression [35,57,59,60].

3.3 Effect of apigenin on cell signaling pathways

Cancer cells have the ability to modify important cell signaling pathways to ensure their progression, proliferation and metastasis. APG has shown to be able to modulate the PI3K/AKT/mTOR intracellular signaling pathway, which is involved in cellular processes such as cell growth, proliferation and survival [63].

The PI3K/AKT and mTOR signaling pathway are two interdependent pathways that play an important role in apoptosis inhibition. Active AKT has been reported in various types of cancer for a reduction and inactivation of proapoptotic factors including BAD and procaspase-9 [64]. APG has been demonstrated an *in vitro* and *in vivo* inhibitory effect on AKT kinase. Moreover, APG has induced AMPK activation [65–67], which directly phosphorylates the mTOR binding partner Raptor, inhibiting mTOR activity. Also, AMPK can phosphorylate TSC2 and enhances its ability to turn off mTOR activity [68,69].

In human keratinocytes, Tong et al. (2012) demonstrated that APG can inhibit AKT activity and induce AMPK activation independently because neither inhibition of AKT activity nor overexpression of constitutively active AKT had any effect on AMPK activation [70]. APG has also reduced the expression of mTOR in different cancer cells [33,71].

Other signaling pathways used by cancer cells include MAPK/ERK, NF-κB, Wnt/β-catenin and JAK/STAT signaling pathways. The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway starts at the cell membrane receptor upon its stimulation by extracellular mitogenic signals (e.g., growth factors, differentiation factors). Then, the MAPK/ERK cascade is sequentially activated by phosphorylation mechanisms. ERK1/2 at the terminal kinases can translocate to the nucleus to regulate transcription events affecting cell death, tumor progression, angiogenesis, and metastasis [72]. Several studies with different types of cancer cells have suggested that APG suppresses the phosphorylation of ERK, blocking its activity and thus

apoptosis is induced (Chen et al., 2016; Hasnat et al., 2015; Shao et al., 2013; Woo et al., 2020; Zhao et al., 2017).

The NF- κ B signaling pathway is also affected in cancer cells, affecting both cell survival and proliferation. In an inactive state, NF- κ B remains in the cytoplasm as a heterodimer that binds to I κ B α . When ligands such as TNF, FasL and TRAIL activate the IKK complex, phosphorylation and degradation of I κ B α are observed. Then, NF- κ B is released and translocated to the nucleus, leading to the activation of pro-survival (BCL-2 and BCL-XL, survivin, XIAP) and cell cycle-related genes (cyclin D1), inhibiting cell death [77]. APG has been reported as an inhibitor of NF- κ B signaling pathway due to its ability to block the phosphorylation and degradation of I κ B α by inhibiting IKK activation [38,74,78–80].

In addition to the signaling pathways mentioned above, several studies reported that APG is involved in other signaling pathways such as JAK/STAT [37,81,82], Wnt/ β -catenin [34,83,84] and transforming growth factor- β (TGF- β) [85].

4. Nano-formulations

Nowadays, most of the clinically used chemotherapeutic drugs are plant-derived agents or their semi-synthetic derivatives [86,87]. Despite the high activity of APG against cancer, as already mentioned, its clinical application is limited due to its low aqueous solubility, low oral bioavailability and short retention time in biological systems. Furthermore, the rapid metabolism causes modifications in its structure, decreasing the antitumor activity [88].

Nano-formulations have promisingly overcome the limitations of conventional drug delivery systems, providing targeted drug delivery, improving bioavailability, aqueous solubility, biodistribution, controlling the drug release rate as well as the occurrence of specific side effects [87,89–91]. For cancer applications, nanoparticles can improve the selectivity of drugs for tumor cells and microenvironment while sparing healthy cells and tissues [92]. Nano-formulations also protect active compounds from environmental factors such as fluctuation in pH, enzymatic attack and potential biochemical degradation [93].

Nanoparticles can be designed to target the desired cells by passive or active targeting. Particle size and size distribution should be carefully selected because these parameters can affect pharmacokinetics, biodistribution and safety. Small-sized NPs (<20-30 nm) are quickly depurated by the excretory system while particles with larger

sizes (>200 nm) are captured by the mononuclear phagocytic system. For cancer applications, nanoparticles presenting a specific particle size distribution may present a preferential accumulation in tumor tissues. Surface properties such as charge, hydrophobicity and functional groups are also critical factors that affect the interaction with biological molecules, playing an important role in the stability of nanoparticles and opsonization process [87,89–91]. Surface modifications may provide specific targeting properties or stimuli-sensitive responses, which is relevant in anti-cancer therapies.

A major concern during the development of NPs is the immunogenicity given that they can act as antigenic agents. Hypersensitivity reactions, anaphylactic shock as well as humoral and cellular immune responses can be observed [87,94,95]. Thrombogenicity and hemolysis have already been reported [91,96]. In this context, particles can be opsonized by plasma proteins and key materials (e.g., natural lipid or polymers) can be selected to minimize or avoid these complications.

Nanocarriers can not only enhance the therapeutic effect of drugs but also overcome resistance to chemotherapeutic agents [92]. Tumors develop resistance to drugs through many mechanisms including drug efflux or the downregulation of uptake mechanisms [97]. Nanoparticles can overcome this limitation by providing an alternative route of cellular internalization.

Overall, nanoparticles targeted for the treatment of solid tumors are administered systemically and accumulate in the tumor tissues through the enhanced permeability and retention (EPR) effect. This event occurs as the result of leaky tumor vasculature and poor lymphatic drainage [98]. A summary of advantages and disadvantages of main nanocarriers exploited for the incorporation of APG is presented in Table 1.

Table 1. Advantages and disadvantages of main nanocarriers exploited for apigenin.

Nanocarrier type	Advantages	Disadvantages
Liposomes	• Hydrophilic and lipophilic agents can be efficiently incorporated • Encapsulation of macromolecules • Biocompatible and biodegradable systems (structure analog to cell membrane) • High cell interaction/internalization	• Leakage of encapsulated molecule during storage • Complex and high-cost production • Difficulty in large scale manufacturing and sterilization • Lipid oxidation/hydrolysis • Batch to batch variation

Polymeric NP	• Passive targeting in tumoral tissues • Controlled/sustained drug release • Tunable chemical and physical properties depending on the selected polymer • Reproducible results when synthetic polymers are prioritized • Tunable surface properties (targeted drug delivery) • More stable than lipid systems	• Short half-life • Toxicological issues (solvent residue in formulation or use of non-biodegradable polymers) • Batch to batch variation when natural polymers are prioritized
	• Size and shape uniformity • Specific properties (e.g., conductivity) • Multiple shapes • Tunable size, pharmacokinetics and biodistribution	• Toxicity

4.1 Polymeric nanoparticles

Polymeric nanoparticles are colloidal systems composed of natural or synthetic polymers [92]. According to the morphological structure and drug distribution, they may be distinguished into nanocapsules and nanospheres. The nanocapsules are composed of an oil core where the active compound can be dissolved, surrounded by a polymeric shell that controls the molecule release. Nanospheres, in turn, present a continuous polymeric network where the drug can be trapped inside and/or adsorbed on its surface [99].

The wide use of these nanocarriers is associated with the potential of providing a controlled and sustained release of drugs. In addition, they can protect molecules with biological activity against environmental factors as well as improve the bioavailability and therapeutic index [100,101]. A summary of the main polymeric NPs of APG developed for cancer application is presented in Table 2.

Table 2. Polymeric nanoparticles of apigenin with antitumoral activity.

Physicochemical characterization (size, zeta potential, EE)	Preparation method	Cancer type	<i>In vitro</i> release results	<i>In vitro</i> or <i>in vivo</i> activity results	Reference
100.2 nm -12.2 mV 87.7%	Solvent displacement	Skin melanoma	Initial release burst up to 16 h ($\pm$ 50%) followed by a controlled release for up to 3 days.	IC ₅₀ (A375 cells): 15 and 25 μ M for NPs and free APG, respectively.	Das et al. (2013)

243 nm	Precipitation	Ovarian	60% of APG were released within 12 h followed by a controlled release for up to 3 days.	Reduction of cell viability in 70% (OVCAR4 cells)	Yu et al. (2017)
270 nm -4.84 mV	Multiple emulsion solvent evaporation	Hepatocellular	Initial release burst up to 6 h ($\pm$ 24%) followed by a controlled release for up to 60 days.	IC ₅₀ (HepG2 cells): 20.3 and 40.7 μ M for NPs and free APG, respectively. IC ₅₀ (Huh-7 cells): 31.4 and 44.4 μ M for NPs and free APG, respectively. Murine model: control of tumor development after 12 weeks, hepatic enzyme levels were restored.	Bhattacharya et al., (2018)
PLGA NPs 110 nm -25.0 mV 70.3% Galactose/PLGA NPs 129 nm -14 mV 75.4%	Nanoprecipitation	Hepatocellular	Initial release burst followed by a sustained release for up to 8 days.	IC ₅₀ (HepG2 cells): 15.9, 40.2 and 60.1 μ M for galactose/PLGA NPs, PLGA NPs and free APG, respectively. Murine model: reduction in nodule formation, downregulation of MMP2 and MMP9, induction of apoptosis. Galactose/PLGA NPs were more effective.	Ganguly et al. (2021)
PLGA NPs 92 nm -16.4 mV 50.5% NPs functionalized with DMSA 93 nm -18.9 mV 48.8%	Nanoprecipitation	Melanoma lung metastasis	Initial release burst followed by a sustained release for 30 days.	IC ₅₀ (B16F10 cells): 11.5-times lower for DMSA-APG-NPs than free APG after 48 h. IC ₅₀ (A549 cells): 10.8-times lower for DMSA-APG-NPs than free APG after 48h. Functionalization with DMSA resulted in increased antitumoral activity. Mice model: NPs functionalized with DMSA increased plasma and lung AUC values by 4.43 and 9.24 times when compared to free APG, respectively.	Sen et al. (2021)

NPs = nanoparticles; IC₅₀ = half-maximal inhibitory concentration. Cell culture conditions may explain differences in IC₅₀ for free APG.

Das et al. (2013), for example, developed polymeric nanoparticles for melanoma aiming to improve the activity, photostability and release profile of APG. NPs were prepared through the solvent displacement method and poly(lactic-co-glycolide) (PLGA) was selected as the polymer due to its high biodegradability and biocompatibility. NPs showed a spherical shape, a mean particle diameter of 100.2 nm (DLS analysis), a

polydispersity index (PDI) lower than 0.3, a zeta potential of -12.2 mV and an encapsulation efficiency (EE) of 87.7%. These formulation parameters were kept stable over 60 days of storage. Improved photostability of APG was also achieved after nanoencapsulation. A biphasic release profile of APG from NPs was found, presenting an initial release burst up to 16 h (close to 50%) followed by a controlled release for up to 3 days (between 50-70% of the initial APG amount). Initially, an APG diffusion through the polymer matrix would be observed followed by degradation of the polymer matrix. A375 melanoma cells and HaCaT keratinocytes were selected for antitumoral and cytotoxicity assays, respectively. The treatment of A375 cells with APG-loaded nanoparticles and free APG resulted in a dependent-concentration effect. IC₅₀ values of 15 and 25 μ M were found for APG-loaded nanoparticles and free APG, respectively. No cytotoxic effect on HaCaT cells was observed, suggesting a selective action for this flavonoid (cell viability was higher than 90% for both samples tested at concentrations lower than 25 μ M). The nanoencapsulation enhanced the interaction of APG with DNA, which could explain an increased antitumoral activity via apoptotic mechanisms. The mitochondrial dysfunction events and breakdown of the oxidative phosphorylation system in tumoral cells were maintained after APG nanoencapsulation [8].

The anti-cancer activity of APG/PLGA nanoparticles and other flavonoids was also evaluated by Yu et al. (2017) in an ovarian cancer cell line. NPs were prepared by precipitation method, showing a spherical shape, an average diameter of 243 nm (DLS analysis) and 75 μ g of APG per mg of PLGA. In PBS (pH=7.4), 60% of the APG was released within the first 12 h of incubation followed by a controlled release for up to 3 days ($\pm$ 70% of the initial loaded APG amount). OVCAR4 cell line was used to investigate interactions of APG with p53 genes (one of the major oncogenes that regulate cancer occurrence and progression). The HPV16 E6 gene was transfected in these cells to prevent p53 production. Cells were exposed to X-rays to confirm the mutation. Cell viability and growth as well as the impact on G0/G1 phase were tested in both mutated and non-mutated cancer cells. APG-loaded nanoparticles reduced cancer cell viability (about 70% after 96 h), even more significant in p53 dysfunctional cells, suggesting that this inhibition is associated with p53 gene functions. APG also inhibited cell growth, but this reduction was not significant. Both transfected and non-transfected cells enhanced arrest in G0/G1 phase, suggesting that other genetic pathways, not only p53, are also involved in anti-cancer activity. In all assays, APG nanoparticles were more effective

than free APG. This fact was attributed to the low solubility of the APG in the cell culture medium, which would affect the interaction with cells [102].

Once the PLGA has already been approved by the Food and Drug Administration (FDA) for intravenous administration, Bhattacharya et al. (2018) prepared APG-loaded nanoparticles with this polymer by exploiting this route. In this new study, the authors considered the effect of APG on hepatocellular cancer. Polymeric nanoparticles were prepared by the multiple emulsion solvent evaporation technique and showed an average particle size of 270 nm, zeta potential of -4.84 mV and low drug loading efficiency (19.14%). When added to PBS (pH=7.4), the systems presented an initial burst release of APG (24% in 6 h) followed by a sustained drug release pattern (52% in 60 days). A faster release of APG was found in the acid medium, which values were 79.06 and 88.54% in pH 5 and 3, respectively. Two different human hepatocellular carcinoma cell lines (HepG2 and Huh-7) were considered for *in vitro* activity assays. In HepG2 cells, IC_{50} values for APG-loaded nanoparticles and free APG were 5.5 and 11 $\mu\text{g/mL}$, respectively. In Huh-7 cells, in turn, this parameter was 12 and 8.5 $\mu\text{g/mL}$ for free and encapsulated APG, respectively. Therefore, the nanoencapsulation had a positive impact on the flavonoid activity. Scintigraphy studies showed a preferential hepatic distribution of NPs when compared to free APG, which was also supported by pharmacokinetic studies. Plasma and hepatic clearance values of APG were reduced after nanoencapsulation. *In vivo* assays in rats with hepatocellular carcinoma confirmed the greater activity of NPs compared to free compound. Macroscopic analysis of liver tissues demonstrated that treatment with NPs (20 mg/kg single i.v. dose/week) for 12 weeks successfully controlled the tumor development in these animals. During the histological evaluation, NPs showed to be more effective in maintaining the hepatocellular architecture than free APG. The activity of enzymes associated with liver function was also monitored, and the treatment with APG NPs was shown to be able to restore the physiological levels [103].

Ganguly et al. (2021) compared the performance of two types of polymeric NPs designed for hepatocellular cancer treatment. Once this cancer is mostly diagnosed at an advanced stage, a target-specific delivery of chemotherapeutic agents should be prioritized. For this reason, galactose-polymer conjugates were prepared. The galactose can be recognized by asialoglycoprotein receptors present in liver cells and thus this approach could improve drug targeting to the liver. APG-loaded NPs composed of PLGA or galactose/PLGA conjugates were prepared by the nanoprecipitation technique. PLGA

was galactosylated by a simple esterification reaction. APG-loaded NPs presented a particle size of 110 nm, PDI of 0.041 and zeta potential of -25.0 mV. For NPs with galactose/PLGA conjugates (GAL-PLGA-APG), a mean particle size of 129.0 nm, PDI of 0.059 and zeta potential value of -14.0 mV were found. APG-loaded NPs with and without galactose showed an EE of 75.4 and 70.3%, respectively. *In vitro* release assays revealed a biphasic release profile characterized by an initial burst of APG followed by a sustained release for approximately 8 days. The antiproliferative effect was evaluated in HepG2-cells and was more pronounced after 48 h of incubation. IC₅₀ values of 15.9, 40.2 and 60.1 μ M were found for GAL-PLGA-APG, PLGA-APG and free APG, respectively. This increased cytotoxicity for nanoparticles with galactose was attributed to higher cell uptake. In a murine model of liver carcinoma, GAL-PLGA-APG exhibited a more significant reduction of nodule formation, downregulation of matrix metalloproteinases and induction of apoptosis in the liver compared to APG-loaded PLGA NPs and free APG. Histological analyses demonstrated that GAL-PLGA NPs could significantly reduce hepatic lesions, abnormal cells, and glycogen deposition in the liver as well as restore hepatic architecture to physiological levels [50].

Sen et al. (2021) considered a similar approach in the treatment of pulmonary metastatic melanoma. APG/PLGA NPs were functionalized with meso-2,3-dimercaptosuccinic acid (DMSA), which leads to preferential deposition of NPs in the lungs. NPs were prepared by the nanoprecipitation technique and then conjugated with DMSA by the EDC/NHS coupling protocol. The average particle size, PDI, EE and zeta potential of unconjugated and DMSA-conjugated NPs were 92/93 nm, 0.237/0.093, 50.5/48.8% and -16.4/-18.9 mV, respectively. These characteristics were maintained for 6 months after storage at both -20 and 4°C. *In vitro* release assays performed in PBS pH 7.4 containing 2-hydroxypropyl- β -cyclodextrin as solubilizing agent revealed a biphasic release profile characterized by an initial burst release of APG followed by a sustained release for 30 days with 53.95% of released APG. Overall, DMSA-functionalized and non-functionalized PLGA NPs showed a similar release profile. The *in vitro* antitumoral activity was performed in B16F10 murine melanoma cells and A549 human lung adenocarcinoma cells. In A549 cells, the IC₅₀ values of free APG were 7.8 and 10.8 times higher than DMSA-APG-NPs after 24 and 48 h of incubation, respectively. In the B16F10 cells, the IC₅₀ values of free APG were 3.4 and 11.5 times higher than those for DMSA-APG-NPs after 24 and 48 h of incubation, respectively. DMSA-functionalized NPs had greater antitumoral activity than non-functionalized NPs, which was attributed to the

different cell internalization rates (conformed by confocal microscopy analyses). In a 3D tumor spheroid model, DMSA-conjugated NPs also showed more penetration in tumoral mass than unconjugated NPs. Both NPs provided higher depolarization of mitochondrial transmembrane potential and enhanced levels of caspase activity compared to the control. They also provided an upregulation of p-53. Reduction in COX-2 levels provided by NPs suggests prevention of melanoma cell migration and invasion. *In vivo* pharmacokinetic assays were performed in a mice model (C57Bl/6 strain) and revealed a sustained APG release after an oral administration. In fact, DMSA-APG-NPs maintained a steady blood level of APG in plasma for approximately 25 h. The values of C_{max} , half-life and AUC in plasma and lungs were higher for the DMSA-APG-NPs than free APG and APG-NPs. Plasma and lung AUC values were remarkably high (2.25 and 4.43 times and 5.93 and 9.24 times, respectively) following treatment with APG-NPs and DMSA-conjugated formulations when compared to those with free APG. The treatment with DMSA-APG-NPs significantly reduced the number of tumor nodules on the lung surfaces of the mice. Scintigraphy imaging studies showed the following tissue distribution order: liver>kidney>lungs>spleen. Histopathological analysis and other biochemical toxicity parameters showed no toxicity for all tested samples [104].

4.2 Aptamer-polymer nanoparticle complexes

Aptamers are small oligonucleic acids or peptide molecules that bind to their target with high specificity. They can be classified as nucleic acid aptamers and peptide aptamers depending on the random sequence pools used to generate them [105].

Dutta et al. (2018) functionalized APG-NPs with an aptamer targeted to the EpCAM receptor, which is a surface biomarker overexpressed on colorectal carcinoma cells. Modification of the aptamer at the 3'-amino group was performed to link the peptide to the carboxyl group of PLGA. This chemical modification was confirmed by gel electrophoresis. The phosphodiester bond was replaced with a phosphorothioate backbone to prevent digestion by DNases in plasma. NPs were initially prepared by the multiple emulsion solvent evaporation technique and then conjugated with a modified aptamer under specific conditions. The APG nanoparticle demonstrated a smooth spherical surface, an average particle size of 204.55 nm, a zeta potential of -8.55 mV and an EE of 73.11%. After its conjugation with the modified aptamer, these parameters changed to 226.86 nm, -5.37 mV and 69.68%, respectively. The low zeta potential (<30 mV) requires a suspension reconstitution before injection and this size is compatible with

the diffusion through endothelial cell gap. These particles maintained stable particle size and zeta potential after storage in refrigerated conditions (2–8 °C) for 6 months. *In vitro* release studies showed a sustained release pattern over 60 days. NP internalization was confirmed by microscopy. The cellular uptake percentage of NPs with and without aptamer was 97 and 49.2%, respectively. The *in vitro* antiproliferative activity was evaluated in HCT-116 and HT-29 cells. NPs with the aptamer demonstrated the highest cytotoxicity. After 48 h, IC₅₀ values were 15.4 and 35.8 µM for HCT-116 and HT-29 cells, respectively. For the APG NP without the aptamer, in turn, a IC₅₀ value of 23.5 µM was found for HCT-116 cells whereas HT29 cells exhibited an IC₅₀ of 40.5 µM. NPs with aptamer also reduced the cell activity of GSH, which increases ROS generation with the subsequent apoptosis cascade activation. Scintigraphy analyses demonstrated that NPs with aptamer improved the distribution of APG in colon and colorectal regions. Moreover, animals treated with these nanoparticles presented the most significant reduction in tumor size and ulcerative regions. Taken together, these results suggest that NPs with aptamers are more effective than free APG and APG-loaded NPs in colorectal cancer [106].

4.3 Lipid-based nanosystems

Lipid-based nanosystems such as liposomes, nanoemulsions and nanostructured lipid carriers have received great attention in cancer treatment due to their high biocompatibility, the ability to improve drug solubility and bioavailability and increased efficacy [107]. A summary of these main systems loaded with APG is presented in Table 3.

Table 3. Main lipid-based nanocarriers loaded with APG for cancer applications.

Type of lipid system	Physicochemical characterization (size, zeta potential, EE)	Preparation method	Cancer type	<i>In vitro</i> or <i>in vivo</i> activity results	Reference
Liposomes	~100 nm -6.8 mV	Thin-film hydration	Lung	20% inhibition of cell growth (A549 cells) after treatment with 10 µM of APG. Murine model: accumulation in tumoral sites	Jin et al. (2017)

	103.2 nm 89.74%	Lipid film hydration	Colorectal	IC ₅₀ (HT-29 cells): 26.71 and 45.96 μ M for NPs and free APG, respectively. IC ₅₀ (HCT-15 cells): 28.22 and 43.28 μ M for NPs and free APG, respectively. Murine model: tumor volume reduction; reduction of expression of biomarkers associated with vascularization and cell proliferation.	Banerjee et al. (2017)
	Without 5-FLU 98.17 nm -3.74 mV 89.98% With 5-FLU 105.2 nm -3.84 mV 81.13%	Thin-film hydration	Colorectal	Without 5-FLU IC ₅₀ (HT-29 cells): 26.71 μ M IC ₅₀ (HCT-15 cells): 28.22 μ M With 5-FLU IC ₅₀ (HT-29 cells): 10.26 μ M IC ₅₀ (HCT-15 cells): 7.42 μ M HR-29 and HCT-15: G2/M arrest induction Mice: reduction in cell proliferation, tumor regression and inhibition of tumor vasculature.	Sen et al. (2019)
Nanostructured lipid carrier	NLC without surface modification 75.60 nm +11.6 mV ~70% NLC functionalized with hyaluronic acid (HA) 88.54 nm +6.42 mV ~70%	Hot homogenizer with ultrasonication	Lung	IC ₅₀ (A549 cells): 23.51, 35.4 and 64.44 μ M for free APG, APG-NLCs and HA-APG NLCs, respectively. HA-APG NLCs increased cells in the apoptotic phase and contributed to a reduction in MRP2, HO1 and Bcl-2.	Mahmoudi et al. (2019)
Lipid nanocapsule	46.1 nm -28.18 mV 95.86%	Phase inversion	Hepatocellular and breast	Enhanced cytotoxic activity against HepG2 and MCF-7 cells.	Ding et al. (2013)
Nanoemulsion	138.21 nm -30.7 mV	High speed homogenization	Skin	A431 cells Nanoemulsions were less effective than free APG.	Jangdey et al. (2017)

Phospholipid micelle	137.1 nm -12.94 mV 87.35%	Thin film hydration	Lung	IC ₅₀ (A549 cells): 18.5 and 3.7 μ M after 24 and 47 h, respectively. Murine model: tumor inhibition rate of 72.9%.	Munyendo et al. (2013)
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4.3.1 Liposomes

Liposomes are spherical vesicles composed of one or more phospholipid bilayer(s) and an aqueous core that allows the encapsulation of hydrophilic and hydrophobic compounds in a single vesicle. They are considered passive targeted drug-delivery systems due to the enhanced permeability and retention (ERP) effect that may be observed [113–115].

Jin et al. (2017) developed APG-loaded liposomes for lung cancer. D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) was selected as a formulation constituent since it acts as a solubilizer agent, inhibits P-glycoprotein-mediated multidrug resistance, and enhances the bioavailability of anticancer drugs. In addition to evaluating the performance of APG liposomes, the synergistic apoptotic effect of this nanocarrier and tyroservatide (YSV), a bioactive tripeptide obtained from the spleen of pigs, was considered. APG-TPGS liposomes were prepared by the thin-film hydration method resulting in uniform spheres with a mean diameter of $\sim$ 100 nm and zeta potential of -6.8 mV. The therapeutic potential was investigated in lung cancer cells A549. The monotherapy with liposomes APG-loaded (2.7 μ g/mL) inhibited the growth of A549 cells by 20% while the combined therapy with these liposomes and tyroservatide (270 μ g/mL) showed an inhibition percentage of 50%. Liposomes increased the cell internalization rate of APG. Fluorescence imaging studies indicated that liposomes start to accumulate in tumor zones 4 h after their injection in the tail vein of nude mice, achieving a maximum effect after 8 h. A549 tumor-bearing nude mice were considered to confirm the efficacy of APG-TPGS liposome and YSV combination in lung cancer treatment. When these agents were tested individually, the APG showed higher activity than YSV. APG-TPGS liposome and YSV combination, in turn, demonstrated the strongest antitumor effects, with a more significant necrotic effect [116].

Banerjee; Banerjee & Mandal (2017), in turn, developed liposomes of APG to treat colorectal cancer. These nanocarriers were prepared by the lipid film hydration method using 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) as the phospholipid to increase the interaction with cells. Liposomes loaded with APG had a spherical shape,

uniform (PDI= 0.175), an average particle size of 103.2 nm and EE of 89.74%. The anticancer activity was tested by considering HCT-15 cells, HT-29 cells and an *in vivo* tumor xenograft model (murine model). For free APG, an IC₅₀ value of ~45.96 μ M was obtained in HT-29 cells whereas this parameter was ~43.28 μ M in HCT-15 cells. The nanoencapsulation increased the *in vitro* activity, changing the IC₅₀ values to 26.71 and 28.22 μ M in HT-29 and HCT-15 cells, respectively. *In vivo*, both free and encapsulated APG reduced the tumor volume significantly. Liposomes were more effective than free APG, which was attributed to the EPR effect of the nanoparticle. Histological examination in the tumor tissues showed that APG-loaded liposomes provided a regression in the intricacy of the vascular architecture and reduced adipocyte (providing energy for increased tumor growth) and pericyte population. The treatment with liposomes also reduced the expression level of CD-31 and Ki-67, which are biomarkers associated with vascularization and cell proliferation [108].

This same research team developed a novel liposomal formulation by combining APG and 5-fluorouracil (5-FLU) for the same application. Liposomes were prepared by a modified thin film hydration method by considering the same phospholipid. The mean particle diameter of APG-loaded liposomes and APG/5-FLU-loaded-liposomes was 98.17 (PDI= 0.187) and 105.2 nm (PDI= 0.165). Liposomes with APG or AGP/5-FLU showed a similar surface charge, which was -3.74 and -3.84 mV, respectively. APG-loaded liposomes presented an EE of 89.98% for this flavonoid, which was reduced to 81.13% after combination with 5-FLU. In this last system, the EE for 5-FLU was 35.12%. The hydrophilic characteristic of 5-FLU may explain this low encapsulation rate. The same *in vitro* and *in vivo* assays as well as cell and animal models reported in the previous study were considered for antitumoral activity. The IC₅₀ values for APG-loaded liposomes were ~26.71 and ~28.22 μ M against HT-29 and HCT-15 (both colorectal cancer cell lines), respectively. Nanoencapsulation almost doubled the antitumor activity. In presence of 5-FLU, IC₅₀ values of APG-loaded liposomes were even lower (10.26 and 7.42 μ M in HT-29 and HCT-15, respectively). Non-specific endocytosis could justify the increased antitumoral activity of AGP-loaded liposomes compared to free APG. APG liposomes also demonstrated greater G2-M arrest induction than 5-FLU liposomes. In the same way, APG liposomes induced greater cellular reproductive death than 5-FLU liposomes in clonogenic assays. *In vivo* assays demonstrated maximal tumor regression for dual drug-loaded liposomes, confirming the synergistic action of these compounds. In addition, this system resulted in a reduction in the intricacy of the vascular architecture,

cell proliferation (reduced expression of Ki-67) as well as adipocyte and pericyte density as observed in the previous study [117].

4.3.1.1. Transfersomes

Transfersomes are ultra-deformable vesicles composed of water, surfactant and phospholipids in which an aqueous core is surrounded by a complex lipid bilayer. These vesicles have the ability to deform and cross narrow constrictions of 5 to 10 times less than their own diameter without any measurable drug loss [118].

Jangdey et al. (2017) developed APG-loaded transfersomes for skin cancer by a modified rotary evaporation sonication technique using Tween 80 as the surfactant. The optimized transfersomes presented a mean particle size of 35.41 nm (DLS analysis), a zeta potential of -14.26 mV, PDI of 0.253 and EE of 84.24%. The systems were stable at 4 and 25°C for 6 months. *In vitro* release studies using PBS pH 5.5 resulted in APG release of 9.4% within 2 h, followed by a sustained release (61.2% of APG was released within 24 h). *Ex vivo* permeation studies with Swiss albino mice skin were also performed. The skin permeation flux of APG from the optimized transfersome was significantly higher than a pure APG suspension (6.68 versus 5.43 $\mu\text{g}/\text{cm}^2/\text{h}$). In the same way, greater cutaneous retention was found for transfersomes (0.79 versus 0.58%) [119]. Although the authors have designed this formulation for skin cancer, activity assays have not yet been carried out.

4.3.2. Nanostructured lipid carriers

Nanostructured lipid carriers (NLC) emerged as an upgrading of solid lipid nanoparticles due to the partial replacement of solid lipids by liquid lipids. As a consequence, a greater disorder in the internal structure of these nanocarriers is observed (less organized crystalline structure), resulting in gaps for drug accommodation and encapsulation. Drug expulsion is also minimized during the formulation storage [120].

Hyaluronic acid(HA)-based nanostructured lipid carriers (NLCs) were considered to enhance the efficacy of APG as a nuclear factor E2-related factor 2 (Nrf2) inhibitor agent. Overexpression of Nrf2 contributes to the chemotherapeutic resistance in non-small-cell lung cancer (NSCLC). HA has a high affinity for glycoprotein CD44, which is a potent targeting ligand in lung cancer cells. APG-NLCs were prepared by hot homogenization followed by ultrasonication method. The lipid phase was composed of Precirol® ATO 5, Miglyol-812 and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine

(DOPE). Poloxamer 407 was included in the aqueous phase as the surfactant. HA was adsorbed on the particle surface through electrostatic interactions with the positive charge of DOPE. APG-NLCs presented a particle size of 75.60 nm, which increased to 88.54 nm after the addition of HA. The zeta potential values of APG-NLCs and HA-APG-NLCs were +11.6 and +6.42 mV, respectively. The EE of HA-APG-NLCs and APG-NLCs was approximately 70%. The APG was released faster from APG-NLCs than HA-APG-NLCs and APG release was slower in physiological (pH 7.4) than in acidic conditions (pH 5.5). As already mentioned, APG is more soluble in an acid medium. A sustained release was achieved considering that approximately 80% of APG was released up to 80 h. The antitumoral activity was evaluated in A549 cells. The IC_{50} values of free APG, APG-NLCs and HA-APG-NLCs were 64.44, 35.4 and 23.51 μ M, respectively. The improved intracellular APG accumulation by ligand-receptor recognition (confirmed by flow cytometry) and nanosized carrier uptake may explain this higher activity of HA-APG-NLCs. HA-APG-NLCs increased the population of cells in the apoptotic phase to 36.64% whereas the combination of APG-NLCs and docetaxel (chemotherapy agent widely used in non-small-cell lung cancer) led 57.37% of the cells to apoptosis. HA-APG-NLCs also remarkably decreased mRNA levels of MRP2, HO1, and Bcl-2, which are target genes in cancer. This formulation showed to be promising for lung cancer; however, *in vivo* assays still need to be performed [109].

4.3.3 Lipid nanocapsules

Lipid nanocapsules (LNC) are systems presenting a core-shell arrangement of the constituents with a liquid reservoir (in general, oils) as the core, which can be coated by a protective membrane. This protective membrane is mainly composed of polymer chains that may also be found in the surfactant [121].

Ding et al. (2013) developed and characterized lipid nanocapsules containing APG (APG-LNC), including anti-proliferative activity assays against human hepatocellular carcinoma cell line (HepG2) and human breast cancer cell line (MCF-7). APG-LNCs were prepared by phase inversion method and the optimized formulation was composed of 40% surfactant (Solutol HS 15), 25% oil phase (medium chain triglycerides) and 35% water. APG-LNCs showed spherical shape without aggregation, a mean particle size of 46.1 nm, a zeta potential of -28.18 mV and an EE of 95.86%. After incorporation in LNC, the solubility of APG increased from 2.16 μ g/mL (literature data) to 5.88 mg/mL. Approximately 43% of APG was released from LNC within the first 6 h and 80% after

48 h, indicating a sustained release. APG-LNC enhanced the cytotoxic activity against HepG2 and MCF-7 compared to free APG [110].

4.3.4 Nanoemulsions

Nanoemulsions (NEs) are fine oil-in-water dispersions that present a particle size between 20 and 500 nm. Unlike microemulsions, NEs can be prepared using low surfactant concentrations (3-10%) [122]. The small droplet size avoids physical destabilization caused by gravitational separation, flocculation and/or coalescence [123,124]; however, instability phenomena are more common than in other particulate systems. NE is a suitable vehicle for APG due to the ability of improving both aqueous solubility and bioavailability. The lipid nature of these systems improves the interaction with the cell bilayer. NEs also improve the skin penetration of drugs without the need to include chemical absorption enhancers. Oily constituents may improve the skin hydration whereas surfactants and co-surfactants can play a role as penetration enhancers [125].

Jangdey, Gupta & Saraf (2017) developed a Carbopol® gel containing APG-loaded NEs for UV-induced skin carcinoma, which is a cancer type characterized by a high incidence rate. Cutaneous malignant melanoma represents the most lethal form of skin cancer. NEs were prepared using high-speed homogenization, resulting in oil droplets with 138.31 nm. The zeta potential of the NEs was -30.7 mV, which slightly increased after its incorporation in gel formulations (-31.9 mV). In stability studies performed during 90 days at 4 and 25°C, no significant changes in APG content, droplet size, and zeta potential were found. Moreover, phase separation and flocculation were not observed. Taken together, these findings suggest that the purposed formulation is stable. In release assays performed in modified Franz diffusion cells and cellophane membrane, a burst release phase corresponding to approximately 10–15% of APG was found within 2 h with subsequent sustained release. Approximately 50% of APG was released within 12 h, which was maintained at this level up to 24 h. *Ex vivo* skin permeation and deposition studies were performed in abdominal goat skin. The gel containing APG-loaded NE and APG-free suspension showed a permeation flux of 6.68 and 5.43 $\mu\text{g}/\text{cm}^2/\text{h}$ and APG retention of 0.74 and 0.55%, respectively. Free APG inhibited the proliferation of epidermoid carcinoma cell line A431 in 18.28, 23.34, and 32.50% at 100, 200, and 400 $\mu\text{g}/\text{mL}$. After nanoencapsulation, only the concentration of 400 $\mu\text{g}/\text{mL}$ reduced the cell viability [111]. The slow release of APG from the systems could explain these findings.

4.4 Nanosuspensions

Nanosuspensions consist of poorly water-soluble drug suspended in dispersion without any matrix material. This approach is used to enhance the solubility of drugs that are poorly soluble in water. The increase in solubility results in a higher rate of flooding of the active compound and consequently the maximum plasma level is reached faster. The reduced particle size allows the intravenous application of poorly soluble drugs, avoiding the blockade of blood capillaries [126].

Wu et al. (2017) applied the liquid antisolvent precipitation method to prepare APG nanosuspensions aiming to improve both the solubility and dissolution of this flavonoid. Under optimal conditions, APG nanosuspensions exhibited a near-spherical shape and a mean particle size of 159.2 nm. Solid-state techniques demonstrated that APG is found in an amorphous state inside the particles. The dissolution rate of the APG nanoparticles was 6.08 and 6.14 times that of the free APG in artificial gastric juice (pH 1.2) and artificial intestinal juice (pH 6.8), respectively. In oral bioavailability studies, the value of AUC for APG nanoparticles was 4.96 times higher than that of the free APG. Considering that previous literature indicated activity of APG against liver cancer, HepG2 cells were considered in activity assays. The IC_{50} values of APG nanoparticles and free APG were 89.33 and 216.84 $\mu\text{g/mL}$, respectively. In view of these results, the authors concluded that nanoencapsulation of APG improves both the pharmacokinetics profile and activity [127].

4.5 Micelles

Micelles are spherical amphiphilic structures that have a hydrophobic core and a hydrophilic shell [128]. These systems are gaining increasing attention particularly due to improved solubility and bioavailability of hydrophobic drugs. The reduced particle size increases diffusion of molecules through the intestine and enhanced permeability and retention (EPR) effect in tumoral therapies [129].

4.5.1 Polymer micelles

Polymer chains can also self-assemble forming micelle-like particles. The inner core can incorporate the APG due to its low solubility, the outer hydrophilic corona stabilizes the particle in contact with aqueous surroundings [130]. Zhai et al. (2013) synthesized APG-loaded polymer micelles composed of Pluronic P123 and Solutol HS

15 by the thin-film dispersion method. The authors selected a polymer combination rather than isolated polymers to achieve greater system stability and APG solubility. Polymer micelles had an average diameter of 16.9 nm, a zeta potential of -5.87 mV and an EE of 96.36%. This reduced particle size increases the systemic circulation time as it prevents or reduces the recognition by the reticuloendothelial system and allows an efficient accumulation of NPs in tumor tissues *via* the EPR effect. *In vitro* release assays with PBS containing 20% (v/v) ethanol resulted in 84% of APG released from micelles within 36 h, indicating a sustained release profile. HepG3 and MCF-7 cells were considered to investigate the antitumoral activity. The inhibition rate of APG-loaded polymeric micelles on both HepG2 and MCF-7 cells was higher than free APG. In HepG2 and MCF-7 cells, the IC_{50} values were 5.57/3.75 and 20.19/16.62 $\mu\text{g/mL}$ for APG-loaded polymeric micelles and free APG, respectively [131].

4.5.2 Phospholipid micelles

Phospholipid micelles can also be considered to improve the bioavailability of poor solubility drugs [132]; however, these systems have been considered very unstable in plasma. For this reason, Munyendo et al. (2013) combined D- α -tocopherol acid polyethylene glycol 1000 succinate (TPGS) with phospholipid micelles. In addition to the ability to interact with phospholipids, TPGS micelles can escape from the reticuloendothelial system and inhibit intestinal efflux. Mixed micelles containing APG (TPGS-Ph-APG) were prepared by the thin film hydration method resulting in spherical particles with a particle size of 137.1 nm, a PDI of 0.12, a zeta potential of -12.94 mV and EE of 87.35%. These systems were kept stable for more than 90 days after a freeze-drying process. The amount of released APG after 6 h was approximately 50 and 70% from TPGS-Ap-Ph micelles in simulated gastro-intestinal fluid and PBS (pH 7.4), respectively. A plateau was achieved after 15 h in both release media. *In situ* absorption studies demonstrated the highest absorption in the ileum, followed by the midsmall intestine, jejunum and colon. An enhancement ratio higher than 2 was achieved with TPGS/APG micelles, showing that this agent improves the oral absorption of APG. During the *in vitro* anti-tumor assays in A549 cells, IC_{50} values of Ph-APG were 5 and 1 $\mu\text{g/mL}$ after 24 and 48 h, respectively. The free APG, in turn, was less active, where IC_{50} values of 10 and 5 $\mu\text{g/mL}$ were found for these same incubation periods. For *in vivo* assays, the oral administration of TPGS-Ph-APG and APG-Ph (3 mg kg^{-1} day $^{-1}$) was performed on day 7 post-tumor implantation. Mice treated with TPGS-Ph-APG micelles

showed a tumor inhibition rate of 72.9% while APG-Ph micelles presented an inhibition of only 19.5%. The instability of APG-Ph in biological systems would be the reason for these differences. The rapid release of APG leads to glucuronidation and sulfation events. In summary, the inclusion of TPGS into phospholipid micelles improves system stability, and avoids the rapid metabolization of APG, impacting both absorption and antitumor activity [112].

4.6 Inorganic nanoparticles

4.6.1 Gold nanoparticles

Gold nanoparticles (AuNPs) have received attention in cancer phototherapy due to their tunable and highly sensitive optical and electronic properties (surface plasmon resonance). This phenomenon is a consequence of the collective coherent oscillation of free electrons at the surfaces of the AuNPs generated from the electromagnetic field of light. The irradiation of AuNPs with visible light leads to light energy absorption that rapidly releases heat into the local environment, which has been exploited for the selective laser photothermal therapy of cancer. AuNPs have been functionalized with ligands (e.g., antibodies, folic acid, etc.) to ensure cancer cell targeting [133,134]. Nonetheless, Patra et al. (2007) reported that AuNPs without a conjugated chemical group or biomolecule attached to their surface have the ability to target some cancer cell lines and lead them to apoptosis in a selective mode. Although the exact target of the AuNPs within cancer cells remains unclear, induction of apoptosis of cancer cells via cell cycle arrest and mitochondrial dysfunction have been suggested as probable mechanisms [135–137].

Rajendran et al. (2015) developed gold nanoparticles containing APG (APG-AuNPs) since the -OH and C=O groups present in APG can reduce Au^{3+} ions to form and stabilize AuNPs and evaluated its activity against epidermoid squamous carcinoma cells (A431). After a pH adjustment, hydrogen tetrachloroaurate was added dropwise, under agitation, to a solution of apigenin at room temperature. Spherical particles with uniform size, an average diameter of 22.2 nm and a zeta potential value of -32.2 mV were obtained. The antitumoral activity of these APG-AuNPs was evaluated in both A431 and SiHa cells. In A431 cells, the IC_{50} values of APG-AuNPs were 180, 130.3 and 95.4 $\mu\text{g mL}^{-1}$ at 24, 48 and 72 h, respectively. SiHa cells treated with 200 $\mu\text{g mL}^{-1}$ of APG-AuNPs showed a cell viability of 52.7, 44.2 and 31.8% after 24, 48 and 72 h, respectively. No cytotoxicity was found when HaCat cells (normal cells) were treated with this sample, suggesting a

selective action for the compound. In fluorescence microscopy analyses, A431 cells exposed to APG-AuNPs showed crescent-shaped nuclei and chromatin condensation with membrane damage, which is characteristic of apoptosis. In the same context, DNA prepared from APG-AuNPs treated A431 cells showed a ladder-like pattern in agarose gel electrophoresis, indicating the presence of fragmented DNA. Flow cytometry results also demonstrated the occurrence of early and late apoptotic cells. In the chick embryo chorioallantoic membrane assay, APG-AuNPs inhibited the formation of new blood vessels and subbranches. In summary, APG-AuNPs showed to be biocompatible, with anti-angiogenic properties and induced apoptosis in tumoral cells, suggesting that these systems can be effective against skin cancer [3].

Jiang et al. (2017), in turn, combined gold nanoparticles of APG (APG-AuNPs) with X-ray radiation to treat lung cancer cells. The gold nanoparticles were synthesized via reducing gold salts in presence of APG. These systems presented an average diameter of 20 to 30 nm and zeta potential of -20 mV. AuNPs were able to enhance X-ray absorbance to eliminate cancer cells. Although the APG-AuNPs or X-ray alone can induce the apoptosis of the A549 cells and reduce cell proliferation rate, the combination of these treatments was more effective. In the same way, the use of these two therapies increased the ratio of cells in G0/G1 phases and free radicals [7].

4.6.2 Silica nanoparticles

The mesoporous silica nanoparticles (MSN) are characterized by low solubility and dissolution rate in water, which can sustain the release of poorly water-soluble drugs [138]. Advantages of these systems include the enormous surface area, high amount of pores, pore size with narrow distribution and high physicochemical stability [139].

APG was incorporated in mesoporous silica nanoparticles (APG-MSN) by a physical absorption method in a ratio of 1:1 (w/w) aiming to improve both solubility and bioavailability. Particle size, zeta potential, and EE were 49 nm, -15.0 mV, and 42.27%, respectively. After 48 h, the solubility of APG in an aqueous medium containing 0.4% sodium dodecyl sulfate was 25.11 µg/mL at 37°C [140]. Different solid-state characterization techniques showed that most of the APG was adsorbed in the pores of MSN. *In vitro* release and *in vivo* bioavailability studies were also performed. The accumulative release of APG-MSN was 2.37 times higher than free APG. *In vivo* bioavailability assays showed that the area under the curve for the APG-MSN was 8.32 times higher than free APG, suggesting that APG-MSN improved oral bioavailability

[140]. Although these results are promising, activity studies were not performed with these systems.

4.7. Protein-based nanoparticles

Nanodispersions of whey protein isolate (WPI) loaded with APG were developed by using a pH-cycle method (a free-solvent method) for application in colorectal cancer. The WPI was selected as it is an abundant, sustainable, and inexpensive material. The particles presented EE values ranging from 93.2 to 98.2%, size diameter between 180 and 240 nm and a surface charge close to -20 mV. Nanodispersions enhanced the uptake of APG by HCT-116 and HT-29 cells; however, this fact did not result in an increase of anti-proliferative activity when compared to free apigenin. An increased cell cycle arrest in the sub-G1 phase was also observed, which indicate the occurrence of apoptosis and then inhibition of tumoral cell growth. *In vivo* biodistribution assays were performed in mice and showed a greater amount of APG in blood and colon mucosa after nanoencapsulation. In view of these results, the authors suggest the development of functional beverages with these particles for cancer prevention [141].

5. Final considerations

Nanoparticles have shown to be promising vehicles for the incorporation of APG for application in cancer prevention and treatment. In addition to increased solubility, dissolution and bioavailability; they can improve the biodistribution of APG in tumor cells, increase both APG uptake into cells and cell death by apoptosis. The enhanced permeability and retention (EPR) effect and surface functionalization allow that nanoparticles achieve tumor regions more easily, improving activity/efficacy. The reduced particle size also increases the cell internalization rate (endocytosis), facilitating the interaction of APG with DNA and thus apoptosis.

Until now, liposomes and polymeric NPs have been the most studied nanocarriers for the incorporation of APG. Liposomes show advantages in terms of cell internalization, toxicity and compatibility with the intravenous route (the main administration route for antitumoral agents). Nevertheless, stability problems such as vesicle fusion and oxidation phenomena are common during the storage. Polymeric NPs can overcome this limitation, besides providing a more sustained release for the APG; however, less interaction of these particles with tumoral cells is expected. The surface of

these NPs can be modified to improve this aspect, as reported in few studies presented in this review, but challenges in terms of large-scale production and cost would be found.

Cytotoxicity assays of APG nanoparticles in normal and cancer cells have also indicated a relatively high selectivity of action of the APG, bringing perspectives in terms of safer therapies. In the same context, studies associating antitumoral drugs already used clinically with APG nanoparticles have also shown the possibility of reducing the dose of drug administrated due to a synergism of action, which would minimize adverse effects. *In vivo* studies presented very promising results such as the reduction of tumor volume or neovascularization, encouraging trials with humans. Future studies could also consider the application of these systems in a perspective of cancer prevention.

Funding Statement

The authors thank the Brazilian governmental funding agencies (CAPES and CNPq).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT author statement

Izi Vieira Nunes Cunha: Writing - Original Draft.

Angela Machado Campos: Review & Editing.

Thiago Caon: Conceptualization; Writing - Review & Editing; Supervision.

Abbreviations

BCL-2: B-cell lymphoma 2; BAX: BCL-2-associated X protein

BAK: BCL-2 antagonist killer 1

BIM: BCL-2-like protein 11

BID: BH3 interacting domain death agonist

PUMA: p53 upregulated modulator of apoptosis

BCL-XL: B-cell lymphoma-extra large

BCL-W: BCL-2-like protein 2

MCL1: myeloid cell leukemia 1

ETC: electron transport chain

UCP: uncoupling protein

MNR: Mre11–Rad50–Nbs1

CDKs: cyclin-dependent kinases

ATM: ataxia telangiectasia-mutated

ATR: ataxia telangiectasia-mutated and rad3-related

CHK1: checkpoint kinase 1

CHK2: checkpoint kinase 2

CDC25A: cell division cycle 25A

CDK2: cyclin dependent kinase 2

CDC45: cell division cycle 45

CDC2: cell division control protein 2

PI3K: phosphatidylinositol 3-kinase

AKT: protein kinase B

mTOR: mammalian target of rapamycin

NF- κ B: nuclear factor kappa B

JAK/STAT: janus kinase/signal transducer and activator of transcription

IC₅₀: half-maximal inhibitory concentration

C_{max}: maximum plasma concentration

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5 CAPÍTULO III - EFEITO DA COMPOSIÇÃO DOS INVASOMAS NA FLUIDEZ DE MEMBRANA, ESTABILIDADE E INTERAÇÕES COM A PELE

Considerando que invasomas são sistemas ainda pouco estudados, este capítulo trouxe como objetivo central a avaliação das propriedades biofísicas destas vesículas bem como interações com a pele após modificações na composição. Vesículas com e sem colesterol, limoneno e etanol foram preparadas para comparação. Para a caracterização das formulações, empregaram-se métodos espectroscópicos e de espalhamento de luz. O produto deste capítulo é um artigo científico, que se encontra sob avaliação no periódico *International Journal of Pharmaceutics*

**EFFECT OF INVASOME COMPOSITION ON MEMBRANE FLUIDITY,
VESICLE STABILITY AND SKIN INTERACTIONS**

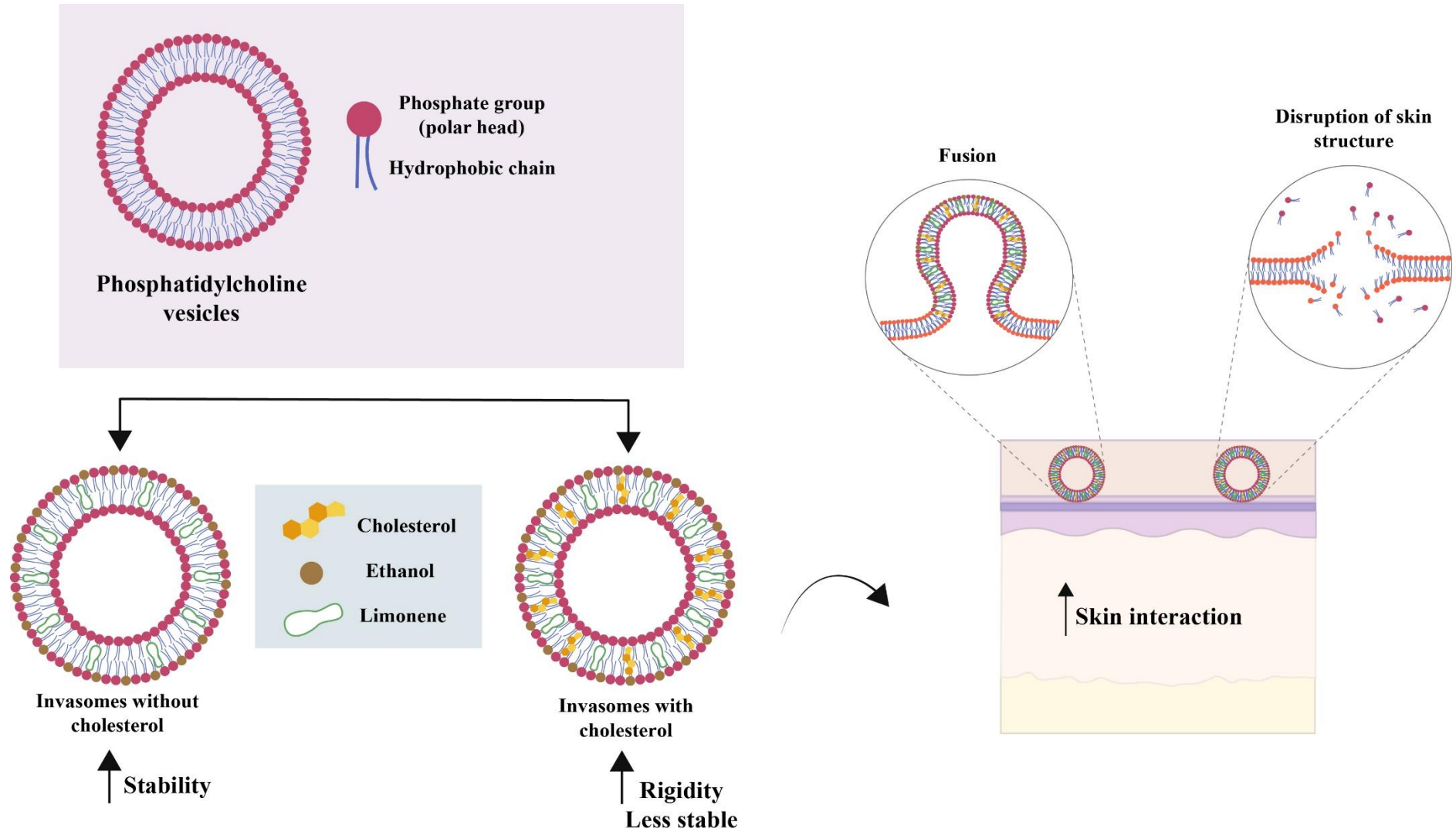
Izi Vieira Nunes Cunha^a, Angela Machado Campos^a, Adriana Passarella Gerola^b,
Thiago Caon^a

^a Pharmaceutical Sciences Department, Federal University of Santa Catarina,
Florianópolis 88040-900, SC, Brazil

^b Chemistry Department, Federal University of Santa Catarina, Florianópolis 88040-
900, SC, Brazil

[#]Corresponding author: caon.thiago@ufsc.br; thiagocaon@gmail.com

GRAPHICAL ABSTRACT



ABSTRACT

Invasomes represent a particular type of liposome composed of ethanol and terpenes with the proposal to increase the percutaneous permeation of drugs. Cholesterol is often found in liposomes to ensure greater control of drug release, plasma stability and increased cellular uptake. Each new compound added to the phospholipid membrane can affect its fluidity and vesicle stability as well as interactions with the skin, which was exploited in this study by considering light scattering and spectroscopic techniques. The addition of high cholesterol concentrations (from PC:CHOL 5:0.5) into the phosphatidylcholine vesicles led to increased membrane rigidity and a greater degree of disorder in skin lipid domains in a concentration-dependent manner. On the other hand, less stable vesicles were obtained. Ethanol, in turn, resulted in more flexible and larger vesicles. The effect of limonene on membrane rigidity was dependent on the composition of the vesicle. This last agent was crucial to obtain more monodisperse vesicles (low PDI values) and it also showed a synergistic action with cholesterol in the disruption of lipid domains in the skin. Stability analyzes showed that invasomes are more stable when compared to liposomes. Cholesterol-free invasomes showed to be stable for up to 40 days at room temperature. Therefore, further studies should be performed to ensure that vesicles containing cholesterol can be effectively stabilized considering that this agent improves the interaction of lipid vesicles with the skin.

Keywords: invasomes; rigidity; limonene; stability; skin interaction.

1. INTRODUCTION

Invasomes are liposomal vesicles composed of phosphatidylcholine, ethanol, and one or a mixture of terpenes. The combination of these agents have demonstrated to enhance the percutaneous penetration of drugs when compared to the conventional liposomes (Chen et al., 2011; El-Tokhy et al., 2021). The superior properties of these invasomes have been related to the presence of ethanol and terpenes in their composition, which act synergistically on percutaneous absorption of drugs (Dragicevic-Curic et al., 2008, 2008; Shah et al., 2015). Permeation enhancement mechanisms on the stratum corneum (SC) include lipid extraction, increased lipid fluidity, higher solubility of the drug as well as changes in skin hydration, putative pore pathway or even in keratinized protein structure (Van Der Merwe and Riviere, 2005; Williams and Barry, 2004).

The SC is composed of corneocytes surrounded by intercellular lipids, which contribute to skin barrier properties. The interior of corneocytes is filled with agglutinated-keratin fibers. Ethanol penetrates the SC due to its low molecular size, causing the desaturation of the soft keratin structure by loosening fibril packing in the corneocytes. Low concentration of this agent disturbs the short lamellar structures of SC, leading an irregular structure. Although not impact on the hydrocarbon chain packing is observed after treatment with ethanol, it interacts with polar portions of SC phospholipids, causing a swelling effect (Horita et al., 2015).

The ability of improving percutaneous penetration of terpenes depends on factors such as lipophilicity, size and chirality, energy of vaporization, boiling point and degree of unsaturation (Kumar et al., 2022). In general, terpenes play a role as absorption enhancer agents by interacting with lipophilic tails of intercellular lipids as well as polar head groups, affecting both intercellular and transcellular pathways (Chen et al., 2016). Terpenes are able to extract or fluidize SC lipids, weakening the skin barrier (Ahad et al., 2011a, 2011b; Wang et al., 2017) or even affect the hydrogen bonds between ceramide molecules. Ceramides are SC proteins strongly organized via hydrogen bonds, resulting in a network that enhances the barrier properties. The tight network can be loosened by terpenes through functional groups that donate or accept hydrogen bonds (e.g., hydroxyl groups) (Wang et al., 2017). Terpenes dissolved in intercellular lipid domains can also improve drug portioning into the SC (Chantasart et al., 2009) or lead to the formation of a molecular complex that increases drug concentration in the SC (Cui et al., 2011). Modeling studies, in turn, suggest that hydrocarbon terpenes interact with drugs via donor/acceptor interactions, Van der Waals forces and HBD (hidrogen bond donor)- π

interactions while oxygen-containing terpenes interact with drugs through the establishment of hydrogen bonds (Drakulić et al., 2008).

Several mechanisms of invasome-skin interaction have been reported in the literature. A widely accepted hypothesis suggests a vesicle disintegration on the skin surface and then their constituents disrupt intercellular lipid lamellae, leading to the formation of channel-like penetration pathways through the skin. These pores allow drugs to circulate freely through the skin (Chen et al., 2011; Dragicevic-Curic et al., 2008; Honeywell-Nguyen et al., 2004; Ota et al., 2003; Vaddi et al., 2002). Another theory proposes that small intact vesicles could penetrate the SC via a transepidermal osmotic gradient. In this last situation, elastic membrane proprieties would be expected (Cevc et al., 2002; Cevc and Blume, 1992; Honeywell-Nguyen et al., 2004).

Although invasomes have improved the cutaneous permeation of drugs such as curcumin, isradipine, nimesulide and avanafil (Ahmed and Badr-Eldin, 2019; Badran, 2012; Chen et al., 2012; Qadri et al., 2017), they are considered unstable systems. This fact has been particularly associated with the hydrolysis and oxidation of phospholipids, which limit the storage time and conditions (Ingvarsson et al., 2011).

As vesicle deformability increases, their stability decreases. The flexibility of membranes may contribute to the processes of vesicle aggregation and the formation of giant onion-like multilamellar structures (Carreras et al., 2020; Coldren et al., 2006; Romero and Morilla, 2015). Flexible vesicles are also more affected by temperature, reducing stability over storage time (Hussain et al., 2017).

The addition of cholesterol into these vesicles, a rigid steroid molecule, has been alternatively considered to overcome these limitations. In fact, its incorporation into phospholipid-based systems has contributed to increase the stability and encapsulation efficiency of drugs (Abdulbaqi et al., 2016). The presence of cholesterol in liposomes impacts fluidity, permeation, membrane strength, elasticity, lipid transition temperature, phospholipid packing, and plasma stability (Ermilova and Lyubartsev, 2019; Ohvo-Rekilä et al., 2002; Song et al., 2022). Although the application of invasomes to improve the percutaneous penetration of drugs has been widely used, few studies have been dedicated to investigating how the vesicle composition affects the biophysical properties (rigidity, stability) and interactions with human skin. For this reason, a series of vesicles (liposomes and invasomes) presenting different constitutions was prepared in this study. Secondly, accelerated stability assays of these vesicles were performed to define the

more stable systems. The equipment LUMiSizer[®] was considered in this last step, a still little-explored technique in the evaluation of the stability of liposomes.

2. MATERIAL AND METHODS

2.4. Materials

Lipoid S100, non-hydrogenated soybean phosphatidylcholine, (PC $\geq$ 94.0%) was kindly donated from Lipid (Campinas, Brazil). Cholesterol (CHOL $\geq$ 99%) and limonene were purchased from Sigma-Aldrich (St. Louis, US). Ethanol and chloroform were obtained from Vetec Química Fina Ltda. (Duque de Caxias, Brazil). Phosphate buffered (PBS) saline was prepared with 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 136.9 mM NaCl, 2.7 mM KCl and adjusted to pH 7.4 with 1 M HCl. Krebs-Ringer bicarbonate buffer was prepared with 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 2.5 mM CaCl₂·2H₂O, 1.0 mM MgCl₂·6H₂O, 5.5 mM glucose and adjusted to pH 7.4 with 1 M HCl. Ultrapure water was obtained from a Milli-Q[®] purification system (Millipore, Milford, MA, USA).

2.2. Preparation of invasomes

Liposomes and invasomes were prepared by the thin-film evaporation method with sonication treatment (Dragicevic-Curic et al., 2008) (Table 1). Briefly, PC was mixed with CHOL in the ratios of 5:0; 5:0.25; 5:0.5; 5:1; 5:2 (w/w), which were dissolved in chloroform. For invasome preparation, limonene was added to the solution containing PC and CHOL in chloroform. A lipid film was obtained after the solvent elimination using a rotary evaporator at 50°C, 150 rpm and 206 mbar. The film was then rehydrated with 4 mL of PBS buffer without and with ethanol 3% (w/v) for 40 min and vortexed for 2 min. Finally, a probe sonicator (UP200S Ultrasonic Processors, Hielscher Ultrasonics GmbH, Germany) was considered to reduce the size of liposomes. The vesicles were sonicated considering pulses of 8 s on and 2 s off (total time = 6 min), an amplitude of 40%, 200 W in an ice water bath. The formulations were stored in glass flasks protected from light at room temperature.

Table 1. Composition of the vesicles.

Formulation	Composition			
	Phosphatidylcholine	Cholesterol	Ethanol	Limonene
PC:CHOL 5:0	2 mM	-	3%	-
PC:CHOL 5:0.25	2 mM	0.2 mM	3%	-
PC:CHOL 5:0.5	2 mM	0.4 mM	3%	-
PC:CHOL 5:1	2 mM	0.8 mM	3%	-
PC:CHOL 5:2	2 mM	1.6 mM	3%	-
IV:CHOL 5:0	2 mM	-	3%	0.5%
IV:CHOL 5:0.25	2 mM	0.2 mM	3%	0.5%
IV:CHOL 5:0.5	2 mM	0.4 mM	3%	0.5%
IV:CHOL 5:1	2 mM	0.8 mM	3%	0.5%
IV:CHOL 5:2	2 mM	1.6 mM	3%	0.5%
IV LIM 0.5%	2 mM	0.8 mM	3%	0.5%
IV LIM 1%	2 mM	0.8 mM	3%	1%
PC:CHOL 5:1 without ETOH	2 mM	0.8 mM	-	-
IV:CHOL 5:1 without ETOH	2 mM	0.8 mM	-	0.5%

2.3. Particle size, polydispersity index and zeta potential

The mean droplet size and polydispersity index (PDI) of colloidal suspensions were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK). Analyses were performed at 25 °C selecting 90° as the scattering angle. Zeta potential (ZP) values were obtained from electrophoretic mobility measurements. All analyses were carried out in triplicate.

2.4. Fluorescence spectroscopy

For the evaluation of membrane rigidity, the method reported by Chwastek *et al.* (2020) was considered. Initially, a stock solution of 9-(2,2-dicyanovinyl)julolidine (DCVJ) in DMSO was prepared, [DCVJ]= 2.0 mM. Afterwards, 2 µL of the DCVJ stock solution were added to 2.0 mL of the colloidal suspension containing the vesicles described in Table 1, resulting in a DCVJ concentration of 2.0 µM and a DMSO percentage of less than 1%. After 15 min, fluorescence measurements were performed in a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Australia). DCVJ was excited at 440 nm using a xenon flash lamp as an excitation source, and the emission spectra was recorded in the range of 460-650 nm. The probe used (DCVJ) is a well-characterized TICT probe with fluorescence emission in the visible range of the light

spectrum. The photophysical properties of DCVJ are sensitive to the viscosity of the medium and thus it can be used to estimate viscosity in lipid membranes. In the singlet excited state, the molecule undergoes rapid rotation around the single bond that connects the julolidene and dicyanovinyl subunits, acting as a molecular rotor. Given that internal rotation and fluorescence emission are competitive processes, in non-viscous solvents and excited singlet state, internal rotation is preferred and fluorescence emission is reduced. Therefore, the DCVJ is weakly fluorescent in a fluid solution whereas the increase in the viscosity of the medium makes this internal rotation more difficult, favoring the deactivation of the excited state by fluorescence emission (Jee et al., 2009). For the evaluation of membrane viscosity (i.e., rigidity) of liposomes and invasomes, the emission intensity of DCVJ incorporated into liposomes/invasomes was compared to that of a solvent of known viscosity (methanol).

2.5. *UV-visible spectroscopy*

Instability events were monitored by UV-visible spectroscopy at 600 nm (Cary® 50 UV-Vis, Varian, USA). Immediately after preparation of the vesicles (see composition in Table 1), approximately 2 mL of each colloidal suspension was analyzed in a spectrophotometer at intervals of 5, 30 and 60 min for 33 h. The technique measures the changes in scattered (turbidimetry) or absorbed (absorbance) light by the vesicles. Turbidimetry is a well-established method for assessing colloidal aggregation, particularly micron-sized particles. In this technique, the signal changes are measured over time and indicate changes in the particle volume fraction and size, which translates into instability events such as aggregation or sedimentation (Ray et al., 2015).

2.6. *Accelerated stability studies*

The accelerated stability of the vesicles was evaluated by the LUMiSizer® equipment (LUM GmbH, Berlin, Germany). In this technique, centrifugal sedimentation is applied in colloidal dispersions, which allows obtaining a stability ranking of dispersions. The centrifugal force accelerates the occurrence of instability phenomena such as sedimentation, flocculation and creaming. The technique measures the intensity of transmitted light as a function of time and position over the entire sample length in a cuvette during centrifugation. For this analysis, samples were placed in rectangular polycarbonate cuvettes (optical path of 2.0 mm) and centrifuged at 450 rpm for 2 h at 25°C to capture 750 transmission profiles at an interval of 10 s.

Stable colloidal dispersions show the formation of a flatbed under a centrifugal field while aggregated particles usually show a step profile. Differences on particle size explain variations in sedimentation/creaming velocity. The red and green profiles represent transmissions detected at earlier and later time, respectively. Under the action of a centrifugal force, vesicles migrate gradually and lead to density variations at different positions in the sample cell, which is reflected by transmission variations. The density of transmission profiles reflects the time required for sample phase separation. More dense profiles indicate that longer intervals are required for phase separation (Caddeo et al., 2013).

The instability index, a factor calculated by the software SEPView[®], was used to compare the stability of developed colloidal suspensions. The value “0” indicates no separation or change in light transmission whereas “1” represents a complete segregation of phases.

2.7. Long-term stability studies

The long-term stability of vesicles was evaluated by considering measurements of size and PDI in different time intervals (immediately after preparations and after 24 h, 48 h, 72 h, 15 and 40 days of preparation). The formulations were stored in glass flasks protected from light at room temperature. Dynamic light scattering measurements were performed in a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK) as described above.

2.8. Vesicle-skin interaction studies

The human skin was obtained from abdominoplasty surgeries and stored in Krebs-Ringer buffer (pH 7.4) until tissue preparation for assays. The study was approved by the Research Ethics Committee of the University (CAE number: 87349418.7.0000.0121). The SC was carefully separated from other underlying skin layers and divided into sections of approximately 20 mg with a scalpel. SC samples were submerged in 4 mL of each formulation (PC:CHOL 5:0, PC:CHOL 5:0.5, PC:CHOL 5:2, IV:CHOL 5:0 and IV:CHOL 5:2) or PBS (control) for 24 h. FT-IR spectra were recorded in a frequency range from 600 to 4,000 cm^{-1} on a Perkin-Elmer Frontier equipment (Waltham, USA) with the collection of 16 scans at a resolution of 4 cm^{-1} . Reduction or displacement of spectral bands indicates disorders in lipid or protein domains of the skin.

2.9. Statistical analysis

Results were analyzed by using GraphPad Prism version 8. One-way ANOVA with post-hoc Tukey was considered for multiple comparisons. *p*-values of less than 0.05 were considered statistically significant.

3. RESULTS

3.1. Particle size, PDI and surface charge

Liposomes and invasomes containing different PC:CHOL ratios (5:0, 5:0.25, 5:0.5, 5:1, 5:2) were prepared to evaluate the effect of CHOL on mean particle diameter, PDI and surface charge.

The liposomes composed of only PC and CHOL did not show uniform vesicle size distribution (high PDI values), making difficult the analysis of CHOL effect on the vesicle size. On the other hand, lower PDI values were found when limonene was added to the formulations (invasomes), which indicates the existence of homogeneous sized vesicles and therefore allows analysis of changes in size. In invasomes, no significant differences in the vesicle size were found among formulations when CHOL ratio was increased (ANOVA/Tukey; $p > 0.05$). Similar findings were found by Shaker et al. (2017), who reported that low cholesterol ratios ($< 5:1.6$ PC:CHOL) did not significantly increase liposome particle size compared to the control (cholesterol-free formulation) (Shaker et al., 2017).

The effect of limonene on vesicle size, PDI and zeta potential was also evaluated. When vesicles with limonene 0.5% (w/v) and without this agent were compared, no significant difference in the size was found (ANOVA/Tukey; $p > 0.05$). On the other hand, a significant increase in vesicle size was found for vesicles prepared with higher concentrations of limonene (1%; w/v). The presence of limonene was crucial to reduce the PDI values, but this agent did not affect the surface charge. Similar findings were found by Dragicevic-Curic et al. (2008). These authors observed that an increase in the amount of terpenes from 0.5 to 1% leads to an increase in vesicle size as well as a reduction in PDI values (Dragicevic-Curic et al., 2008).

The presence of ethanol seems positively affect the mean particle size ($p < 0.05$). Similar results were found by Komatsu and Okada (1995), who reported that the presence of ethanol above 44 mg/mL leads to an increase in vesicle size by inducing aggregation and membrane fusion (Komatsu and Okada, 1995).

Table 2. Size, polydispersity index and zeta potential of developed colloidal suspensions.

Formulation	Size	PDI	Zeta Potential
PC:CHOL 5:0	245.7±6*	0.8	-1.0
PC:CHOL 5:0.25	249.8±7.8*	0.8	-1.3
PC:CHOL 5:0.5	255±6,5*	0.8	-0.7
PC:CHOL 5:1	235.7±8.7*	0.8	-1.1
PC:CHOL 5:2	245.5	0.8	-1.5
IV:CHOL 5:0	153.8±17.6*	0.4	-0.6
IV:CHOL 5:0.25	154.5	0.3	-1.4
IV:CHOL 5:0.5	175.0±0.5*	0.3	-3.4
IV:CHOL 5:1	164.5±25*	0.3	-1.0
IV LIM 0.5%	136.1±4.4*	0.3	-1.4
IV LIM 1%	322.5±14.6*	0.4	-2.7
PC:CHOL 5:1 without ETOH	159.9±3.6*	0.6	-3.3
IV:CHOL 5:1 without ETOH	136.1±4.4*	0.3	-1,4

*n=3

3.2 Rigidity of the vesicles

The relative brightness of DCVJ incorporated into liposomes/invasomes was compared to that of solvents of known viscosity to evaluate the vesicle rigidity. The position of the fluorescence emission band of DCVJ can also be correlated with the solvent polarity (Fig. 2). Fig. 2 shows the high sensitivity of DCVJ to the cholesterol content present in membranes, indicating its preferential localization in the proximity of membrane cholesterol as already reported in the literature (Chwastek et al., 2020). The low emission intensity of DCVJ was observed in methanol (Figure 2A) as high fluidity of the solvent favors the deactivation of the excited state by internal rotation. In the presence of PC:CHOL 5:0 liposomes, the emission intensity of DCVJ increased considerably, due to its incorporation into the liposome's rigid membrane. In this situation, the microviscous environment prevents the deactivation by internal rotation. The emission intensity at 488 nm with increasing cholesterol content is shown in Fig. 2B. The PC:CHOL 5:0.25 formulation showed no significant differences in terms of membrane rigidity when compared to the cholesterol-free formulation (PC:CHOL 5:0). In fact, this result corroborates with the literature, where researchers report that cholesterol concentrations below 10% have no effect on membrane rigidity (Kaddah et al., 2018). From this concentration, the higher the cholesterol content, the greater the

intensity of the DCVJ emission peak, suggesting that an increase in cholesterol concentration causes an increase in membrane rigidity.

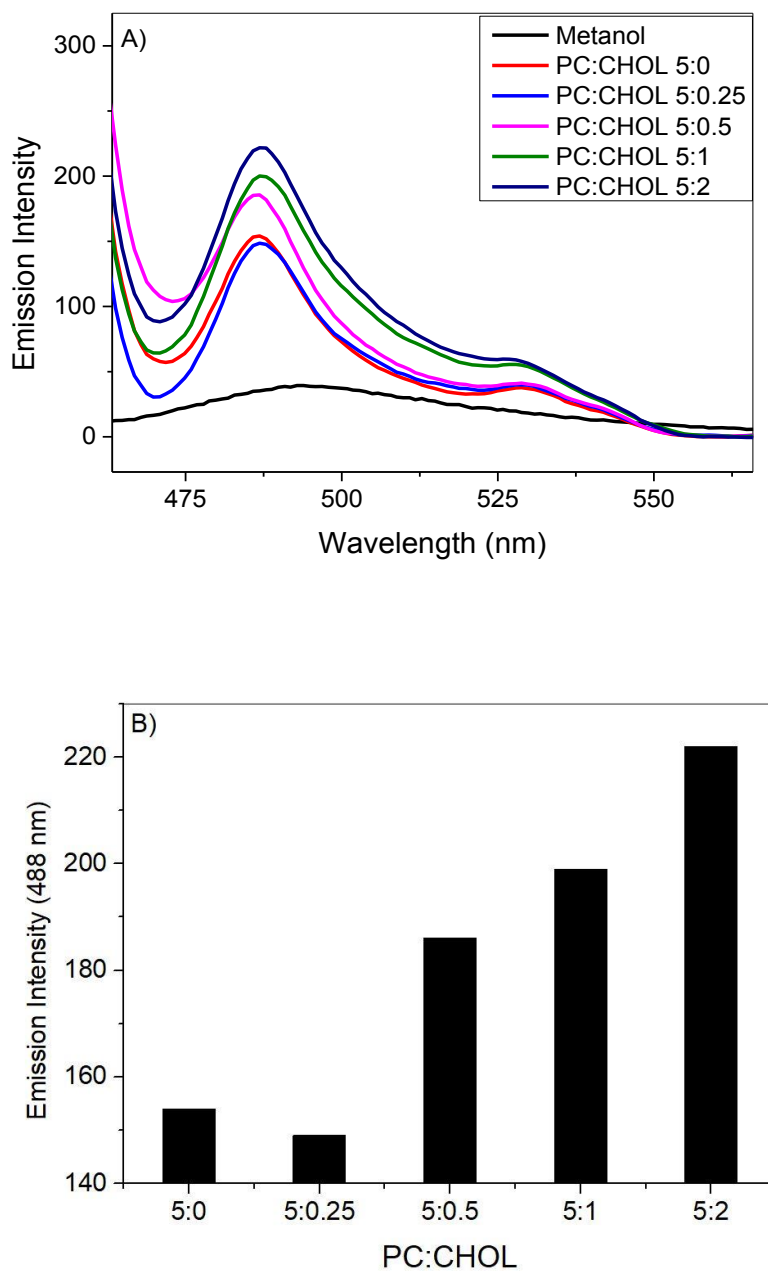


Fig. 2. A) Fluorescence emission spectra of DCVJ in methanol and liposomes with different cholesterol content. B) Emission intensity at 488 nm as a function of PC:CHOL. λ_{exc} = 440 nm.

The effect of limonene and ethanol on the vesicle rigidity was also evaluated. The concentration of these agents was fixed at 0.5 (w/v), 3 (w/v) and 10% (w/w) for limonene, ethanol and cholesterol, respectively. Fig. 3-4 shows the results obtained by analyzing vesicles with different compositions. The presence of ethanol has a positive

effect on membrane fluidity (Fig. 3A) even in vesicles composed of cholesterol (Fig. 3B). These results are in agreement with the literature, which shows that ethanol increases the fluidity of lipids in the vesicle structure, leading to a less rigid structure than conventional liposomes (Babaie et al., 2020). On the other hand, an increase in membrane rigidity was observed when ethanol was added to vesicles containing limonene (Fig. 3C and 3D). Vesicles with limonene and without ethanol (Fig. 4A), in turn, did not affect the membrane fluidity significantly. A similar result was found by Brennan *et al.* (2013) in fluorescence anisotropy assays (Brennan et al., 2013). In this same way, the presence of limonene did not affect the membrane fluidity in PC:CHOL vesicles (Fig. 4B).

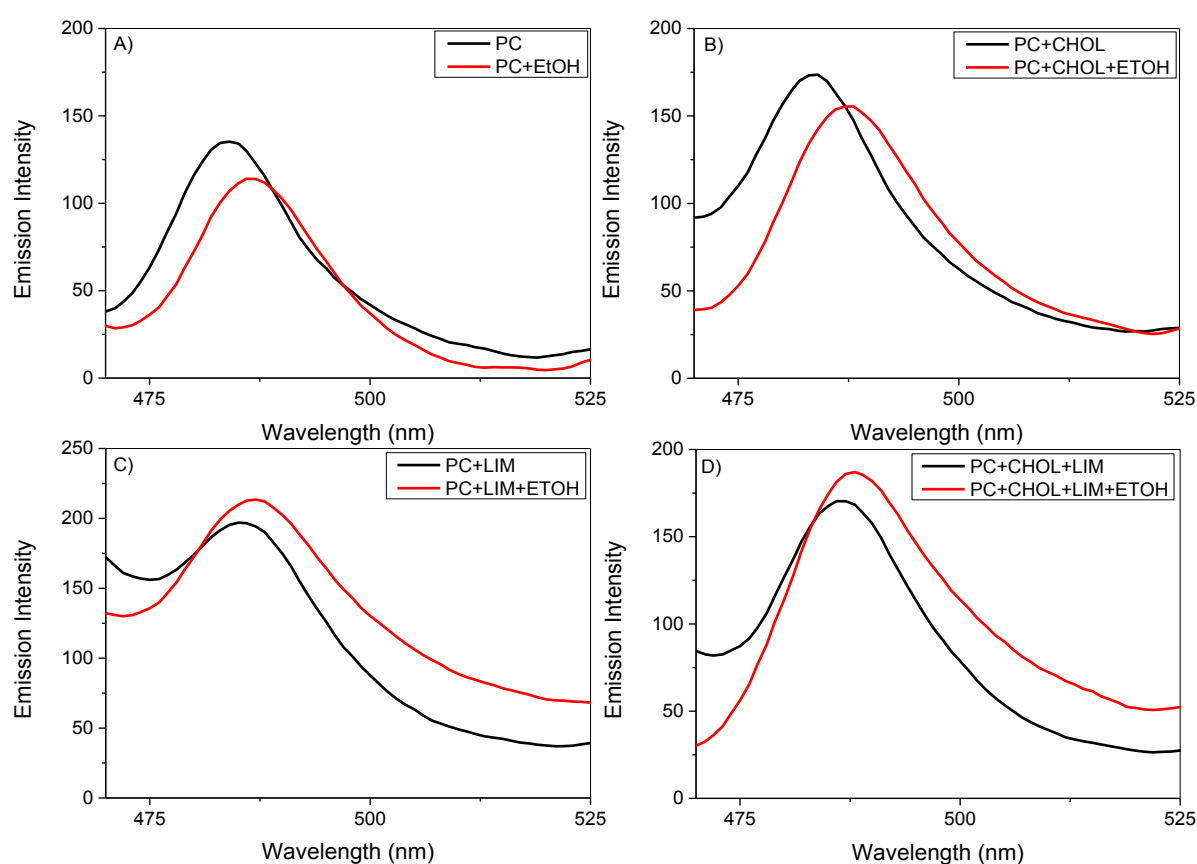


Fig. 3. Fluorescence emission spectra of DCVJ incorporated into vesicles with and without ethanol. The concentration of limonene, ethanol and cholesterol was fixed at 0.5, 3 and 10%, respectively; λ_{exc} = 440 nm.

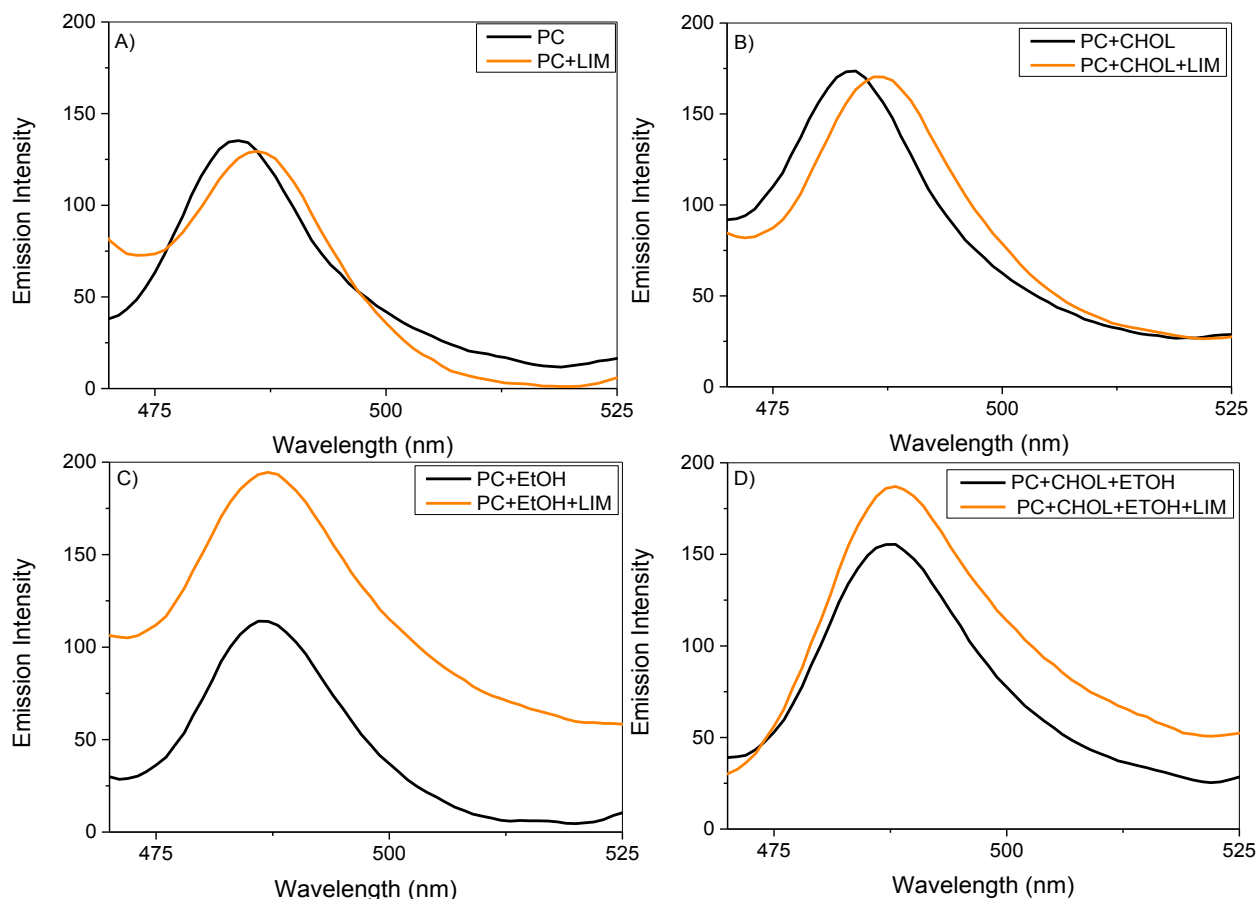


Fig. 4. Fluorescence emission spectra of DCVJ incorporated into vesicles with and without limonene. The concentration of limonene, ethanol and cholesterol was fixed at 0.5, 3 and 10%, respectively; $\lambda_{\text{exc}} = 440$ nm.

For the invasomes containing ethanol and limonene (Fig. 4C-D) with and without cholesterol, an increase in the intensity of fluorescence emission of DCVJ was observed. This result indicates an increase in local microviscosity due to the reduction in the deactivation of the excited state of the probe by internal rotation (Babaie et al., 2020). It is interesting to note that in the analyzed concentration, limonene does not change the local microviscosity of the vesicle, while ethanol decreases the rigidity of the microenvironment. On the other hand, the presence of the two additives leads to a cooperative effect that result in an increase in local microviscosity.

Although the intensity of fluorescence emission for DCVJ is independent of the polarity of the medium, emission wavelength shifts are observed. A bathochromic decoupling to longer wavelengths with increasing solvent polarity is observed for this probe due to the stabilization of the excited state in more polar solvents (Kung and Reed, 1986). As shown in Fig. 2, the emission peak of DCVJ in lipid membranes is similar in the absence and presence of cholesterol. In methanol, a bathochromic shift of 10 nm is found, indicating that the micropolarity of the membrane in the region where the probe is

incorporated is lower than the polarity of methanol. In other words, the probe is located in hydrophobic regions resulting from hydrocarbon chains (Chwastek et al., 2020). Although cholesterol presence in membranes did not affect the emission wavelength (Fig. 2), i.e., the local micropolarity of the membranes, the presence of ethanol (Fig. 3A) and limonene (Fig. 4A) led to a small bathochromic shift for DCVJ, indicating an increase in the micropolarity of the invasomes (Fig. 3 and 4). The increase in local micropolarity in the presence of ethanol (Fig. 3) is expected due to the greater polarity of ethanol in relation to the hydrophobic chains of phospholipids. On the other hand, the increase in local micropolarity in the presence of limonene (Fig. 4) cannot be explained directly, and must be related to the formation of hydrophilic clusters or the penetration of water molecules into the lipid layers of vesicles (Nesterkina et al., 2022).

3.3 Instability events by the relative light scattering

UV-visible spectroscopy was considered for initial stability studies. Colloidal aggregates smaller than 800 nm scatter light in the UV-Vis region, an effect known as Rayleigh scattering. In this study, the absorption at 700 nm was evaluated as no chromophoric molecule in this region is found. Therefore, it is assumed that the absorption intensity is proportional to the sample scattering. Furthermore, the scattering intensity increases with increasing particle size. For pure PC vesicles and vesicles with ethanol and cholesterol, an intense reduction in absorption was observed in times shorter than 500 min due to precipitation of the vesicles. The precipitation effect can be observed visibly, which can be related to the high PDI values already reported (Table 2).

On the other hand, the vesicles showed to be stable for longer than 1000 min in the presence of limonene (Fig. 5A-B). A distinct effect was observed for PC vesicles in the presence of limonene and cholesterol (Fig. 5C-D), with a slight increase in absorption over time. For these systems, a kinetic of vesicle reorganization is found after a few hours, resulting in an increase in particle size (Table 3).

Interestingly, the presence of limonene and ethanol in the vesicles seems to play a synergistic effect on stability. The vesicle size over time is kept when this combination is considered (Fig. 5C). The analysis of size and PDI over time (after preparation, 24 h, 48 h, 72 h, 15 and 40 days) also confirmed this hypothesis (Table 3).

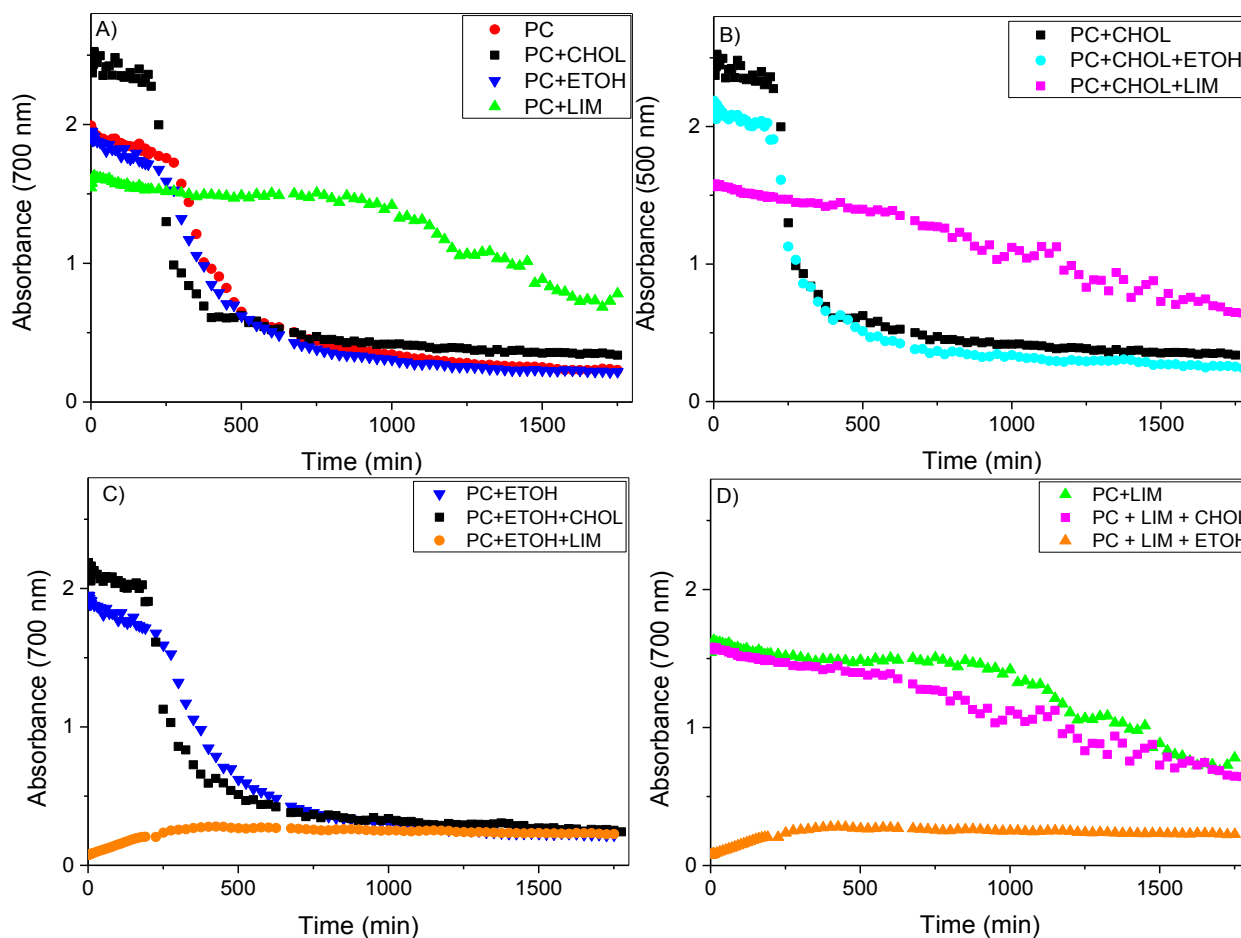


Fig. 5. Absorption kinetics at 700 nm for different colloidal suspensions (A-D). The concentration of limonene, ethanol and cholesterol was fixed at 0.5, 3 and 10%, respectively.

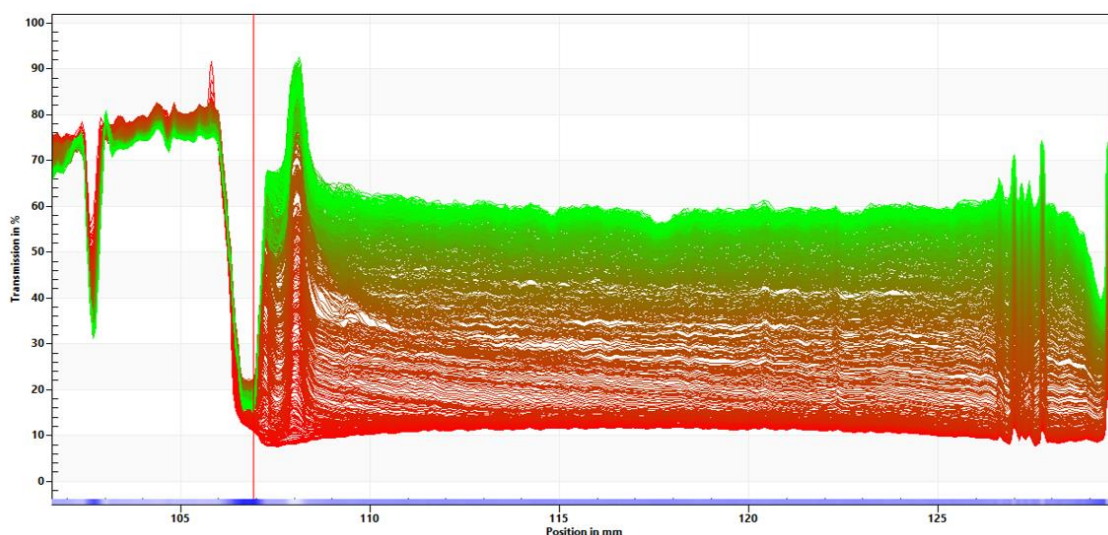
3.5. Accelerated stability analysis

As already mentioned, few stability studies by exploiting the equipment LUMiSizer® may be found in literature. On the other hand, this technique can be useful to discriminate more stable formulations in an initial screening after definition of a specific protocol. Optimal conditions for the assays were presented in the method section. Fig 6 illustrates the transmission profile of vesicles after changes in their composition. The red and green profiles represent transmissions detected in earlier and later time, respectively. Under the action of centrifugal force, vesicles migrate gradually and leads to density variations at different positions in the sample cell, which is reflected by transmission variations. The density of transmission profiles reflects the time required for

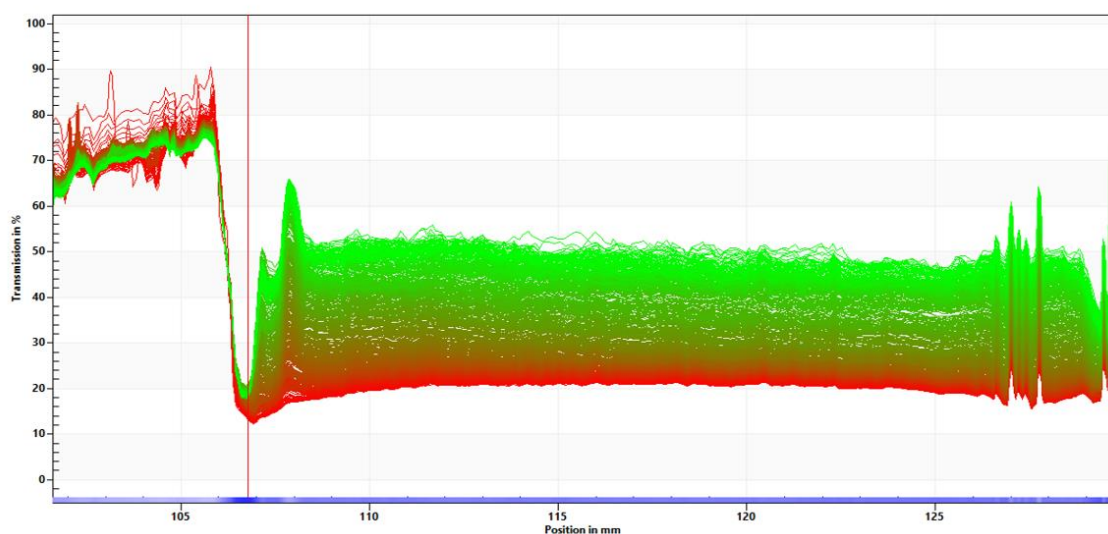
sample phase separation. More dense profile mean that more time is necessary for phase separation.

The addition of cholesterol led to a decrease in centrifugal stability while the addition of limonene, particularly in the presence of ethanol, had an opposite effect as already observed in previous analyses. PC vesicles only with limonene presented a lower instability index than the vesicles without limonene (0.40 vs. 0.62), suggesting a synergistic action between limonene and ethanol on stability. PC+LIM+ETOH was the most physically stable formulation. Vesicles with CHOL showed a slight improvement in dispersion stability (instability index=0.56) when compared to PC vesicles (instability index=0.62) (ANOVA/ Tukey's test, $p<0.05$).

A



B



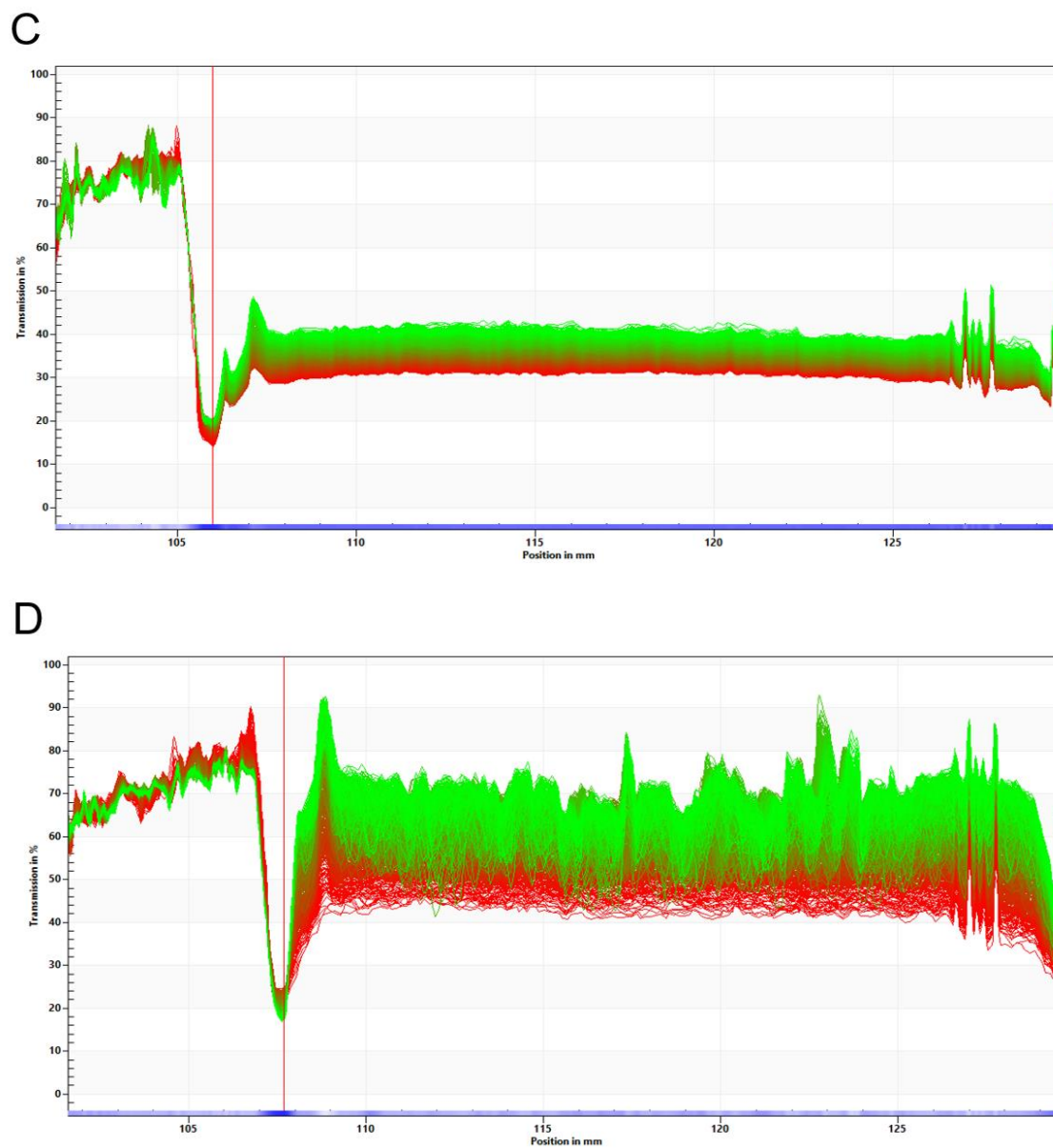


Fig. 6. Transmission profiles of PC (A); PC+LIM (B); PC+LIM+ETOH (C); PC+LIM+ETOH+CHOL (D) vesicle.

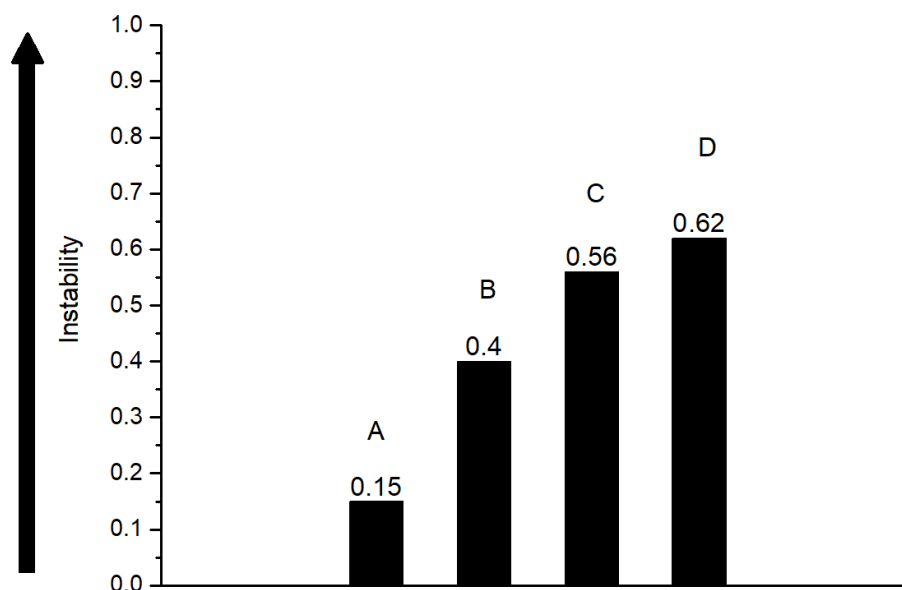


Fig. 7. Instability index of vesicles varying their constitution A) PC+LIM+ETOH; B) PC+LIM; C) PC+LIM+ETOH+CHOL; D) PC.

3.4 Long-term stability studies

The stability of invasomes composed of PC, ethanol and limonene was confirmed by size and PDI analyzes in different intervals. No significant changes in these parameters were found for different storage times (Table 3).

Table 3. Size and polydispersity index of formulation PC+LIM+ETOH over time.

	0	24 h	48 h	72 h	15 days	40 days
Size	161.8±16.4	183.1±9.2	180.4±3.0	165.4±14.7	189.1±6.8	193.4±13.6
PDI	0.4	0.3	0.3	0.3	0.3	0.4

$p > 0.05$

3.6. Vesicle-skin interaction studies

FTIR is a technique able to provide information on the disorder degree in skin protein and lipid domains. Asymmetric and symmetric CH vibrations attributed to SC lipids are found at 2,920 and 2,850 cm^{-1} , respectively (Vaddi et al., 2002). As the amount of lipids in the skin varies, the intensity of these bands varies proportionally. Lipid extraction by chemical absorption enhancers (CAEs) results in a decrease in band intensity (Levang et al., 1999). CAEs can also fluidize the SC lipids, which result in the

shift of CH₂ stretching bands to higher wavenumber values (*trans* to *gauche* conformation) and an increase in bandwidth (Golden et al., 1986; Suhonen et al., 1999). The amide I and II stretching vibrations of SC proteins are found at 1,650 and 1,550 cm⁻¹, respectively. The amide I band is the result of C=O stretching vibrations whereas the amide II band is related to C-N stretching and N-H bending vibrations. These two bands, particularly the amide I band, shift to higher or lower frequencies according to the change in protein conformation (Vaddi et al., 2002).

The FTIR spectra of SC treated with vesicles composed only of PC and ethanol did not show differences compared to the control (tissue in PBS buffer). As the ratio of cholesterol increased in the vesicles, a reduction in the intensity of lipid bands was observed. Similar findings were found after treatment with invasomes. Invasomes with CHOL also showed a reduction in the intensity of lipid bands compared to the control tissue, indicating a greater tissue disruption. Invasomes showed to be more effective to change de skin barrier properties than liposomes, suggesting that CHOL and limonene act synergistically. On the other hand, the amount of cholesterol seems to have a more significant impact on the lipid disorder than limonene (Fig. 8). No significant changes in polar domains were observed after different treatments.

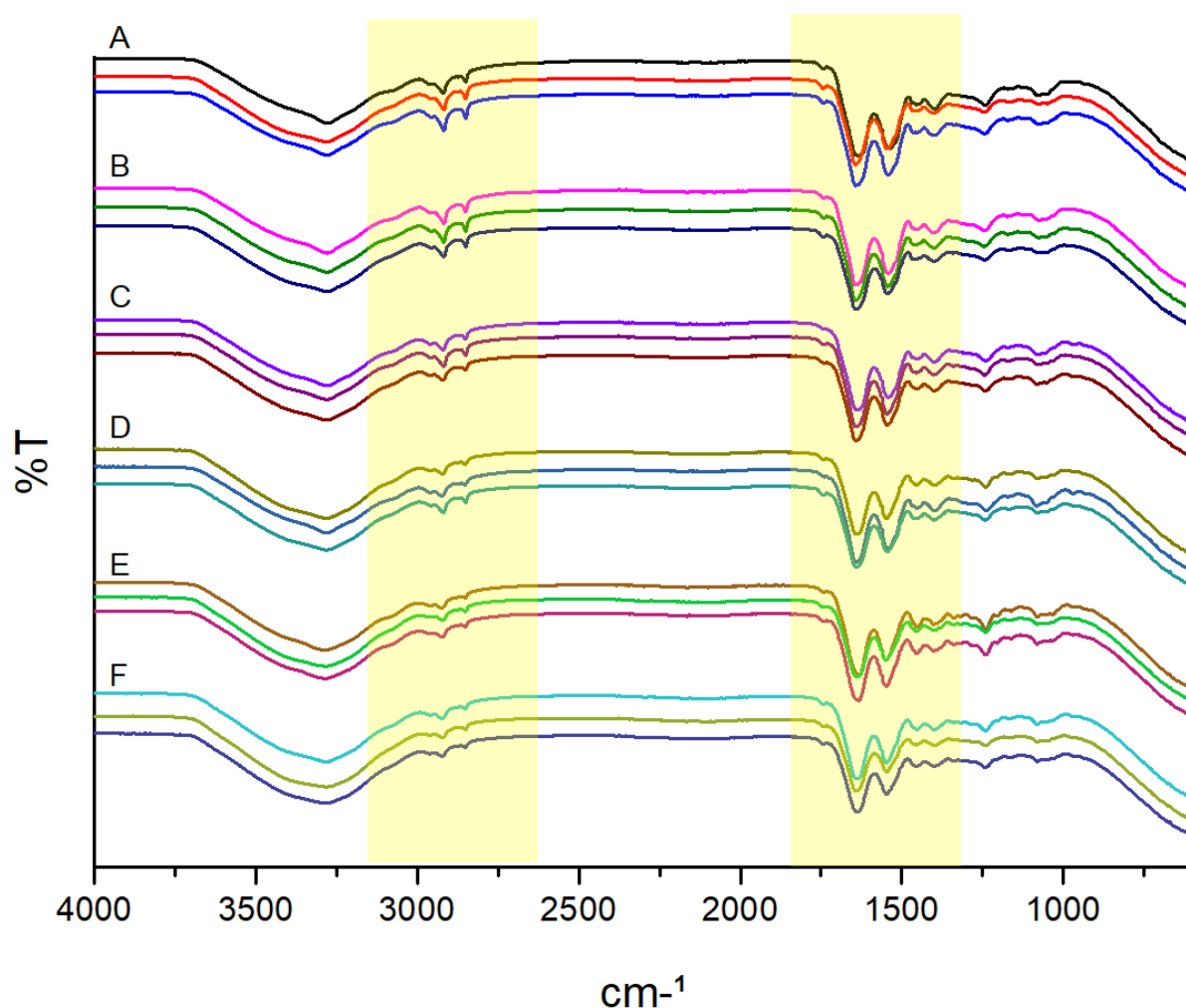


Fig. 8. FT-IR spectra of stratum corneum treated with liposomes and invasomes. PBS buffer (A); PC:CHOL 5:0+ETOH (B); PC:CHOL 5:0.5+ETOH (C); PC:CHOL 5:2+ETOH (D); IV:CHOL 5:0 (E); IV:CHOL 5:2 (F).

4. DISCUSSION

Invasomes have been shown to be effective in the topical delivery of a variety of drugs (Nangare and Dugam, 2020). In these systems, one or more terpenes are mixed with phospholipids and ethanol. The formation of these structures is mainly governed by nonpolar interactions between hydrocarbon chains of phospholipids (Mertins, 2008). Each new molecule added to the phospholipid membrane can affect the biophysical properties of vesicles, impacting biological effects. In this study, the effect of vesicle composition on stability and membrane fluidity was evaluated by considering light scattering and spectroscopic techniques. Human stratum corneum, the main skin barrier, was treated with colloidal suspensions with and without cholesterol and/or limonene to understand how these agents affect its lipid and protein domains. Conventional liposomes

were prepared as control formulations for comparison purposes. Limonene, the monoterpene selected for invasome preparation, was added into the organic phase due to its limited aqueous solubility. A solubilizing agent should be included in the formulation if the aqueous phase had been prioritized, which could result in mixed systems (e.g. mixed micelles, micelles and liposomes, etc.) instead of invasomes.

The presence of limonene was crucial to improving the stability of phosphatidylcholine (PC) vesicles. In fact, invasomas showed lower PDI values than conventional liposomes, indicating a more monodisperse population of particles. Limonene has known antioxidant activity (Akhavan-Mahdavi et al., 2022; Murali et al., 2013), which would contribute to reducing the oxidation of PC, a common event in liposomes. When ethanol is added to PC vesicles with limonene, even greater stability was achieved. The literature indicates that this agent can stabilize vesicles by electrostatic mechanisms when it increases the surface charge (Zhou et al., 2018), which was not observed in our study. The ethanol content used to prepare the vesicles would not be sufficient to modify this parameter. An increase in membrane rigidity could be the reason for the improved stability of vesicles prepared with ethanol and limonene. These agents interact with different regions of the phospholipid molecule (limonene interacts with hydrophobic region whereas ethanol with polar head), reducing the molecular mobility. As a consequence, degradation reactions would be minimized or even avoided as molecular reactive sites of PC would be less exposed.

Although cholesterol concentrations above 10% resulted in an increased vesicle rigidity, a marginal contribution of this agent on stability was observed. In accelerated stability studies, control vesicles (only PC) had a higher index of instability than colloidal suspensions containing PC and cholesterol. On the other hand, the presence of cholesterol in PC vesicles with limonene and ethanol had a negative impact on the stability. The competitive effects of cholesterol and limonene could explain these findings. Both molecules interact with the phospholipid hydrophobic acyl chain through hydrogen bonds and hydrophobic interactions due to their hydrophobic nature (Joshi et al., 2021; Nakhaei et al., 2021). In the presence of cholesterol, the acyl chains are further apart, weakening interactions of limonene with the acyl chain. As a consequence, these vesicles are more unstable than their cholesterol-free counterpart. A slight increase in the size of invasomes is observed when cholesterol is added, particularly in larger proportions, supporting this hypothesis.

Rigidity is one of the most important biophysical properties affecting the performance of liposomes as drug delivery systems. Plasma stability, drug release, hydrophobicity and cellular uptake may be adjusted by changing this parameter (Stern et al., 2017; Wu et al., 2019). Several studies have demonstrated that an increase in membrane rigidity provided by cholesterol contributes to greater plasma stability and cellular uptake (Bijsterbosch et al., 2000; Ran et al., 2013; Wang et al., 2010). Greater control of drug release from vesicles and encapsulation efficiency can be also achieved with more rigid liposomal vesicles (Liu et al., 2022). As is already known, cholesterol increases membrane rigidity only when used in larger proportions. In this study, an increase in membrane rigidity was achieved from a PC:CHOL ratio of 5:0.5. The effect of ethanol and limonene on the membrane fluidity was also evaluated. The presence of ethanol in PC vesicles reduced the membrane rigidity even in cholesterol-containing vesicles, an effect that has already been reported (Patra et al., 2006). Ethanol is preferentially accommodated in the vicinity of the lipid headgroup region (Holte and Gawrisch, 1997), causing a disorder in the polar domains of lipids. This fact can explain the increase in the vesicle particle observed after its addition. In fact, Prata et al. (2006) reported an increase in area per lipid after the addition of alcohols. A similar disorder effect would be expected in biological membranes (Gupta et al., 2020), which justify its application as an absorption enhancer. Ethanol has been often combined with absorption enhancers that act on lipid domains precisely to provide a greater disorder in the membrane by different action mechanisms (Kumar et al., 2022). Although the presence of limonene lead to a reduction in the membrane rigidity in vesicles when few constituents were considered, an opposite effect was observed for systems composed of PC, CHOL, ethanol and limonene (basic constituents of invasomes).

Interestingly, the presence of cholesterol in both liposomes and invasomas contributed to increasing the degree of disorder in lipid domains of human SC even at low concentrations of this agent. The higher the proportion of cholesterol in the vesicles, the greater the degree of lipid disorder, a concentration-dependent effect. When proportions between 20 and 40% of cholesterol are present in a bilayer of phospholipids, the formation of called '*rafts*' is reported. These structures are gigantic agglomerations of cholesterol molecules, which form '*clusters*' navigating through the phospholipid bilayer, affecting the entire dynamics present in the membrane (Bennett et al., 2009). Previous studies also showed that the addition of at least 20% of cholesterol (5:2 ratio) in liposomes significantly increases the transport of molecules through the skin (Coderch et al., 2000).

Limonene, as already reported in the literature, also contributed to an increased lipid disorder. This monoterpene acts as a spacer in lipid packaging, increasing the disorder in more ordered lipid domains (Mendanha et al., 2017)

5. CONCLUSION

The biophysical properties of highly organized lipid membranes showed to be strongly affected by the addition of new molecules. Cholesterol acts by modifying membrane fluidity, leading to an increased membrane rigidity at concentrations above 10% (w/w). No positive effect on the stability of the vesicles was achieved with this agent and high PDI values were found. On the other hand, cholesterol increased the degree of lipid disorder in a concentration-dependent manner. Another benefit achieved with this agent was an increase in the degree of lipid disorder of the stratum corneum, which would facilitate the transport of drugs incorporated in these vesicles. The addition of limonene, in turn, reduced the PDI values of phosphatidylcholine vesicles and thus it was crucial to improve the stability of the vesicles. This agent also improved the interaction of vesicles with the stratum corneum, particularly lipid domains. Low concentrations of ethanol (3% w/v) led to an increase in vesicle size and decreased the membrane rigidity even in the presence of cholesterol.

In the light of these findings, it is possible to state that the inclusion of new molecules into the phosphatidylcholine vesicles results in important changes in their characteristics, affecting both stability and skin interaction. Therefore, the incorporation of molecules into liposomal membranes should be performed be carried out with caution in order to achieve the desired effect (e.g. high permeation rate, sustained drug release, increased stability).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We kindly thank the Brazilian governmental agencies CNPq/MCTI and CAPES/MEC for financial support for this project and student scholarship, respectively. A.P.G. thanks

INCT-Catálise, FAPESC (TO 2019TR0847) and CNPq (427008/2018-2) for the financial support. A.P.G. thanks the CNPq for her research productivity fellowship 312029/2021-7. I.V.N.C. and T.C. thank the company Lipid (Campinas) for the phosphatidylcholine (Lipoid S100) donation.

CRedit author statement

Izi Vieira Nunes Cunha: Conceptualization, Methodology, Writing - original draft, Investigation, Data curation; **Angela Machado Campos:** Methodology, Data curation; **Adriana Passarella Gerola:** Investigation, Data curation, Review & Editing; **Thiago Caon:** Conceptualization, Supervision, Methodology, Writing - review & editing, Project administration, Funding acquisition.

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6 CAPÍTULO IV – DESENVOLVIMENTO DE INVASOMAS DE APIGENINA COM POTENCIAL ANTI-MELANOMA

Neste capítulo, a APG foi incorporada em lipossomas e invasomas, realizando-se todos os ensaios de caracterização mencionados anteriormente bem como a avaliação da eficiência de encapsulação e da permeação/retenção cutânea. A APG é um flavonoide com ação antitumoral multidirecionada que também tem demonstrado alta seletividade a células tumorais (importante para redução de efeitos colaterais), razões que motivaram a sua escolha para a incorporação nestes nanocarreadores. Com os invasomas, espera-se alcançar as camadas mais profundas da pele, acessando o sítio tumoral mais facilmente. O produto deste capítulo é um artigo científico, que será submetido para apreciação no periódico *International Journal of Pharmaceutics*.

DEVELOPMENT OF APIGENIN-LOADED INVASOMES WITH ANTI-MELANOMA POTENTIAL

Izi Vieira Nunes Cunha^a, Ingrid Vicente Farias^a, Debora Fretes Argenta^a, Adriana Passarella Gerola^b, Angela Machado Campos^a, Thiago Caon^a

^aDepartment of Pharmaceutical Sciences, ^bDepartment of Chemistry; Federal University of Santa Catarina, Florianopolis, 88040-900, SC, Brazil

[#]Corresponding author: caon.thiago@ufsc.br; thiagocaon@gmail.com.

ABSTRACT

The apigenin (APG) is a plant-derived flavonoid that have received increasing attention as potential anti-melanoma molecule due to its action in multiple signaling pathways and high selectivity to cancer cells. It is able of modulating cell proliferation, apoptosis, cell cycle arrest, invasion and metastasis. On the other hand, this flavonoid is characterized by low aqueous solubility and pharmacokinetic problems such as high metabolism rate association with plasma proteins and slow oral absorption. As an approach to overcome these limitations, it was incorporated into invasomes, which are phospholipids vesicles able of reaching deeper regions of the skin due to the presence of one or more terpene in their composition as well as small amounts of ethanol. When compared to conventional liposomes, invasomes showed to be more stable, deformable and able of interacting more effectively with the skin non-polar and polar domains. The presence of limonene, which was the terpene selected for the vesicle composition because of its already reported anti-melanoma action, was crucial to enhance both permeation and retention of APG in the skin. Cholesterol also led to slightly improving the transport of APG in the skin. Taking into account the improved distribution of APG in basal layer of the epidermis (tumor site) and advantages in terms of stability and membrane flexibility, invasomes can be considered promising colloidal carriers for the administration of anti-melanoma agents.

Keywords: invasome; apigenin; melanoma; rigidity; stability; skin interaction.

1. INTRODUCTION

Melanoma is a malignant tumor of melanocytes, which are cells derived from the neural crest and predominantly found in the basal layer of the epidermis. Under physiological conditions, melanocytes are responsible for producing and transferring a dark pigment called melanin to mitotically active keratinocytes. The transferred melanin is concentrated in the perinuclear space of keratinocytes and protects the nucleus from UV radiation damages (Tuong et al., 2012). The pathogenesis of melanoma is complex and has not yet been fully elucidated. It involves interactions between environmental factors, accumulation of sequential genetic alterations, activation of oncogenes, inactivation of tumor suppressor genes, and impaired DNA repair (Leonardi et al., 2018).

Although melanoma is the least common type of skin cancer, it is responsible for approximately 80% of skin cancer-related deaths. In the last update (2012-2018), the National Cancer Institute estimated 99.5% survival for patients diagnosed in early stage (I - II), reducing to 70.6% in stage III and 31.9% in stage IV (NCI, 2022).

Despite the approval of new therapeutic options in the last years, a disease recurrence has been observed in all patients within a period of 9 months (Flaherty, 2010; Flaherty et al., 2010). Targeted therapies and immunotherapy show greater selectivity, but development of tumor resistance and excessive stimulation of the immune system can occur (Berning et al., 2019; Spain et al., 2016). Moreover, targeted therapies are restricted to melanoma patients presenting a BRAF V600E mutation (Hong and Ahn, 2017). Therefore, the investigation of new compounds that help in the treatment of melanoma or studies with combinations of different drugs is extremely relevant (Liu et al., 2019).

Several studies have demonstrated that flavonoids can play a role as modulators of several signaling pathways of cell growth and proliferation as well as in the reduction of reactive oxygen species (ROS) (Asensi et al., 2011; Briguglio et al., 2020; Katiyar, 2011; Nichols and Katiyar, 2010). In addition, these compounds stimulate DNA repair and are able of absorbing UV radiation (Souto et al., 2019). The apigenin (APG), chemically known as 4,5,7,-trihydroxyflavone, is a bioactive compound of this class. In its native form, it is found in vegetables and fruits such as parsley, onions, oranges, chamomile tea, wheat sprouts and other spices (Jeetah et al., 2014; Rajendran et al., 2015). It has attracted interest due to pharmacological properties such as anti-inflammatory, antioxidant, anti-proliferation activity, changes in cellular signaling and apoptosis induction, which suggest an application potential in the cancer (Jiang et al., 2017; Pápay

et al., 2015). APG has been considered in skin, colon, prostate, oral, breast, lung, hepatic, colorectal and pancreatic cancer (Chinembiri et al., 2014; Das et al., 2013; Qin et al., 2016; Sung et al., 2016). In the context of melanoma, APG seems to be effective against tumor cells by inducing apoptosis, cell cycle arrest or even affecting several pathways related to the survival and progression of tumor cells (Woo et al., 2020).

Although the APG is widely distributed in several dietary sources and the daily intake is relatively high, low plasma concentrations are found after an oral administration (Wang et al., 2013). Moreover, its efficacy is limited due to its low aqueous solubility, which leads to poor intestinal absorption (Pápay et al., 2015). A topical administration as well as the nanotechnology can be alternatively considered to overcome these limitations. Considering the barrier properties of the skin and solid tumor, flexible colloidal systems or composed of chemical penetration enhancer represent a promising alternative. In this study, invasomes were purposed for the incorporation of apigenin. These systems are composed of phosphatidylcholine, ethanol, and one or more terpenes. These last agents play a synergistic action, enhancing the percutaneous penetration of drugs compared to the conventional liposomes (Chen et al., 2011; El-Tokhy et al., 2021).

Different mechanisms of invasome-skin interaction have been reported in the literature. A hypothesis suggests that vesicles disintegrate on the skin surface and then their constituents disrupt skin intercellular lipid lamellae, leading to the formation of channel-like penetration pathways (Chen et al., 2011; Dragicevic-curic et al., 2008; Honeywell-Nguyen et al., 2004; Ota et al., 2003; Vaddi et al., 2002). Another well accepted hypothesis proposes that small intact vesicles penetrate the stratum corneum via transepidermal osmotic gradient, requiring elastic/flexible characteristic for the vesicles (Cevc et al., 2002; Cevc and Blume, 1992; Honeywell-Nguyen et al., 2004).

Although the flexibility of membranes can enhance the percutaneous penetration of drugs, vesicle aggregation and formation of giant onion-like multilamellar structures are often observed (Carreras et al., 2020; Coldren et al., 2006; Romero and Morilla, 2015). Temperature changes during formulation storage make these instability phenomena even more common (Hussain et al., 2017). Therefore, the addition of cholesterol has been alternatively considered. Its incorporation into phospholipid-based systems increases the stability and encapsulation efficiency of drugs (Abdulbaqi et al., 2016). The presence of cholesterol in liposomes impacts on fluidity, permeation, membrane strength, elasticity, lipid transition temperature, phospholipid packing, and plasma stability (Ermilova and Lyubartsev, 2019; Ohvo-Rekilä et al., 2002; Song et al., 2022).

Another reason for prioritization of invasomes is that the limonene, the terpene selected for system composition, has already demonstrated an anti-melanoma activity (Alipanah et al., 2021; Vandresen et al., 2014). In fact, it is able of inhibiting tumor initiation, growth, angiogenesis as well as cell apoptosis (Araújo-Filho et al., 2021; Vasconcelos et al., 2020). In addition, this terpene has shown to inhibit metastasis through downregulation of VEGF expression, which is associated with angiogenesis, and matrix metalloproteinases, which are related to tissue invasion processes (Pratheeshkumar et al., 2012). In phase I clinical trials, toxicity events were not reported, suggesting that this compound is very well tolerated (Yu et al., 2018).

Therefore, a synergistic or additive effect of APG and limonene is expected for invasomes. In this study, liposomes and invasomes with and without APG and/or CHOL were prepared by thin-film hydration technique aiming to understand the effect of these constituents on the vesicle stability and rigidity, interaction with skin lipid and protein domains as well as retention in the dermis and epidermis.

2. MATERIAL AND METHODS

2.1. Materials

Lipoid S100, non-hydrogenated soybean phosphatidylcholine ($PC \geq 94.0\%$) was kindly donated from Lipoid GmbH (Germany). Cholesterol ($CHOL \geq 99\%$) and limonene were purchased from Sigma-Aldrich (Brazil). Ethanol and chloroform were obtained from Vetec Química Fina Ltda. (Duque de Caxias, Brazil). Apigenin 98% (*Matricaria* sp.), in its aglycone form, was purchased from Active Caldic (Palhoça, Brazil). Phosphate buffered (PBS) saline was prepared with 10.1 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 136.9 mM NaCl, 2.7 mM KCl and adjusted to pH 7.4 with 1 M HCl. Krebs-Ringer bicarbonate buffer was prepared with 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4 , 25 mM $NaHCO_3$, 2.5 mM $CaCl_2 \cdot 2H_2O$, 1.0 mM $MgCl_2 \cdot 6H_2O$, 5.5 mM glucose and adjusted to pH 7.4 with 1 M HCl. Ultrapure water was obtained from a Milli-Q® purification system (Millipore, Milford, MA, USA).

2.2. Preparation of invasomes

Liposomes and invasomes were prepared by the thin-film evaporation method and sonication (Dragicevic-Curic et al., 2008b) (Table 1). Briefly, PC was mixed with CHOL in the ratio of 5:0.5 (w/w) (CHOL proportion that affect the membrane properties), which were dissolved in chloroform. For invasome preparation, limonene was added to the solution containing PC and CHOL previously solubilized in chloroform. A lipid film was obtained after the solvent elimination in a rotary evaporator at 50°C, 150 rpm and 206 mbar. The film was then rehydrated with 4 mL of PBS buffer containing ethanol 3% (w/v) for 40 min and vortexed for 2 min. Finally, a probe sonicator (UP200S Ultrasonic Processors, Hielscher Ultrasonics GmbH, Germany) was used for reducing the liposome size. The vesicles were sonicated considering pulses of 8 s on and 2 s off (total time = 6 min), an amplitude of 40%, 200 W in an ice water bath. The formulations were stored in glass flasks protected from light at room temperature.

Table 1. Composition of the vesicle

Formulation	Phosphatidylcholine	Cholesterol	Ethanol	Limonene	Apigenin
Liposome (PC)	2 mM	-	3%	-	-
APG Liposome (PC+APG)	2 mM	-	3%	-	100 µg/mL
Invasome (IV)	2 mM	-	3%	0.5%	-
APG Invasome (IV+APG)	2 mM	-	3%	0.5%	100 µg/mL
Invasome+Cholesterol (IV+CHOL)	2 mM	0.4 mM	3%	0.5%	-
APG+CHOL Invasome (IV+CHOL+APG)	2 mM	0.4 mM	3%	0.5%	7 /mL

2.3. Particle characterization

2.3.1. Particle size, polydispersity index and zeta potential

The mean droplet size and polydispersity index (PDI) of colloidal suspensions were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK). Analyses were performed at 25 °C by selecting 90° as the scattering angle. Potential zeta (ZP) values, in turn, were obtained from electrophoretic mobility measurements without any dilution of samples. All analyses were performed in triplicate.

2.3.2. Encapsulation Efficiency

Encapsulation efficiency (EE) was determined through the ultracentrifugation technique using Amicon® Ultra-0.5 30K devices (Millipore, Billerica, MA, US). Briefly, 1 mL of the formulation was added to ultrafiltration units and then centrifuged at 6,200 rpm for 10 min at 25°C. The amount of free APG in the supernatant was determined by HPLC (section 2.9). The EE (%) of APG was calculated by using the Equation 1.

Equation 1.

$$EE\% = \frac{(Total\ amount\ of\ APG - amount\ of\ free\ APG) \times 100}{Total\ amount\ of\ APG}$$

2.4. Fluorescence spectroscopy

For the evaluation of membrane rigidity, the method reported by Chwastek *et al.* (2020) was considered. The colloidal suspension was mixed with a viscosity-sensitive fluorescent dye 9-(2,2-dicyanovinyl)julolidine (DCVJ) in mass ratio of 100:1 and simultaneous measurements of absorbance and fluorescence were performed in a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Australia). Fluorescence was excited at 440 nm using a xenon flash lamp as an excitation source, and the emission spectra in the range of 460-650 nm were analyzed. The probe used (DCVJ) is a well characterized TICT probe with the fluorescence emission in the visible range of the light spectrum. The photophysical properties of DCVJ are sensitive to the viscosity of the medium and thus it can be used to estimate the viscosity in lipid membranes. In the singlet excited state, the molecule undergoes rapid rotation around the single bond that connects the julolidene and dicyanovinyl subunits, acting as a molecular rotor. Given that internal rotation and fluorescence emission are competitive processes, in non-viscous solvents and in the excited singlet state, internal rotation is preferred and fluorescence emission is reduced. Therefore, the DCVJ is weakly fluorescent marker in a fluid solution whereas the increase in the viscosity of the medium makes this internal rotation more difficult, favoring the deactivation of the excited state by fluorescence emission (Jee *et al.*, 2009). For the evaluation of membrane viscosity (i.e., rigidity) of liposomes and invasomes, the

emission intensity of DCVJ incorporated into liposomes/invasomes was compared to that of a solvent of known viscosity (methanol).

2.5. *Stability studies*

2.5.1. *UV-visible spectroscopy*

Instability events were monitored by UV-visible spectroscopy at 600 nm (Cary® 50 UV-Vis, Varian, Australia). Immediately after preparation, approximately 2 mL of each colloidal suspension was analyzed in a spectrophotometer at intervals of 5, 30 and 60 min for 33 h. The technique measures the changes in light scattered (turbidimetry) or absorbed (absorbance) by the vesicles. Turbidimetry is a well-established method for the evaluation of colloidal aggregation, particularly micron-sized particles. In this technique, the signal changes are measured over time and indicate changes in the particle volume fraction and size, which translates into instability events such as aggregation or sedimentation (Ray et al., 2015).

2.5.2. *Accelerated stability studies*

Accelerated stability of the vesicles was evaluated by the equipment LUMiSizer (LUM GmbH, Berlin, Germany). In this technique, a centrifugal sedimentation is applied in colloidal dispersions, which allows to obtain a stability ranking of dispersions. The centrifugal force accelerates the occurrence of instability phenomena such as sedimentation, flocculation and creaming. The technique measures the intensity of transmitted light as a function of time and position over the entire sample length in a cuvette during centrifugation. For this analysis, samples were placed in rectangular polycarbonate cuvettes (optical path of 2.0 mm) and centrifuged at 450 rpm for 2 h at 25°C to capture 750 transmission profiles at an interval of 10s.

Stable colloidal dispersions show the formation of a flatbed under a centrifugal field while aggregated particles usually show a step-profile. Differences on the particle size explain variations in sedimentation velocity. The red and green profiles represent transmissions detected in earlier and later time, respectively. Under the action of a centrifugal force, vesicles migrate gradually and leads to density variations at different positions in the sample cell, which is reflected by transmission variations. The density of transmission profiles reflects the time required for sample phase separation. More dense

profiles indicate that longer intervals are required for phase separation (Caddeo et al., 2013).

The instability index, a factor calculated by the software SEPView[®], was used to compare the stability of developed colloidal suspensions. The value “0” indicates no separation or change in light transmission whereas “1” represents a complete segregation of phases.

2.5.3. Storage stability studies

The storage stability of vesicles was evaluated by considering measurements of size and PDI in different time intervals (immediately after preparation and after 24 h, 48 h, 72 h, and 15 days of preparation). The vesicles were stored in glass flasks protected from light at room temperature. Measurements were performed by DLS using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK) as described above.

2.6.1 Ex vivo skin permeation studies

The cutaneous permeation/retention assays of vesicles were performed in Franz-type diffusion cells presenting a diffusion area of 1.77 cm². The human skin was initially collected from abdominoplasty surgeries and stored in Krebs-Ringer buffer (pH 7.4) until tissue preparation. The study was approved by the Research Ethics Committee of the University (CAE number: 87349418.7.0000.0121). Subcutaneous fat was completely removed using a scalpel and the tissue of interest was washed with Krebs-Ringer buffer (pH 7.4) before storage at -20 °C. Before permeation assays, the frozen tissue was equilibrated with PBS pH 7.4 for 30 min considering that hydration level of tissue can affect the permeation rate of molecules. The skin was mounted at the interface of the donor and receptor chamber with the stratum corneum facing upwards. The receptor chamber was filled with 10 mL of phosphate buffer containing 30 % ethanol to maintain the sink condition. After adding 2 mL of each formulation (200 µg/mL) in the donor chamber, the system was kept at 37 °C under continuous stirring at 270 rpm. After the end of the permeation assay (24 h), aliquots of 400 µL were withdrawn from the receptor chamber and the amount of APG retained in skin layers (dermis and epidermis) was also analyzed. The excess of formulation on the skin was removed and the epidermis was separated from the dermis using a scalpel. APG was extracted from the tissues with 3 mL of acetonitrile, followed by overnight storage to increase the extraction efficiency. In the

next day, the tissues were vortexed for 10 min, sonicated for 30 min, centrifuged for 30 min at 6,200 rpm and finally the supernatant was filtered and analyzed by HPLC.

2.6.2. *AT-IR analysis of stratum corneum*

The stratum corneum (SC) was carefully separated from other underlying skin layers with a scalpel and divided into sections of approximately 20 mg. The tissues were then submerged in 4 mL of each formulation (PC, PC:CHOL 5:0.5, PC:CHOL 5:2, IV, IV:CHOL 5:2) or PBS (control) for 24 h. FT-IR spectra were recorded in a frequency range from 600 to 4,000 cm^{-1} on a Perkin-Elmer Frontier equipment (Waltham, USA) with the collection of 16 scans at a resolution of 4 cm^{-1} . Reduction or displacement of spectral bands indicate disorders in lipid or protein domains of the skin. Results were expressed as the mean spectrum of three different samples for each treatment.

2.9. *HPLC analyses*

The quantification of APG found in formulations and permeation studies was performed in a Perkin-Elmer Series 200 HPLC system (Waltham, MA, US) equipped with a UV detector, pump, autoinjector and online degasser. A C18 column (250 × 4.6 mm, 5 μm particle size; Perkin Elmer, Shelton, USA) linked to a guard column of the same packing material (KJ0-4282, Phenomenex, Torrance, CA, USA) was considered as a stationary phase. The mobile phase was composed of methanol and water acidified with formic acid (pH 5.5) in a ratio of 70:30 (v/v), respectively. Elution was performed in an isocratic mode at a flow rate of 1.0 mL min^{-1} with a sample injection volume of 20 μL and a total sample acquisition time of 10 min. The wavelength of the detector was set to 320 nm. The method showed to be linear in the range 0.5 to 100 $\mu\text{g mL}^{-1}$ ($y = 32741x - 38641$, $R^2=0.9977$); precise (intraday and interday relative standard deviations lower than 2.5 and 3%, respectively), accurate (recovery values higher than 98%) and specific.

2.10. *Statistical analysis*

Results were analyzed by using GraphPad Prism version 8 software (San Diego, CA). One-way ANOVA with post-hoc Tukey was considered for multiple comparisons. p -values lower than 0.05 indicated significant statistic difference among the groups.

3. RESULTS

3.1. Formulation characterization

3.1.1. Particle size, Pdl, surface charge and encapsulation efficiency

The liposomes containing only PC did not show uniform vesicle size distribution (high PDI values), which makes difficult to analyze the effect of APG on the vesicle size.

For invasomes with and without CHOL, the presence of APG led a slight increase in vesicle size, suggesting that this active compound is distributed between the phospholipid chains. Pawlikowska-Pawlęga et al. (2013) also observed that the addition of APG leads to an increase in vesicle size. When the CHOL was added, a larger vesicle size was observed, which can be associated with its distribution into the hydrophobic chain of lipids and large molecular volume. Limonene, in turn, did not impact in vesicle size; however, it resulted in more uniform systems given that lower Pdl values were obtained. Dragicevic-Curic et al. (2008) also found a reduction in Pdl when the amount of terpenes ranged from 0.5 to 1% (Dragicevic-Curic et al., 2008a).

Encapsulation efficiency (EE) values higher than 98% were found for all vesicles, suggesting a high affinity of APG by phosphatidylcholine vesicles as already reported in other studies (Banerjee et al., 2017; Sen et al., 2019). APG is able of interacting with both polar and hydrophobic sites of phospholipids via hydrogen bonds and/or hydrophobic interactions (Pawlikowska-Pawlęga et al., 2013), which explain the high EE values even in presence of other agents.

3.2. Evaluation of the biophysical properties of the membrane

Table 2. Size, polydispersity index and zeta potential of liposomes and invasomes.

Formulation	Size	PdI	Zeta Potential (mV)
Liposome (PC)	245.7 ± 6.0	0.8	-1.0
APG Liposome (PC+APG)	155.7 ± 18.2	0.4	-1.5
Invasome (IV)	153.8 ± 17.6	0.4	-0.6
APG Invasome (IV+APG)	157.4 ± 8.7	0.4	-1.1
Invasome+Cholesterol (IV+CHOL)	175.0 ± 0.5	0.3	-3.4
APG+CHOL Invasome (IV+CHOL+APG)	183.7 ± 10.9	0.3	-1.5

(n=3; mean ± S.D.)

3.2.1 Membrane rigidity

The relative brightness of DCVJ incorporated into liposomes/invasomes was compared to that of solvents of known viscosity to evaluate the degree of vesicle rigidity. In methanol, which presents high fluidity, a low intensity of DCVJ emission was found (Fig. 1A), indicating that the deactivation of the excited state occurs by internal rotation.

For the liposomes, the emission intensity of DCVJ increased significantly due to its incorporation into the liposome rigid membrane. In this situation, the microviscous environment prevents the deactivation by internal rotation. The incorporation of APG into liposomes (Fig. 1 A-B) reduced the fluorescence and then membrane rigidity even in the presence of CHOL. Similar findings were found when other fluorescent probes such as DPH (1,6-diphenyl-1,3,5-hexatriene) (Dwiecki et al., 2023) and Laurdan (6-lauroyl-N,N-dimethyl-2-naphthylamine) (Veiko et al., 2023) were considered. DPH is hydrophobic molecule located in the acyl chains of lipid membranes (Dwiecki et al., 2023) whereas the Laurdan is preferentially accommodated at the level of the phospholipid glycerin moiety of the membrane (Veiko et al., 2023). In both studies, the APG led a transition of the membrane from a solid ordered phase into a more disordered phase. Given that APG is able to cause changes in fluorescence intensity from these two probes, an interaction with both polar and hydrophobic domains are expected. In fact, the molecule is composed of chemical groups able of establishing hydrogen bonds (hydroxyl groups) and hydrophobic interactions (hydrocarbon rings).

Interestingly, the incorporation of APG into invasomes, which are recognizably more flexible vesicles, has an opposite effect, increasing membrane rigidity (Fig. 1C). In this new situation, APG would reduce the molecular mobility of lipids. In the presence of CHOL, no significant difference in vesicle rigidity was found after addition of APG (Fig. 1D).

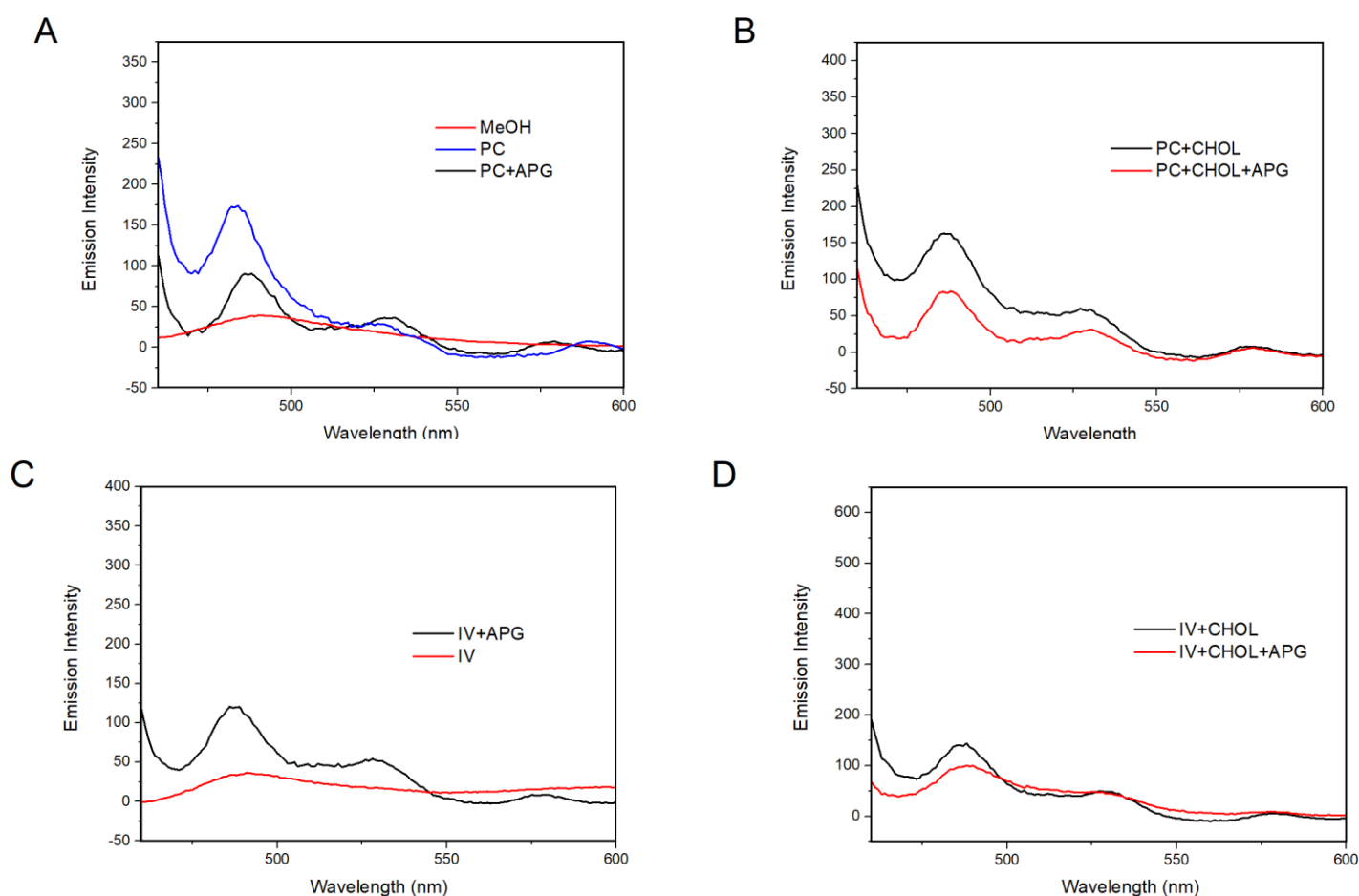


Figure 1. Fluorescence emission spectra of DCVJ incorporated into liposomes and invasomes. $\lambda_{\text{exc}} = 440$ nm.

3.3. Stability studies

3.3.1. UV-visible spectroscopy

UV-visible spectroscopy was considered for initial stability studies based on the Rayleigh scattering effect, where aggregates smaller than 800 nm scatter light in the UV-Vis region. In this study, the absorption at 700 nm was evaluated as no chromophores able of absorbing in this region are found. Therefore, it is assumed that the intensity of absorption is proportional to the scattering of the sample. In other words, the intensity of scattering increases proportionally with increasing particle size.

APG liposomes with and without CHOL (Fig. 2 A-B) showed a significant reduction in the absorption in a shorter time due to precipitation events. The presence of

aggregates was macroscopically observed, which is also supported by high PDI values already reported (Table 1). Invasomes, in turn, showed to be more stable than liposomes (Fig. 2C). The incorporation of CHOL into invasomes without APG negatively affected the stability, showing a rapid reduction in UV absorption intensity. CHOL invasomes with APG, in turn, showed an increased stability (Fig. 2D). APG could approximate the phospholipid chains through the formation of hydrogen and hydrophobic bonds given the increased stability and rigidity (reported in the previous section) of vesicles.

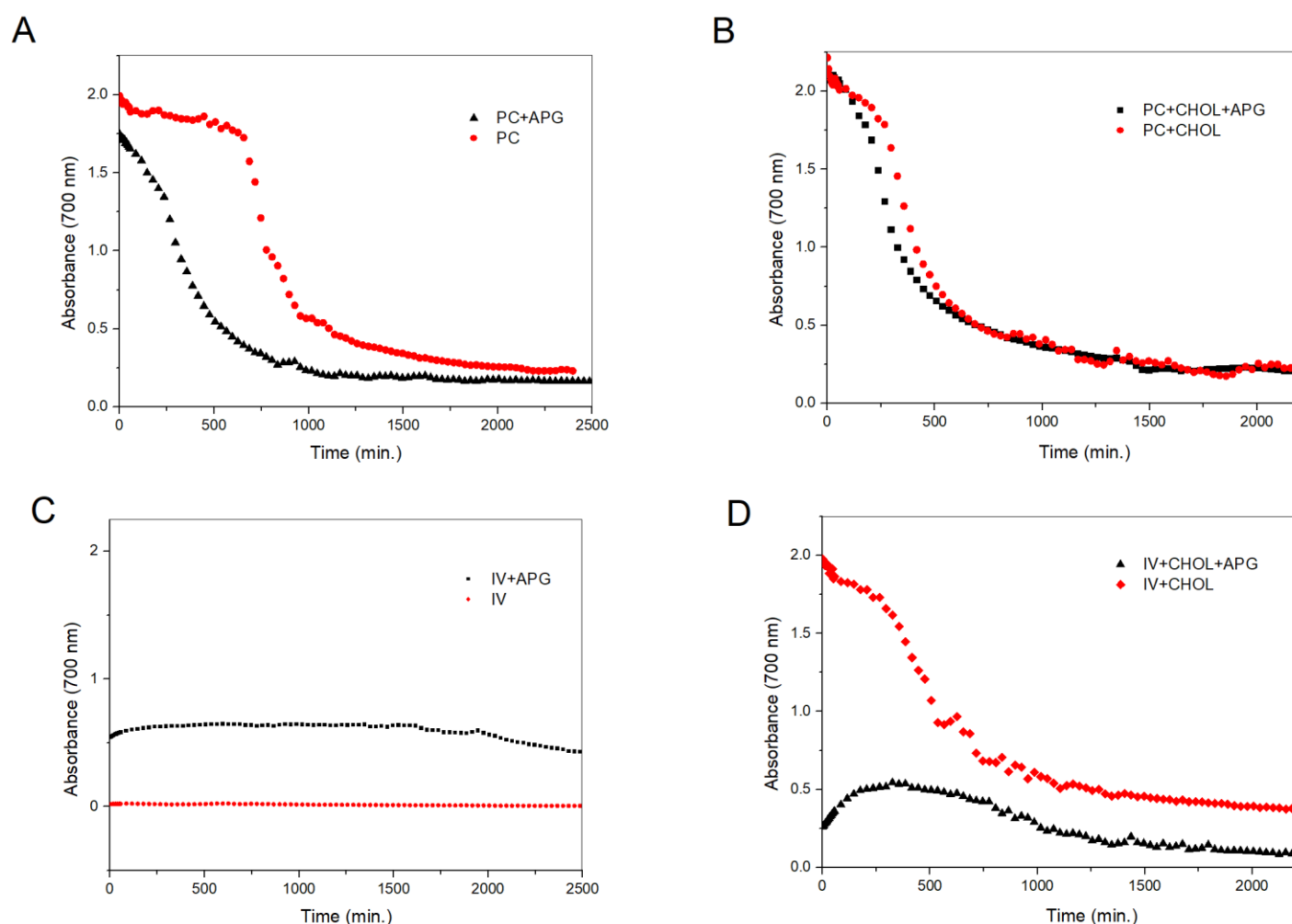


Figure 2. UV-absorption kinetics at 700 nm for different colloidal suspensions (A-D).

3.3.2. Accelerated stability studies

Liposomes showed less stability to centrifugation than invasomes. The addition of APG led to an increased stability in liposomes, but it had a negative effect on stability of invasomes without CHOL. On the other hand, invasomes with CHOL and APG

showed the highest stability to centrifugation. The presence of two molecules in a same system could increase the number of chemical interactions with phospholipids, reducing the lipid molecular mobility and thus an improved vesicle stability would be observed.

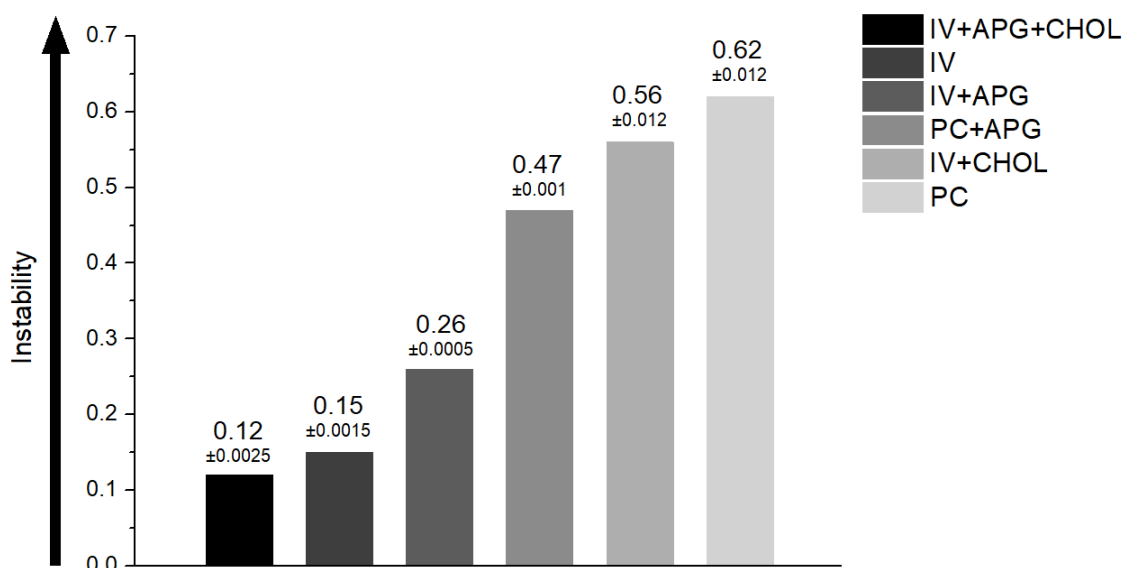


Figure 3. Instability index of vesicles obtained from LUMiSizer® analyses (n=4; mean ± S.D.).

3.3.3. Storage stability studies

Storage stability studies of APG invasomes were also performed by analyzing the size and PDI over different time periods. No significant changes in these parameters were found for different storage times at room conditions (Table 3).

Table 3. Size and polydispersity index of formulation IV+APG over time.

	0 h	24 h	48 h	72 h	15 days
Size	157.9±8.7	197.6±19.3	177.1±12.2	175.9±15.5	207.7±15.4
PDI	0.4±0.1	0.5±0.1	0.5±0.1	0.5±0.1	0.5±0.1

$p > 0.05$

3.4 Ex vivo skin permeation studies

The effect of limonene and CHOL on the permeation and retention of APG through the human skin was also evaluated. The assays were performed in a receptor fluid

composed of PBS (pH 7.4) and ethanol (70:30 v/v) to maintain the sink condition. The long experimental time was defined based on other literature studies (Adnan et al., 2023; Jangdey et al., 2017). APG+CHOL liposomes, APG invasomes and APG+CHOL invasomes led to an amount of APG permeated when compared to liposomes containing only APG (Fig. 4). Both CHOL and limonene positively impacted on the transport rate of APG in human skin, but a more significant was found for the limonene.

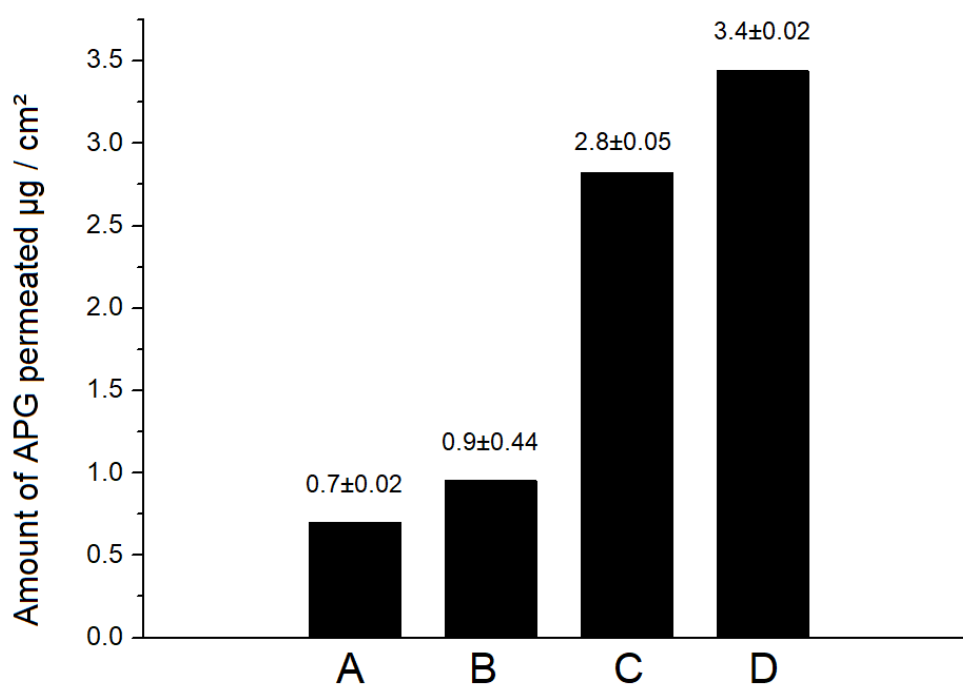


Figure 4. Amount of APG permeated after 24 h. APG liposomes (A), APG+CHOL liposomes (B), APG invasomes (C) and APG+CHOL invasomes (D) ($n=4$).

After 24 h, the amount of APG retained in epidermis was 2.29 ± 0.25 , 2.14 ± 0.04 , 8.26 ± 0.14 , 10.24 ± 0.21 $\mu\text{g cm}^{-2}$ for APG liposomes, APG+CHOL liposomes, APG invasomes and APG+CHOL invasomes, respectively (Fig. 5). In dermis, these values were 2.0 ± 0.01 , 2.07 ± 0.01 , 8.29 ± 0.17 , 10.15 ± 0.04 $\mu\text{g cm}^{-2}$, respectively. The invasomes provided a higher APG retention in both epidermis and dermis compared to the liposomes. In fact, several authors have already demonstrated that invasomes are able of increasing the cutaneous permeation of compounds through the skin (Ahmed and Badr-Eldin, 2019; Babaie et al., 2020; Cevc et al., 2002; Dragicevic-Curic et al., 2008b; Nangare and Dugam, 2020). The presence of limonene was essential to improve APG permeation. In addition, the incorporation of CHOL appears to increase the interaction of

vesicles with the skin, which would result in an increased permeation of APG. López-Pinto et al. (2005) also observed that the presence of CHOL in liposomes contributed to a deeper penetration of drugs in the skin. The mechanism proposed by the authors in that the CHOL would stabilize the size of the vesicles, avoiding coalescence and size increase, which would compromise the transport of compounds through the skin (López-Pinto et al., 2005).

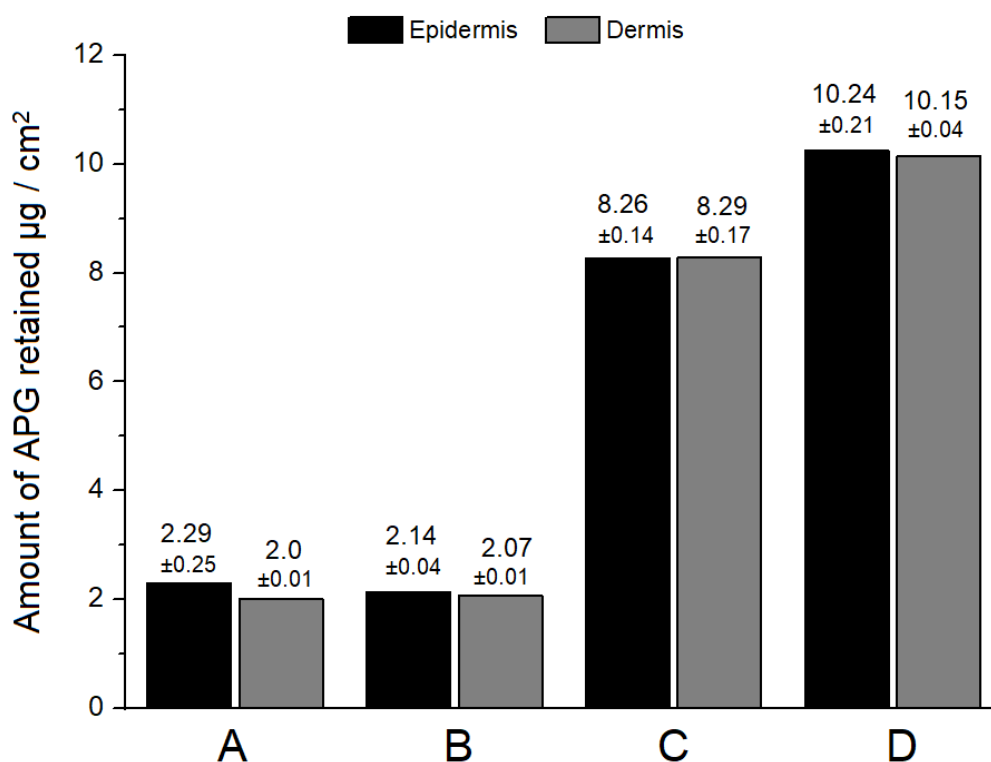


Figure 5. Amount of APG retained in the human dermis and epidermis after 24 h. APG liposomes (A), APG+CHOL liposomes (B), APG invasomes (C), APG+CHOL invasomes (D) ($n=4$).

3.5 Vesicle-skin interaction studies

FT-IR is a technique able to provide information on the disorder degree in skin protein and lipid domains. Asymmetric and symmetric CH vibrations attributed to SC lipids are found at 2,920 and 2,850 cm^{-1} , respectively (Vaddi et al., 2002). As the amount of lipids in the skin varies, the intensity of these bands varies proportionally. Lipid extraction by chemical absorption enhancers such as limonene results in a decrease in band intensity (Levang et al., 1999). These compounds can also fluidize the SC lipids, which result in the shift of CH_2 stretching bands to higher wavenumber values (*trans* to *gauche* conformation) and an increase in bandwidth (Golden et al., 1986; Suhonen et al., 1999). Changing in protein conformation can also be observed by amide I and II

stretching vibrations at 1,650 and 1,550 cm^{-1} , respectively. The amide I band is the result of C=O stretching vibrations whereas the amide II band is related to C-N stretching and N-H bending vibrations. In particular, the amide I band shifts to higher or lower frequencies according to the change in protein conformation (Vaddi et al., 2002).

The FT-IR spectra of SC treated with APG liposomes did not show differences compared to the control (tissue in PBS buffer). As the ratio of cholesterol increased in the vesicles, a reduction in the intensity of lipid bands was observed. Similar findings were found after treatment with invasomes. Invasomes with CHOL also showed a reduction in the intensity of lipid bands compared to the control tissue, indicating a tissue disruption. Invasomes with CHOL showed to be more effective to change the skin barrier properties than liposomes with CHOL, suggesting that CHOL and limonene would play a synergistic action. No significant changes in polar domains were observed after different treatments.

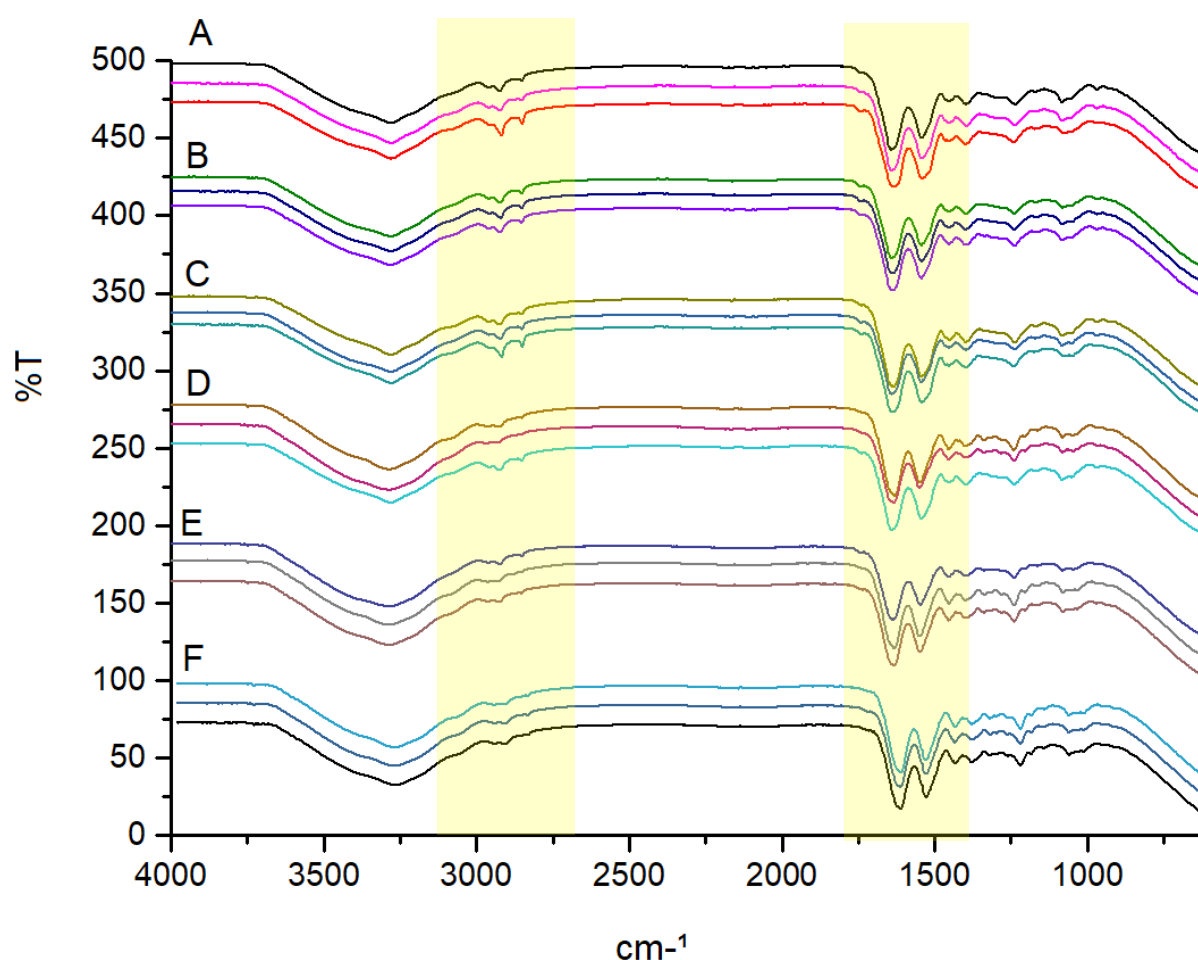


Fig. 8. FT-IR spectra of stratum corneum treated with liposomes and invasomes. PBS buffer (A); APG liposomes (B); PC:CHOL 5:0.5+APG (C); PC:CHOL 5:2+APG (D); APG invasome (E); IV:CHOL 5:2+APG (F).

4. DISCUSSION

Melanoma is a highly aggressive and potentially fatal form of skin cancer, which is responsible for a significant proportion of cancer-related deaths worldwide. Although conventional therapies such as surgical excision, immunotherapy and radiotherapy have been considered in the treatment of melanoma, the use of these approaches is not always effective (Tuong et al., 2012). Development of resistance and adverse side effects are common, which have motivated the investigation of new therapeutical options (Asensi et al., 2011).

APG has been considered to treat different types of cancer due to its capacity of modulating cell proliferation, apoptosis, cell cycle arrest, invasion, metastasis and autophagy by regulating different cell signaling pathways (Hasnat et al., 2015; WOO et al., 2020; Zhao et al., 2017). In addition to its action on multiple targets, this molecule is highly selectivity for cancer cells compared to healthy cells, a relevant aspect considering that the current antineoplastic therapies are very toxic. This fact could also motivate the daily use of APG from a cancer prevention perspective. In spite of that, low aqueous solubility and pharmacokinetic problems such as slow oral absorption, high metabolization and association rate with plasma proteins are found for the APG (Wang et al., 2019). As an approach to overcome these problems, its incorporation into liposomes and a topical administration were purposed here.

Considering that melanocytes, the cells affected in melanoma, reside in the basal layer of epidermis and that the uncontrolled growth of cells or high cell density can limit the diffusion of active compounds into tumoral sites, a particular type of liposomes known as “invasome” was selected as drug delivery system or nanocarrier. The addition of both terpene and ethanol to the primary structure of phospholipids results in more flexible vesicles, improving the interaction with lipid domains of the skin and thus the penetration of active compounds into the skin (Babaie et al., 2020) or even into the tumoral site.

In addition to affect the biophysical properties (e.g., rigidity) of vesicle and lipid domains of the skin, the limonene, which was the terpene selected for this study, has already demonstrated anti-melanoma activity (Alipanah et al., 2021; Vandresen et al., 2014). In *in vitro* assays, it has demonstrated to induce apoptosis and inhibit proliferation of cancer cells. Another noteworthy finding is that this compound is able to sensitize melanoma cells to other chemotherapy agents by downregulating the expression of ABC family enzymes involved in the drug efflux (associated with resistance mechanisms)

(Menichini et al., 2010). Therefore, an additive or synergistic action between APG and limonene against melanoma cells could be achieved.

The presence of APG increased the phosphatidylcholine (PC) vesicle size, but the CHOL had a more significant impact on this parameter. Differences in terms of molecular mass/rigidity as well as distribution into vesicle could explain these findings. The CHOL is a hydrophobic compound and then it is preferentially distributed in the phospholipid hydrophobic acyl chain via hydrogen bonds and hydrophobic interactions (Nakhaei et al., 2021). Once it is a rigid molecule that present a high molecular weight, an increase in free volume between ordered lipids would be observed after its addition, explaining the larger vesicle size. Several literature studies had already shown a positive effect of CHOL on the liposomal vesicle size (Coderch et al., 2000; Karal et al., 2022; Shaker et al., 2017; Song et al., 2022). APG, in turn, would be distributed in the interface between polar head-group and non-polar hydrocarbon chains as already mentioned. Considering that the free volume in polar region is higher than in hydrocarbon chains and that APG is a more flexible molecule, less impact on vesicle size would be found after its accommodation closed to this region. The high encapsulation efficiency (>98%) represents another evidence for a APG distribution in a distinct domain of the vesicle as this parameter was not modified in presence of other agents such as CHOL and limonene.

The effect of APG on the membrane rigidity/flexibility was dependent on vesicle constitution. Liposomes composed of only PC show a high degree of organization, which is reduced in the presence of APG. Some literature studies have indicated an increased lipid membrane disorder after incorporation of APG (Pawlikowska-Pawlęga et al., 2013; Veiko et al., 2023). In this same way, the presence of limonene in PC vesicles also leads a significant reduction in membrane order. Anisotropy studies with the fluorescent probe DPH suggest that limonene acts as a spacers for the membrane lipid chains (Mendanha et al., 2017), which would explain this phenomenon. The increased flexibility of invasomes has been associated with a higher permeation rate of drugs in skin (Cevc et al., 2002; Romero and Morilla, 2015). On the other hand, invasomes without CHOL had an increase in membrane rigidity after incorporation of APG, which also would explain the increased stability. In this situation, APG would play a role as a linker with the lipid chains, limiting lipid mobility. This behavior is not observed in presence of CHOL. Once this last agent is rigid and has a high molecular size, it increases the free volume between lipids and then interactions of other vesicle constituents with lipids are impaired/weakened.

Invasomes resulted in a higher rate of penetration and retention of APG into the skin than liposomes, which can be explained by the greater flexibility and stability of the vesicle as well as the presence of limonene. This terpene is a known chemical permeation enhancer, which interacts preferentially with skin lipids due to its more hydrophobic nature. In fact, ATR-FTIR analyses sustain this hypothesis as limonene led to an increase in the degree of disorder in lipid domains of human SC. Furthermore, the presence of limonene provides greater flexibility to the vesicles, which suffer deformation and then reach deeper layers of the skin easier (Babaie et al., 2020; Chen et al., 2011; Dragicevic-Curic et al., 2008b). The greater stability of the vesicles, which was confirmed by using different techniques, avoids fusion phenomena during storage, which could increase the size of the vesicles and, therefore, reduce the amount of permeated APG.

The presence of CHOL also contributed to an increased skin lipid disorder and APG penetration and retention. The higher the proportion of CHOL in the vesicles, the greater the degree of lipid disorder in skin, a concentration-dependent effect. Previous studies also demonstrated that the addition of at least 20% of cholesterol (5:2 ratio) in liposomes significantly increases the transport of molecules through the skin (Coderch et al., 2000). CHOL can interact with the keratin found in the SC and weakening the packaging of this barrier structure (Kováčik et al., 2020).

Invasomes should be prioritized for further studies (e.g. anti-melanoma activity) given the advantages over liposomes. Interestingly, these formulations demonstrated to be more stable and flexible. The presence of both cholesterol and limonene enhanced the interactions of vesicles with lipid domains of skin, as observed in ATR-FTIR analyses, which could explain the high cutaneous permeation and retention of APG.

5. FINAL CONSIDERATIONS

Invasomes showed to be more promising systems for topical delivery of APG than conventional liposomes due to several reasons: (1) provided high vesicle stability, which improved even more after incorporation of APG; (2) showed high vesicle deformation or flexibility; (3) more interaction with lipid domains of skin, particularly due to presence of the terpene; (4) increased the amount of APG retained in the epidermis and dermis (3 to 4-times in relation to traditional liposomes), which allows achieving deeper regions of the skin and then accessing the tumor site more easily; (5) the presence of terpene with

recognized antioxidant activity in the vesicle could also minimize lipid oxidation events during storage. The formation of structures analogous to cell membranes would imply highly biocompatible systems, another benefit when compared to colloidal systems such as polymer or inorganic nanoparticles. Further studies should be performed to confirm the synergistic action of APG and limonene against melanoma in order to adjust the concentration of these agents in the vesicle. *In vivo* studies are still encouraged as these molecules are well tolerated from a toxicity point of view as well as the incorporation of other anti-melanoma agents in this carrier.

Acknowledgements

We kindly thank the Brazilian governmental agencies CNPq/MCTI and CAPES/MEC for financial support for this project and student scholarship, respectively. A.P.G. thanks INCT-Catálise, FAPESC (TO 2019TR0847) and CNPq (427008/2018-2) for the financial support. A.P.G. thanks the CNPq for her research productivity fellowship 312029/2021-7. The authors also thank the company Lipid (Campinas) for the phosphatidylcholine (Lipoid S100) donation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Izi Vieira Nunes Cunha: Conceptualization, Methodology, Writing - original draft, Investigation, Data curation; **Angela Machado Campos:** Methodology, Investigation, Data curation; **Adriana Passarella Gerola:** Investigation, Data curation, Review & Editing; **Ingrid Vicente Farias:** Methodology, Investigation, Data curation; **Thiago Caon:** Conceptualization, Supervision, Methodology, Writing - review & editing, Project administration, Funding acquisition.

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7 DISCUSSÃO GERAL

Embora raro, o melanoma representa o tipo de câncer de pele mais agressivo e letal. Mesmo com a aprovação de fármacos que utilizam tecnologias modernas como as terapias direcionadas e imunoterápicos, observa-se que estes medicamentos apresentam limitações em termos de resposta sustentada, o que leva a recidivas. Outra problemática é a capacidade das células tumorais em desenvolver resistência a estas substâncias e/ou ativarem vias de proliferação alternativas (DE LUCA et al., 2017), estimulação excessiva do sistema imunológico bem como especificidade para certos tipos de melanomas (HONG; AHN, 2017). Assim, a pesquisa de novos fármacos se mostra extremamente útil e necessária (LIU et al., 2019).

Compostos naturais, como os polifenóis, passaram a ter relevância clínica devido a atividade antiproliferativa frente a células cancerígenas e efeito antioxidante. A APG tem tido um papel de destaque para o tratamento do melanoma devido a alta seletividade de ação e também pela ação em múltiplos alvos (SOUTO; SAMPAIO; CAMPOS, 2019), uma característica essencial para contornar os mecanismos de resistência tumoral.

Ainda que a APG possa ser encontrada em diversas fontes vegetais que fazem parte da alimentação, é caracterizada por uma baixa biodisponibilidade oral e uma série de outros problemas farmacocinéticos (STEVENS; MAIER, 2016) já mencionados. Neste contexto, esforços tecnológicos e até mesmo de troca da rota de administração têm sido considerados. Nesta dissertação, foi proposta sua incorporação em nanocarreadores e uma aplicação local (na pele) já que a formação do tumor tem início na camada basal da epiderme, que é onde ficam os melanócitos. A incorporação em nanopartículas (NPs) permite contornar problemas de solubilidade, aumentar a estabilidade (apigenina é instável após exposição prolongada a luz), além de garantir uma liberação sustentada e maior penetração nos tecidos (neste caso, na pele). A troca da rota de administração, por sua vez, visa contornar problemas farmacocinéticos tais como o metabolismo de primeira passagem, forte ligação a proteínas plasmáticas e baixa biodisponibilidade.

A fim de conhecer os principais sistemas nanoestruturados já utilizados para a APG, a primeira etapa desta dissertação incluiu uma análise da literatura, que foi sistematizada na forma de um artigo de revisão (**capítulo II**). Vantagens e desvantagens de diferentes NPs poliméricas e baseadas em lipídios foram apresentadas, além do levantamento dos principais mecanismos de ação descritos para este composto ativo. Sistemas baseados em polímeros mostraram uma ação mais prolongada ou sustentada

enquanto que sistemas lipídicos trazem como principais vantagens a alta biocompatibilidade e maior penetração em tecidos. Dado que massas tumorais possuem alta densidade celular e são difíceis de penetrar, optou-se por sistemas lipídicos para a etapa experimental desta dissertação. Estudos com lipossomas mostraram resultados promissores quanto a atividade antitumoral, o que é reforçado pela existência de produtos no mercado que empregam este tipo de tecnologia. Para os produtos lipossomais do mercado, a via parenteral tem sido a mais frequentemente utilizada. A via percutânea, por sua vez, é caracterizada por propriedades de barreira significativas, o que faz jus ao seu papel fisiológico na proteção microbiana, física (ex.: radiação ultravioleta), além de evitar a perda hídrica. Por outro lado, há desafios a serem superados quando é utilizada para fins de administração de moléculas ativas. Os lipossomas têm mostrado alta interação com a pele, particularmente aqueles modificados (ex.: etossomas, niossomas ou invasomas).

Os invasomas apresentam a estrutura básica dos lipossomas, ou seja, bicamadas fosfolipídicas organizadas de forma concêntrica e vesicular, entretanto, incluem pequenas quantidades de etanol e terpeno(s) na sua composição. A combinação destas substâncias resulta em vesículas com maior fluidez de membrana e maior potencial de permeação cutânea em relação aos lipossomas convencionais (EL-TOKHY et al., 2021), um aspecto-chave para esta aplicação, razão da seleção dos mesmos para este trabalho. Enquanto o etanol interage com a porção acila (polar) dos fosfolipídios, o terpeno interage com a cauda hidrofóbica. A presença destes agentes traz mudanças em termos da flexibilidade ou organização dos lipídios que integram estas vesículas, estabilidade, bem como grau de interação com a pele. Assim, antes mesmo de incorporar a apigenina nestes sistemas, estudos avaliando o impacto de cada componente sobre estes parâmetros foram realizados (**capítulo III** desta dissertação).

A rigidez das membranas está intimamente relacionada à permeação cutânea. De maneira geral, quanto maior a flexibilidade das membranas, maior a capacidade das vesículas em se deformar e deslizar entre as células justapostas da epiderme (BABAIE et al., 2020; DRAGICEVIC-CURIC et al., 2008a; HORITA et al., 2015). Entretanto, o aumento de flexibilidade pode resultar em uma menor estabilidade das vesículas devido a maior capacidade de se fusionarem. Além disto, a própria movimentação livre dos fosfolipídios pode permitir maior exposição de sítios susceptíveis aos processos de oxidação (ERMILOVA; LYUBARTSEV, 2019; OHVO-REKILÄ et al., 2002). A partir da literatura, sugere-se que a adição de colesterol às vesículas proporciona maior estabilidade que, por possuir estrutura planar e interagir com os domínios lipídicos, é

capaz de diminuir a mobilidade dos fosfolipídios de membrana, resultando em vesículas com maior rigidez (SONG et al., 2022).

Para avaliar estes aspectos, vesículas com diferentes proporções de colesterol, presença/ausência de etanol e limoneno (terpeno selecionado) foram preparadas pelo método de hidratação do filme lipídico. Nesta técnica, todos os componentes hidrofóbicos são primeiramente solubilizados em um solvente orgânico (clorofórmio foi aqui selecionado), o qual é então evaporado sob pressão reduzida para a formação de um filme seco. Na sequência, esse filme é hidratado com uma solução aquosa (para esta dissertação, utilizou-se uma solução de tampão fosfato com etanol), o que leva a auto-organização dos fosfolipídios e então formação de vesículas compostas por bicamadas fosfolipídicas. Por fim, um tratamento ultrassônico foi considerado para garantir que sistemas mais homogêneos (ou com menor polidispersão) pudessem ser obtidos.

Diferentes abordagens foram consideradas quanto a adição do terpeno durante a formação das vesículas. Em um primeiro momento, sugeriu-se a adição deste componente na fase aquosa tendo em vista as suas propriedades voláteis e a possibilidade de perda na etapa de evaporação. No entanto, a concentração de etanol da vesícula, que é compatível com testes em cultura celular, foi insuficiente para a solubilização completa do limoneno. Alternativamente, outros terpenos de maior solubilidade aquosa foram testados e o mesmo problema foi encontrado. Os polissorbato 20 e 80 também foram incluídos na fase aquosa a fim de melhorar a solubilização dos terpenos, mas testes de caracterização revelaram a presença de sistemas mistos (invasomas, micelas ou combinação de ambos), além de alta polidispersão. Por conta disto, o limoneno foi adicionado na fase orgânica (clorofórmio) junto dos demais constituintes lipofílicos.

Comparativamente aos lipossomas, invasomas mostraram-se mais homogêneos tendo em vista os menores valores de PDI ($<0,3$). O aumento da concentração do limoneno de 0,5 para 1% aumentou o diâmetro médio das vesículas, o que já tinha sido relatado por Dragicevic-Curic et al. (2008). Com uma maior proporção de limoneno, as vesículas ficariam mais flexíveis, facilitando o fusão delas, explicando estes achados. Em ensaios destinados a avaliação da rigidez de membrana, a presença de apenas limoneno não afetou a rigidez das membranas, porém, houve um aumento neste parâmetro na presença do etanol. Este aumento na rigidez das vesículas pode ser explicado por interações em diferentes regiões dos fosfolipídios. O limoneno interage principalmente com as regiões hidrofóbicas enquanto que o etanol interage com as porções polares dos fosfolipídios. Estas interações limitam a movimentação molecular

dos fosfolipídios, resultando em um aumento da rigidez das membranas. Tal achado corrobora com o aumento de estabilidade observado nas formulações que continham limoneno e etanol. De fato, a combinação destas substâncias resultou em vesículas estáveis por 40 dias a temperatura ambiente.

No que diz respeito à adição de colesterol, os dados obtidos vão ao encontro dos relatados na literatura. O colesterol atua modificando a organização das cadeias alquílicas dos fosfolipídios, compactando os mesmos e limitando sua movimentação. Observou-se que concentrações abaixo de 10% não são capazes de alterar significativamente a rigidez das membranas, mas, acima desta concentração é possível obter-se aumento significativo e proporcional a quantidade de colesterol presente (KADDAH et al., 2018). Quanto à estabilidade, a adição deste componente não resultou em melhorias neste parâmetro. Em vesículas de PC com limoneno e etanol, a presença do colesterol impactou negativamente na estabilidade. Interações competitivas entre colesterol e limoneno poderiam explicar estes achados. Na presença do colesterol, as cadeias acila encontra-se mais afastadas devido ao maior volume/área molecular, enfraquecendo interações com o limoneno, o que explica a menor estabilidade.

A presença de etanol reduziu a rigidez de vesículas de fosfatidilcolina mesmo na presença de colesterol. O etanol acomoda-se preferencialmente nas proximidades da cabeça polar de fosfolídeos (HOLTE & GAWRISCH, 1997), causando uma desordem nestes domínios. Isto pode explicar o aumento do tamanho da vesícula após sua inclusão nas vesículas de PC. Prata et al. (2006) também relataram um aumento na área ocupada pelos lipídios após a adição de álcoois, corroborando estes achados.

Interações das vesículas com a pele foram avaliadas por espectroscopia na região do infravermelho (ATR-FTIR). Ainda que o colesterol não tenha resultado em melhoria da estabilidade, um impacto positivo nas interações com a pele foi observado. Quanto maior o teor de colesterol nas vesículas de PC, maior o grau de desordem no estrato córneo (EC). De acordo com Kováčik et al. (2020), como o colesterol é estruturalmente similar às ceramidas que compõe o EC, este composto pode se intercalar entre as cadeias hidrofóbicas de ceramidas, enfraquecendo as propriedades de barreira da pele. A presença do limoneno também impactou positivamente na desordem lipídica do EC. Este terpeno é amplamente descrito na literatura como um promotor de absorção. Seu mecanismo de ação baseia-se principalmente na desestruturação e fluidificação reversível dos lipídios da pele, permitindo a passagem das substâncias através da barreira lipídica (CHEN et al., 2014). Quando combinados, limoneno e colesterol aumentaram ainda mais a interação

das vesículas com a pele como diminuíram bandas referentes aos domínios lipídicos. Desta forma, o colesterol parece ser importante para o aumento da interação com a pele.

A próxima etapa (**capítulo IV**) incluiu a incorporação da APG nas vesículas. Tendo em vista os resultados descritos até o momento, lipossomas e invasomas com e sem colesterol foram considerados para investigar ainda melhor o efeito do limoneno e colesterol nas propriedades das vesículas bem como interações com a pele. Ensaios de permeação cutânea em pele humana foram incorporados nesta nova etapa, além dos ensaios de caracterização já mencionados no capítulo III. Alta taxa de incorporação da APG nestas vesículas foi encontrada (>98%), sugerindo alta afinidade pelo sistema mesmo na presença de outros constituintes. Este resultado vai ao encontro de outros estudos da literatura (BANERJEE; BANERJEE; MANDAL, 2017; SEN; BANERJEE; MANDAL, 2019). O aumento no tamanho das vesículas em decorrência da incorporação da APG sugere uma interferência na organização das cadeias alquílicas da fosfatidilcolina, o que é corroborado pela literatura. Segundo Pawlikowska-Pawlęga e colaboradores (2013), este flavonoide pode interagir tanto com domínios apolares quanto polares dos fosfolipídios, o que foi sugerido por estudos espectroscópicos (FT-IR e RMN) (PAWLIKOWSKA-PAWLĘGA et al., 2013). Grupos hidroxilas do flavonoide levariam ao estabelecimento de ligações de hidrogênio enquanto anéis carbônicos a interações hidrofóbicas. A presença do colesterol também aumentou o tamanho das vesículas (um aumento bem maior que o observado com a APG). Por se tratar de molécula hidrofóbica rígida e de alto peso molecular, se intercala nas cadeias apolares dos fosfolipídeos, o que justifica este resultado.

O efeito do APG na flexibilidade/deformabilidade da membrana foi dependente da constituição da vesícula. Lipossomas compostos apenas por fosfatidilcolina (PC) apresentam alto grau de organização, que é reduzido na presença de APG. Alguns estudos da literatura tem demonstrado um aumento da desordem da membrana lipídica após a incorporação de APG (PAWLIKOWSKA-PAWLĘGA et al., 2013; VEIKO et al., 2023). Da mesma maneira, a presença do limoneno nas vesículas de PC também levou a uma redução significativa no grau de organização das membranas. Estudos de anisotropia com a sonda fluorescente DPH sugerem que o limoneno atua como um espaçador das cadeias lipídicas da membrana (ALIPANAH et al., 2021), o que explicaria esse fenômeno. O aumento da flexibilidade dos invasomas tem sido associado a uma maior taxa de permeação de fármacos na pele (COLDREN et al., 2006; ROMERO; MORILLA, 2015). Por outro lado, os invasomas sem colesterol mostraram um aumento na rigidez da

membrana após a incorporação do APG, o que também explicaria o aumento da estabilidade. Nesta situação, a APG desempenharia um papel como ligante das cadeias lipídicas, limitando a mobilidade lipídica. Este comportamento não é observado quando colesterol é adicionado. Como o colesterol é rígido e possui alto tamanho molecular, um aumento do volume livre entre os lipídios seria observado e então as interações de outros constituintes da vesícula com os lipídios seriam prejudicadas ou enfraquecidas.

Os invasomas resultaram em maior taxa de penetração e retenção de APG na pele que lipossomas, o que pode ser explicado pela maior flexibilidade/deformabilidade e estabilidade da vesícula bem como pela presença de limoneno. Este terpeno já é bem conhecido por seu efeito promotor de absorção, o qual interage preferencialmente com lipídios da pele. Análises de ATR-FTIR sustentam esta hipótese como invasomas reduziram as bandas referentes aos carbonos simétricos e assimétricos relacionadas aos domínios lipídicos de fosfolipídeos, levando a uma desestruturação temporária da pele. Ainda, este terpeno proporciona maior flexibilidade às vesículas, deformando-as e assim alcançam as camadas profundas da pele (BABAIE et al., 2020; CHEN; LIU; FAHR, 2011; DRAGICEVIC-CURIC et al., 2008b). A maior estabilidade das vesículas, confirmada por diferentes métodos, evitaria fenômenos de fusão na estocagem, o que poderia levar ao aumento do tamanho das vesículas e, portanto, reduzir a quantidade de APG permeada.

A presença do colesterol também contribuiu aumentar a permeação e retenção da APG na pele. A literatura aponta que a adição de pelo menos 20% de colesterol (proporção 5:2) em lipossomas aumenta significativamente o transporte percutâneo de moléculas (CODERCH et al., 2000). Acredita-se que o colesterol possa interagir com a queratina encontrada no SC, enfraquecendo o empacotamento desta estrutura de barreira (KOVÁČIK; KOPEČNÁ; VÁVROVÁ, 2020).

Ressalta-se a importância da continuidade deste estudo a fim de avaliar a atividade anti-melanoma da APG combinada ao limoneno, antes e após incorporação em invasomas e lipossomas com e sem colesterol, a fim de confirmar a hipótese de um efeito sinérgico entre estas moléculas bioativas e observar se a composição das vesículas afeta a taxa de internalização celular. Além disto, há perspectivas quanto a encapsulação de outros agentes antitumorais nos invasomas por considerar que o limoneno também pode atuar neste tipo de câncer e sensibilizar células tumorais para uma ação ainda mais efetiva. Dada a alta contabilidade biológica do sistema, espera-se também obter um sistema mais seguro e com a possibilidade de uso inclusive na prevenção do câncer.

8 CONSIDERAÇÕES FINAIS

-Na revisão da literatura realizada, constatou-se que sistemas lipídicos podem ser veículos promissores para aplicação em tumores de pele dada a alta biocompatibilidade e potencial de penetração percutânea, justificando a seleção dos invasomas.

-Em vesículas sem apigenina, concentrações de colesterol superiores a 10% promoveram um aumento proporcional na rigidez das membranas, além de promoverem maior grau de desordem do estrato córneo. Por outro lado, a presença deste agente nestas vesículas não melhorou a estabilidade.

-O limoneno aumentou a flexibilidade/deformabilidade das vesículas, tornando-as mais estáveis e uniformes (reduziu valores de PDI), reforçou interações com a pele e aumentou o tamanho destes sistemas para variações de sua concentração de 0,5 para 1%.

-Baixas concentrações de etanol (3% p/v) resultaram em um aumento do tamanho das vesículas e diminuição da rigidez de membrana mesmo na presença de colesterol.

-A apigenina foi eficientemente incorporada as vesículas, alcançando uma eficiência de encapsulamento superior a 98%, sugerindo alta afinidade pelo sistema mesmo na presença de outros constituintes.

-Invasomas resultaram em maior aumento na permeação e retenção cutânea da apigenina que os lipossomas, o que pode ser atribuído ao efeito promotor de absorção do limoneno. Na presença de colesterol, o efeito de promoção da absorção foi ainda maior.

-Estudos mecanísticos de ATR-FTIR mostraram que limoneno e colesterol interagem com domínios lipídicos do estrato córneo, promovendo maior desordem e fluidização, o que poderia justificar a permeação aumentada na presença destes agentes.

-Invasomas mostraram-se mais promissores que lipossomas convencionais para a incorporação da apigenina tendo em vista a maior estabilidade, deformabilidade/flexibilidade das vesículas e interação com a pele, encorajando a realização de ensaios de atividade antitumoral e/ou incorporação de outros agentes antitumorais.

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