UNIVERSIDADE D
COIMBRA

Bruno Gomes Marques da Costa

Relatório de Estágio e Monografia intitulada "Lipoplexes and Polyplexes as Nucleic Acids Delivery Nanosystems: The Current State and Future Considerations" referentes à unidade curricular "Estágio," sob a orientação do Dr. Luís Miguel Castro e do Professor Doutor António José Ribeiro, apresentados à Faculdade de Farmácia da Universidade de Coimbra para apreciação na prestação de provas públicas do Mestrado Integrado em Ciências Farmacêuticas.

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DECLARAÇÃO DE AUTORIA

Eu, Bruno Gomes Marques da Costa, estudante do Mestrado Integrado em Ciências Farmacêuticas, com o n.º 2014201458, declaro assumir toda a responsabilidade pelo conteúdo do Documento Relatório de Estágio e Monografia intitulada "Lipoplexes and Polyplexes as Nucleic Acids Delivery Nanosystems: The Current State and Future Considerations," apresentado à Faculdade de Farmácia da Universidade de Coimbra no âmbito da unidade de Estágio Curricular.

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Coimbra, 16 de julho de 2021.

Bruno Gomes Marques da Costa

(Bruno Gomes Marques da Costa)

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O meu sincero obrigado a todos!

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RELATÓRIO DE ESTÁGIO EM FARMÁCIA COMUNITÁRIA

FARMÁCIA MAIA

Orientador: Dr. Luís Miguel Castro

ABREVIATURAS

DCI: denominação comum internacional

MICF: Mestrado Integrado em Ciências Farmacêuticas

MNSRM: medicamento não sujeito a receita médica

MNSRM-EF: medicamento não sujeitos a receita médica de dispensa exclusiva em farmácia

SNS: Serviço Nacional de Saúde

SWOT: *Strengths*, *Weaknesses*, *Opportunities*, *Threats*

UE28: 28 estados-membro da União Europeia

INTRODUÇÃO

De acordo com a Diretiva 2013/55/EU, de 20 de novembro, a conclusão do Mestrado Integrado em Ciências Farmacêuticas (MICF) dita a realização de uma componente de estágio de seis meses em farmácia aberta ao público ou num hospital (1). Tal preconiza o desenvolvimento profissional em contexto real para aplicação dos conhecimentos teóricos até então adquiridos. Findado o período de seis meses, é redigido um relatório de estágio.

O estágio por mim realizado em farmácia comunitária decorreu entre janeiro e junho de 2019 na Farmácia Maia, sob a orientação do Dr. Luís Miguel Castro.

Figura 1. Zona de atendimento da Farmácia Maia.

CONTEXTO ATUAL DAS FARMÁCIAS COMUNITÁRIAS

O Centro de Estudos e Avaliação em Saúde da Associação Nacional das Farmácias estimou que, no último semestre de 2018 e primeiro semestre de 2019, a indisponibilidade de medicamentos nas farmácias comunitárias dificultou o acesso aos mesmos a cerca de 3.4 milhões de utentes, forçando até a interrupção indevida da terapêutica de perto de 370 mil desses utentes. O estudo avançado refere ainda que, diariamente, 17.5 mil utentes atendidos nas farmácias em Portugal já se teriam deslocado anteriormente a outra farmácia para, sem sucesso, tentar obter os medicamentos. Destes, cerca de um em cada três permaneceu sem a medicação prescrita (2).

Em termos práticos, este enquadramento é fundamental para acautelarmos a frequência de ocorrência de situações semelhantes, a fim de devidamente acompanhar e encaminhar os utentes afetados.

ANÁLISE SWOT

O presente relatório de estágio em farmácia comunitária consiste numa análise SWOT, uma sigla inglesa que se traduz para pontos fortes (*Strengths*), pontos fracos (*Weaknesses*), oportunidades (*Opportunities*) e ameaças (*Threats*). Esta análise foi dividida em duas partes: ambiente interno e ambiente externo. O ambiente interno incide sobre aspetos internos da farmácia e caracteriza-se por pontos fortes e por pontos fracos, visando o seu reforço e a sua atenuação, respetivamente. Já o ambiente externo, contempla elementos imprevistos ou incontroláveis pela farmácia, permitindo, deste modo, o reconhecimento de tendências que se transpõem para oportunidades e ameaças.

AMBIENTE EXTERNO

Oportunidades

Demografia

De acordo com dados do Eurostat (3), a percentagem da população com mais de 64 anos está a aumentar, colocando, em 2017, Portugal na quarta posição na UE28, em termos de população residente. Num país com tendência para o envelhecimento demográfico, fruto da baixa taxa de natalidade, a par com o aumento da esperança de vida, o papel do farmacêutico é preponderante na prevenção e acompanhamento geriátrico. Assim, num contexto profissional, as boas práticas de farmácia direcionadas a esta faixa da população tornaram-se num paradigma de saúde comunitária de qualidade. Inerente a isto, pude desenvolver a minha apetência pela promoção do envelhecimento saudável e, junto da comunidade sénior polimedicada, auxiliar na gestão da sua saúde.

Formação Contínua

Numa era cada vez mais digital, a oferta de formações de cariz científico é vasta. Mas a mesma não se foca apenas na componente científica. Como pude constatar durante o período de estágio, diferentes plataformas de formação contínua a que tive acesso continham conteúdos sobre marketing, vendas, gestão e liderança. Por isto, torna-se cada vez mais evidente que a formação individual do farmacêutico procura ser multifacetada, com vista a melhorar a prestação de cuidados de excelência à população.

Comunicação com Profissionais de Saúde

Intrínseco ao funcionamento das farmácias, a vertente multidisciplinar manifesta-se. Não basta comunicar com a equipa farmacêutica, pois diariamente é necessário contactar com distribuidores, quer por telefone, quer presencialmente, com delegados de informação médica e com médicos sempre que necessário. A meu ver, um bom profissional de saúde não se restringe somente a saber comunicar com o utente, pois o contacto com os diferentes setores do Serviço Nacional de Saúde (SNS) é inevitável.

Tive oportunidade de estabelecer esse contacto com delegados de informação médica aquando da divulgação dos mais recentes estudos, com médicos para esclarecer quaisquer dúvidas relativas às suas prescrições e, por fim, com os armazenistas por forma a assegurar o

acesso ao medicamento por parte dos utentes. Esta competência social é imperativa na prestação de serviços.

Prescrição Farmacêutica

A prescrição de medicamentos por farmacêuticos poderá aliviar a sobrecarga do SNS na prestação de cuidados de saúde primários (4). Apesar de esta não ser ainda uma realidade em Portugal, o statu quo permite a intervenção do farmacêutico e aplicação de protocolos de dispensa dos chamados medicamentos não sujeitos a receita médica de dispensa exclusiva em farmácia (MNSRM-EF).

Posso afirmar que o número de utentes que se desloca às farmácias antes de procurar um médico é elevado e, muitas vezes, a dispensa de MNSRM-EF, após uma avaliação atenta do caso apresentado, evita a ida do utente ao mesmo.

Ameaças

Erros nas Tomas

A prescrição por denominação comum internacional (DCI), a semelhança na aparência e fonética dos medicamentos e também a falta de medicamentos, podem inadvertidamente ocasionar um aumento no número de erros no momento da toma. Durante o estágio, percecionei a crescente ameaça de duplicação de medicação originada pela falta de medicamentos, muitos deles descritos como os "habituais" [sic]. Apesar de, felizmente, o arsenal terapêutico dispor de diversos medicamentos genéricos e biossimilares, os utentes mais idosos terão maior probabilidade de cometer erros nas tomas por divergências na aparência dos mesmos ou denominação. Isto coloca em causa a segurança dos utentes e, por esse motivo, o farmacêutico deve tomar especial atenção.

A seriedade do problema é ainda desconhecida. No entanto, um grupo da Faculdade de Medicina da Universidade do Porto está a conduzir um estudo precisamente sobre a duplicação de medicação no idoso na zona do Grande Porto (5).

Desvalorização da Competência Farmacêutica

Advindo da publicação do Decreto-Lei n.º 238/2007, de 19 de Junho, a venda de medicamentos não sujeitos a receita médica (MNSRM) alastrou-se por cerca de quatro centenas de locais por todo o país. Esse mesmo Decreto-Lei prevê ainda a ampliação futura do número de medicamentos com a classificação de MNSRM (6). A ameaça surge quando nos locais de venda

não está presente alguém capaz de prestar o devido aconselhamento aos utentes, levando, por vezes, a situações de automedicação desnecessárias.

Embora a sua venda fora das farmácias não seja comparticipada pelo SNS, a medida legislativa ocasionou uma redução dos preços destes medicamentos, pois o poder aquisitivo das grandes superfícies o permite junto dos laboratórios farmacêuticos e armazenistas. Contudo, a competitividade gerada colocou muitas farmácias em desvantagem e até mesmo em risco de insustentabilidade.

Falhas Informáticas

Fortuitamente, erros no SPharm, software informático em uso na farmácia onde estagiei, surgiam. Em situações de falha de comunicação com os sistemas de informação dos Serviços Partilhados do Ministério da Saúde era-nos impossibilitado aceder às prescrições eletrónicas e, por conseguinte, fazer a comparticipação sobre o preço dos medicamentos. Acabamos por, em última instância, solicitar aos utentes para regressarem mais tarde.

O avanço das tecnologias e a sua aplicação traz inúmeros benefícios, tal como a redução dos erros no ato da dispensa dos medicamentos. Apesar disso, a dependência nelas é a sua maior ameaça.

Tabela 1. Sumário da análise do ambiente externo.

Ambiente Externo	
Oportunidades	**Ameaças**
Demografia Formação contínua Comunicação com profissionais de saúde Prescrição farmacêutica	Erros nas tomas Desvalorização da competência farmacêutica Falhas informáticas

AMBIENTE INTERNO

Pontos Fortes

Amostra de Utentes Representativa das Diferentes Faixas

A Farmácia Maia situa-se na Rua do Campo Alegre, no Porto, zona central da cidade. Tratando-se de uma zona já bastante urbanizada, existem ainda três grandes hotéis nas imediações, que resulta numa afluência superior de utentes estrangeiros à farmácia. Conta ainda com duas escolas e uma faculdade igualmente próximas. O contacto com um leque de utentes tão diversificado constitui um ponto forte, pois a função farmacêutica exige um ajuste no discurso para que este seja adaptado a cada utente. Assim, tirei proveito para melhorar a minha capacidade de comunicar com clareza e eficácia.

Integração na Equipa

À chegada àquele que seria o meu local de estágio, encontrei uma equipa já muito dinâmica e sempre disponível. Comecei por desempenhar tarefas no *back office* para me familiarizar com a organização interna da farmácia. Desde o primeiro dia, fui autorizado a assistir de perto quaisquer atendimentos ao público. Rapidamente me concederam autonomia para levar a cabo tarefas como a receção das encomendas, o devido armazenamento dos medicamentos, a leitura diária dos termo-higrómetros, a devolução de produtos e a encomenda de produtos rateados. À terceira semana de estágio, realizei o meu primeiro atendimento, onde a atenção por parte da equipa não foi em momento algum descuidada.

O acompanhamento que recebi no decorrer do estágio foi fulcral para todos os conhecimentos que adquiri e consolidei e, também, para as competências que desenvolvi.

Serviços Farmacêuticos

A Farmácia Maia dispõe dos seguintes serviços: medição da pressão arterial, glicemia, colesterol total e triglicéridos. A participação anterior em rastreios cardiovasculares capacitou-me de conhecimentos acerca de procedimentos corretos a adotar no momento da sua prestação, o que se refletiu no grau de confiança por parte dos utentes. Sempre que pertinente, sugeri um acompanhamento constante dos doentes crónicos, o que não só melhorou a sua qualidade de vida dos mesmos, como também, em alguns casos, os fidelizou. Este acompanhamento mais próximo permitiu-me prevenir e corrigir erros nas tomas dos

medicamentos, assim como entender a perceção do doente do seu estado de saúde e esclarecer quaisquer dúvidas remanescentes.

Entretanto, surgiu a oportunidade de durante uma tarde prestar estes mesmos serviços, no Centro de Convívio de Massarelos. A sensibilização e promoção para a literacia em saúde foram as atividades de destaque. Procurei sensibilizar a população a adotar estilos de vida saudáveis e a controlar os fatores de risco, tais como: hipertensão arterial, hipercolesterolemia, tabagismo, diabetes, sedentarismo ou stress psicossocial. Terminou num balanço bastante positivo por parte dos participantes.

Figura 2. Cartaz elaborado para o rastreio cardiovascular.

SPharm

O *software* de gestão implementado na Farmácia Maia é o SPharm. Apesar de não ser o mais comummente encontrado nas farmácias comunitárias em Portugal, a sua interface é bastante intuitiva. É com este programa que tarefas como desde abrir e atualizar fichas de clientes, encomendar e devolver produtos até resolver notas de crédito são feitas.

O domínio das potencialidades deste *software* representa uma mais-valia, por já possuir conhecimentos básicos de Sifarma® 2000.

Pontos Fracos

Inexperiência Inicial

Não tendo anteriormente qualquer contacto com a realidade das farmácias comunitárias, inicialmente senti-me inseguro no aconselhamento. Após uma introspeção, concluo que a principal fonte de insegurança se prendeu com o desconhecimento acerca de muitos MNSRMs. Estes são muitas vezes a primeira linha de tratamento, sobretudo sintomático (ex.: congestão nasal, tosse seca e irritativa/com expetoração, dores musculares). Apesar de ser um ponto fraco, a minha curva de aprendizagem revela que é perante novas situações que mais aprendo, pois incita a procura pelas melhores soluções para cada utente.

Iliteracia em Saúde

A literacia em saúde é um assunto com crescente relevância (7). Com a facilidade de acesso à informação, procura-se cada vez mais prover os cidadãos de maior capacidade de gestão sobre a sua saúde e responsabilidade, para tomarem as suas decisões com base em informações fundamentadas.

No entanto, constatei que, muitas vezes, os utentes demonstram um total desconhecimento acerca de tópicos como o direito de opção e a reciclagem de medicamentos.

A promulgação do Decreto-Lei n.º 271/2002, de 2 de dezembro, introduziu a prescrição de medicamentos mediante a indicação da DCI, nas situações para as quais existam medicamentos genéricos autorizados. Igualmente de acordo com o mesmo, o farmacêutico deve, obrigatoriamente, informar o utente da existência de medicamentos genéricos comparticipados pelo SNS (8). Sempre que aplicável, concedi a liberdade de opção ao utente e verifiquei que muitos deles desconhecem esse direito e, por vezes, questionam a eficácia dos medicamentos genéricos.

Como referido anteriormente, também a gestão dos medicamentos fora de uso é feita de forma incorreta por uma grande parte dos utentes, pois muitos alegam colocar esses medicamentos no lixo doméstico.

Figura 3. Imagem da campanha criada com o apoio da VALORMED para educar a população acerca do modo correto de conservação dos medicamentos em casa e o que fazer com os medicamentes fora de uso.

O farmacêutico, sem dúvida, desempenha um papel vital na promoção para a literacia em assuntos como os já discutidos e outros. Contudo, não deixo de apontar a baixa recetividade à mudança, mesmo quando baseada em informações fundamentadas, sobretudo por parte dos utentes mais idosos.

Tabela 2. Sumário da análise do ambiente interno.

Ambiente Interno	
Pontos Fortes	**Pontos Fracos**
Amostra de utentes representativa das diferentes faixas Integração na equipa Serviços farmacêuticos SPharm	Inexperiência inicial Iliteracia em saúde

CASOS PRÁTICOS

Caso 1: Duplicação da Medicação

Um senhor entrega duas prescrições, uma manual e outra eletrónica. Uma primeira análise das mesmas permitiu-me identificar a prescrição de dois antibióticos por médicos diferentes. A prescrição manual continha uma embalagem Ciproxina® 500 mg. Já a prescrição eletrónica, entre outros, continha Amoxicilina + Ácido clavulânico 875/125 mg (prescrição por DCI).

Em conversa com o utente, o mesmo refere ter ido ao médico de família solicitar a medicação para o tratamento da sua hipertensão arterial e questionou acerca de uma recente manifestação cutânea. O médico identificou tratar-se de uma infeção, levando à prescrição adicional de outro antibiótico. Mais tarde, a acompanhar a esposa à consulta no hospital, a mesma solicitou uma reavaliação da manifestação, incitando novamente a prescrição manual de um antibiótico. Todavia, o utente não julgou haver duplicação da sua medicação, acrescentando querer "o problema resolvido depressa" [sic].

Passei então a explicar que, apesar de apresentarem diferentes princípios ativos, não seria necessário a toma de ambos para tratar a infeção cutânea. Aconselhei, posteriormente, o utente a optar pelo medicamento prescrito pelo médico de família, para que o acompanhamento do utente seja contínuo.

Caso 2: Obstipação Infantil

Uma senhora desloca-se à farmácia e comenta que o seu filho de 2 anos e meio apresenta dores na barriga, acrescentando que chora quando lhe tocam. Procura algo para aliviar os seus sintomas.

Numa primeira abordagem, procurei saber se apresentava alguma manifestação visível e soube que a criança tinha a barriga inchada. Questionei também há quanto tempo o filho não defecava, à qual respondeu que não o fazia há três dias. Acrescentou ainda que foi à volta do mesmo tempo que alterou o leite habitual.

Estando perante um caso evidente de obstipação, aconselhei o aumento do aporte de fibras na dieta do filho com mais papas de fruta. Além disso, propus nova alteração do leite para um com uma fórmula anti-obstipante. Para o alívio do desconforto abdominal, por conter simeticone, sugeri o Infacalm®, conforme a seguinte posologia: 8 gotas após as três principais refeições e antes de dormir (9). Por fim, como tratamento adjuvante da obstipação, uma colher de sobremesa (10) antes de deitar de Xarope de Maçãs Reinetas® (maçã reineta + manitol + sene), um laxante de contacto suave e de sabor agradável.

Caso 3: Parasitose Canina

Um senhor procura aviar uma receita veterinária, afirmando ter um cão com "bichas" [sic]. A receita contém uma embalagem de Drontal Plus Flavour®, um medicamento veterinário usado para o tratamento de certos tipos de parasitoses.

Ao perceber que se trata de um cão com 3 meses de idade, informei que é comum alguns animais nascerem com parasitas, transmitidos pela progenitora ou através do leite materno. Acrescentei ainda que as infeções parasitárias podem ter origem no contacto com fezes contaminadas, recomendando a repetição futura do tratamento conforme a indicação do médico veterinária. Por fim, considerei pertinente propor a desparasitação de todos os elementos da família, fundamental para a profilaxia das parasitoses.

O agregado familiar que contacta com o cão é composto pela esposa do utente e por uma criança com 11 anos de idade. Logo, aconselhei a suspensão oral de mebendazol 20 mg/ml (Pantelmin® 20 mg/ml suspensão oral) para a criança, com a indicação de tomar 5ml de manhã e à noite, durante 3 dias (11). No caso dos adultos, sugeri a toma de 2 comprimidos de mebendazol 100 mg (Pantelmin® 100 mg comprimidos) de manhã e à noite, igualmente durante 3 dias (11).

CONCLUSÃO

Concluída a análise SWOT do estágio curricular por mim realizado em farmácia comunitária, saliento a importância da formação de qualidade ministrada na Faculdade de Farmácia da Universidade de Coimbra. Não é humanamente possível terminar o MICF com todos os conhecimentos prontos aplicar no dia a dia de um farmacêutico. Além da atualização científica atualmente acontecer um ritmo acelerado, é a depararmo-nos com desafios que continuamente aprendemos. O aspeto mais importante é, na minha opinião, sabermos consultar as fontes de informação certas sempre que necessário.

O desafio de fortalecer a literacia em saúde da população portuguesa é evidente e não compete apenas aos profissionais de saúde darem resposta. Trata-se de um desafio coletivo para sermos uma verdadeira sociedade do século XXI, sobretudo com o envelhecimento progressivo da população. Não será suficiente inovar em terapêuticas para aumentar a esperança média de vida, é imperativo tomar posições preventivas e contribuir para o envelhecimento ativo.

Atualmente, o progresso tecnológico e terapêutico auxilia-nos a prestar cuidados de excelência aos nossos utentes, família e amigos. Para os sabermos utilizar a nosso favor devemos incessantemente procurar sermos também farmacêuticos de excelência.

Tabela 3. Resumo da análise SWOT.

Análise SWOT	
Oportunidades	Ameaças
Demografia Formação contínua Comunicação com profissionais de saúde Prescrição farmacêutica	Erros nas tomas Desvalorização da competência farmacêutica Falhas informáticas
Pontos Fortes	Pontos Fracos
Amostra de utentes representativa das diferentes faixas Integração na equipa Serviços farmacêuticos SPharm	Inexperiência inicial Iliteracia em saúde

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MONOGRAFIA

"LIPOPLEXES AND POLYPLEXES AS NUCLEIC ACIDS DELIVERY NANOSYSTEMS

THE CURRENT STATE AND FUTURE CONSIDERATIONS"

Orientador: Professor Doutor António José Ribeiro

ABSTRACT

Designing safe and effective nucleic acid delivery nanosystems presents a challenge that requires a good understanding of the various biological barriers, whose impact gets frequently neglected during *in vitro* assessments. Hence, the development of nanosystems would benefit from a more thorough physicochemical characterization to establish structure-activity relationships and increase the preclinical data relevance.

As hereafter addressed, both lipoplexes and polyplexes are promising candidates for nucleic acid delivery, mostly due to their lower immunogenicity and toxicity versus viral vectors. Following the systemic administration of both lipoplexes and polyplexes, the formation of a so-called protein corona occurs, thus changing the identity of the vector and impacting its previously studied *in vitro* mechanisms of interaction. Due to those changes, their recognition by the immune system may no longer be similar. As such, there are clear indications that preclinical assays are to be performed under physiological representative conditions, to better account or even predict the highly dynamic interactions in humans.

This review focused on lipoplexes and polyplexes presents a summary of considerations to improve the translation of preclinical studies into functional therapies.

Keywords: lipoplexes, nanosystems, nucleic acids delivery, polyplexes, toxicity.

RESUMO

O desenho de nanossistemas seguros e eficazes para terapia génica apresenta um desafio que requer um bom conhecimento das diferentes barreiras biológicas, cujo impacto é frequentemente negligenciado durante os ensaios *in vitro*. Portanto, o desenvolvimento dos nanossistemas beneficiaria de uma caracterização físico-química mais exaustiva para estabelecer relações estrutura-atividade e aumentar a relevância dos dados pré-clínicos.

Como irá ser abordado de seguido, tanto os lipoplexos como os poliplexos são candidatos promissores para a terapia génca, sobretudo devido à sua baixa imunogenicidade e toxicidade comparativamente aos vetores virais. Após a administração sistémica tanto de lipoplexos como de poliplexos, ocorre a formação da chamada proteína corona, alterando a identidade do vetor e impactando os seus mecanismos de interação previamente estudados *in vitro*. Devido a essas alterações, eles poderão agora ser reconhecidos pelo sistema imunitário. Por isso, existem indicações claras de que os ensaios pré-clínicos devem ser executados sob condições fisiológicas representativas, para melhor acautelar ou mesmo prever as interações altamente dinâmicas.

Este artigo de revisão focado nos lipoplexos e poliplexos apresenta um sumário de considerações para melhorar a translação dos estudos pré-clínicos para terapias funcionais.

Palavras-chave: lipoplexos, nanossistemas, poliplexos, terapia génica, toxicidade.

ABBREVIATIONS

APCs: antigen-presenting cells

apoE: apolipoprotein E

BPEI: branched polyethylenimine

CARPA: complement activation-related pseudoallergy

CD: cyclodextrin

Chol: cholesterol

CM-PLH: carboxymethyl poly(L-histidine)

Dex: dextran

DLS: dynamic light scattering

DOPC: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine

DOPE: 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine

DOTAP: 1,2-dioleoyloxy-3-trimethylammonium-propane

DOTMA: 1,2-di-O-octadecenyl-3-trimethylammonium-propane

DSPC: 1,2-distearoyl-*sn*-glycero-3-phosphocholine

DSPE: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine

DSPE-PEG2000: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]

EPR: enhanced permeability and retention

F: fluorinated

FA: folate

FBS: fetal bovine serum

HODA: histidine modified octadecylamine

HSPC: hydrogenated soy phosphatidylcholine

IgM: immunoglobulin M

Lec: lecithin

LNPs: lipid nanoparticles

LPEI: linear polyethylenimine

NA: not available

NCL: Nanotechnology Characterization LaboratoryNK: natural killer

NTA: nanoparticle tracking analysis

OEI: oligoethylenimine

PBAE: poly(β-amino ester)

PCB: poly(carboxybetaine)

PCD: poly(CBA-DMDPTA)

pDNA: plasmid DNA

PEG: poly(ethylene glycol)

PEI: poly(ethylenimine)

PFF: perfluorooctanoyl fluoride

PLGA: poly(lactic-co-glycolic acid)

PLL: poly-L-lysine

PPD: pH-responsive anionic polymer

PPS: pH-insensitive anionic polymer

PUBAP: Polyurethane containing 1,4-bis(3-aminopropyl)piperazine

SEM: scanning electron microscopy

siRNA: small interfering ribonucleic acid

SS: disulfide link.

TEM: transmission electron microscopy

INTRODUCTION

The usage of nucleic acids has provided new treatment approaches to tackle diseases ranging from heritable [1] to infectious diseases [2] and even cancer [3]. The current methodologies allow us to silence, replace or modulate specific genes, known etiological factors of a series of conditions. To this end, remarkable efforts have been made to improve nucleic acids' delivery efficacy and safety, noticeable through the growing number of products reaching the market [4]. However, very few of those products reach the market, being among the main reasons: their inability to protect their cargo against ubiquitous endonucleases, to prevent nonspecific interactions with proteins and other non-target cells, as well as to offer good transfection efficiency to target cells [5]. Thus, developing non-viral vectors for systemic administration requires knowledge of the biological barriers to overcome.

Given the vast array of non-viral vectors for nucleic acid delivery, this review will focus on cationic lipids and cationic polymers/nucleic acid complexes, known as lipoplexes and polyplexes, respectively (Figure 1). The use of cationic lipids and polymers enables a more prone interaction with the anionic plasma membrane of mammalian cells. Besides, these materials seem overall biocompatible and form complexes with negatively charged nucleic acids rather easily [6]. Combinatorial use of cationic lipids and polymers has also shown promising results in improving nucleic acids delivery efficacy with little cytotoxicity [7, 8], increasing the confidence in these vectors.

Rationally designed non-viral vectors for targeted nucleic acid delivery intend to minimize off-target effects and improve transfection [9]. For example, its modification with targeting moieties, such as transferrin [10], and tumor-specific peptide ligands [11], to enhance intracellular uptake has shown promising *in vitro* results. Nevertheless, before reaching the cells of interest, escape the endosomal compartment and, if required, transverse the nuclear membrane, both lipoplexes and polyplexes must protect their cargo, avoid rapid hepatic and renal clearance, toxicity and immune detection, as well as non-specific interactions [12]. When it comes to translating the most promising *in vitro* results into advancements to the clinical practice, the task gets even harder. By establishing structure-activity relationships through *in vitro* interaction assays that closely mimic the complex biological environment, the development efforts may provide translatable results to *in vivo* experiments. The clinical practice would massively benefit from safer and more effective delivery nanosystems, especially relevant when we need to avoid patients' immune responses.

The present review provides an insight into the current development strategies adopted to obtain safer lipid and polymer-based non-viral vectors for nucleic acid therapy. Also, *in vitro* characterization assays are discussed and further correlated to the hematological and immunological responses *in vivo*. The research achievements covered hint to an encouraging future for lipoplexes and polyplexes as nucleic acid delivery nanosystems.

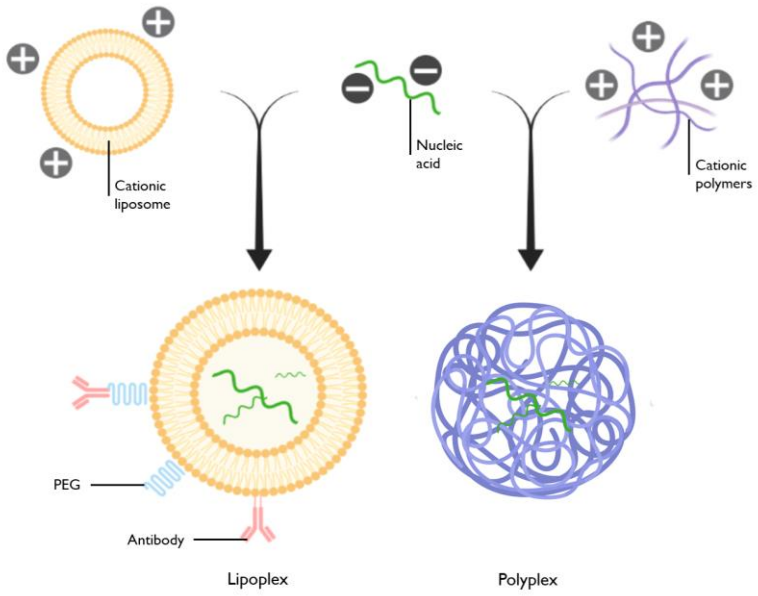

Figure 1. Scheme of the structure of lipoplexes and polyplexes for nucleic acid delivery. Negatively charged nucleic acids naturally interact with cationic lipids to originate lipoplexes. These can suffer surface modifications by grafting a protective polymer, such as PEG, which shields them from opsonizing proteins. They may also, simultaneously or not, bear an antibody covalently coupled to the phospholipids in the membrane, or, preferably, to the distal end of the grafted polymeric chain. On the other hand, polyplexes self-assemble and stabilize through hydrophobic or electrostatic interactions between the cationic polymers and the nucleic acids. The latter, too, are sometimes subject to surface modifications, like PEGylation.

PEG: poly(ethylene glycol).

THE PROBLEM WITH PRESCREENING TOOLS OF NON-VIRAL VECTORS

Regardless of tremendous improvements in lipoplexes and polyplexes' development, their toxicity and immune detection, one of the main obstacles in clinical translation, has never been entirely evaluated. Commonly used biodegradable materials are not immune quiescent and can often result in uncontrollable severe inflammatory responses [13].

Ideally, preclinical studies would allow anticipating any unwanted *in vivo* responses and fine-tuning non-viral vectors at earlier stages to avoid them. Even though it is possible to conduct most assessments *in vitro*, the grouping of those and *in vivo* methods to identify relevant biomarkers that allow us to establish the safety and efficacy profiles is needed to address the complex biological environments [14, 15]. Failure to do so can lead not only to blood incompatibilities, as evidenced by hemolysis and thrombogenicity, but also complement activation (e.g., complement activation-related pseudoallergy or CARPA), cytokine induction (e.g., cytokine storm), and even undesirable immunosuppression [16]. Even with good *in vitro-in vivo* correlations observed for hemolysis, complement-mediated responses, and cytokine-mediated ones [17], the immune system comprises several types of immune cells and other organism's cells. Because of that, it is difficult to assess the immunological reactions *in vitro*. Besides, current synthetic-material-based nanosystems are relatively bulky (>100nm), causing them to accumulate preferably in the liver and tumors [18], as opposed to smaller particles that distribute systemically. Consequently, *in vivo* models are needed to determine their biodistribution patterns. Hence, it is not possible yet to discard animal studies.

Following the systemic administration of the non-viral vectors, numerous barriers arise in a highly dynamic system (Figure 2). Upon entering the bloodstream, the formation of a so-called protein corona is described and seems to perform a vital role in the delivery capabilities of the nanosystems [19]. The coronal components shift over time, being classified as the soft-corona and the hard-corona, as per the transient low-affinity interactions and the permanent high-affinity interactions, respectively, of proteins present in the blood. A nanosystem that passive targets tissues rely on blood vessel permeability, superior in tumors due to the abnormally induced angiogenesis, a phenomenon known as the enhanced permeability and retention effect or EPR for short [20]. But when designing a non-viral vector with active targeting, blockage of the targeting ligand (e.g., transferrin, antibodies) by molecules adsorbed onto the surface needs to be accounted for as it may affect its capability to bind to the target-

receptors, ultimately affecting its biodistribution [21]. Moreover, the existence of the protein corona seems to play a crucial role in the hemolytic and thrombogenic occurrences [22].

Although non-viral vectors can be injected into the target tissue directly to bypass bloodstream's obstacles, other techniques, working towards the systemic administration route, are still emerging. Attempting so unveils a hurdle, considering several animal models react differently compared to humans in the presence of exogenous structures [16]. This review covers some advancements concerning those other techniques later on.

Toxicological studies are also essential as a prescreening tool. These studies should be carried out utilizing serums of different origins, given that the protein corona structure varies accordingly [22]. The formation of the protein corona can originate undesirable immunostimulation, possibly resulting in the secretion of cytokines [23] and inflammatory mediators, as well as activation of the complement cascade [24]. Likewise, it is known properties like particle size, shape, and surface chemistry define their immunological

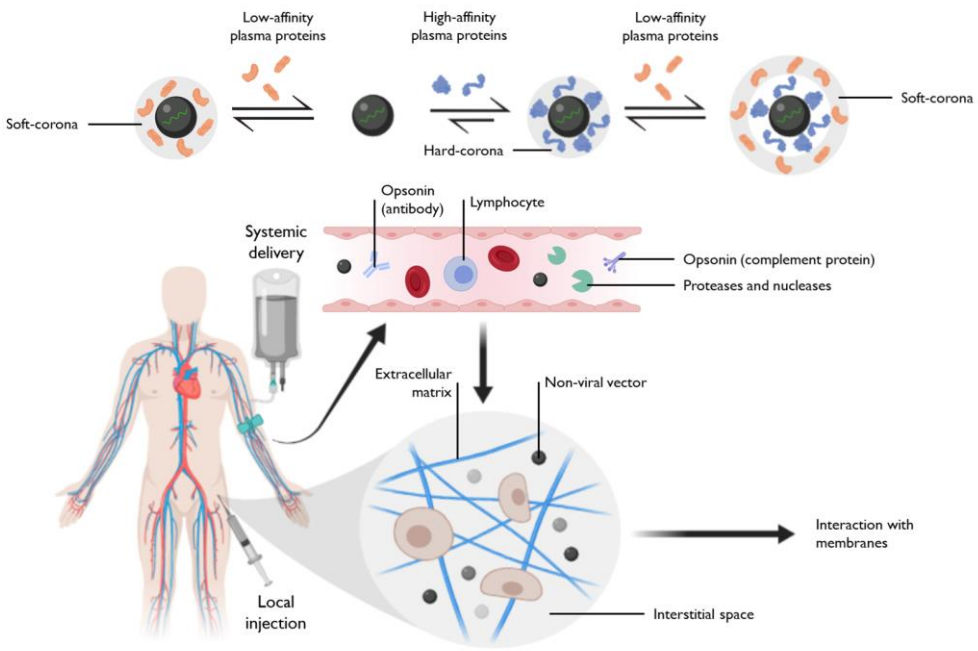

Figure 2. Physiological barriers to *in vivo* systemic delivery of non-viral vectors. After the systemic administration, the non-viral vectors need to extravasate the vascular endothelium (unless it is targeting the endothelial cells), travel across the interstitial space, and reach the target cells' nucleus or cytosol, depending on the cargo. While in the bloodstream, the non-viral vectors acquire a protein corona, which modulates their ability to interact with cell membranes. On top of that, they can also be exposed to opsonins, driving to their clearance by the mononuclear phagocyte system. Concomitantly, plasma circulating proteases and nucleases degrade any unprotected nucleic acids. Escaping these harsh conditions does not get easy since the endothelial cells' junctions prevent bulky non-viral vectors from reaching the interstitial space. After reaching the latter, the extracellular matrix may confine systemically administered non-viral vectors close to the blood vessels' surface and locally injected ones to the site of injection. Lastly, the non-viral vectors need to pass through the cell membrane.

compatibility [25]. So, it is only valid to reiterate the importance of an extensive characterization during the preclinical developments, providing more representative data to subsequent clinical phases of development altogether.

LIPOPLEXES AS DELIVERY NANOSYSTEMS

Cationic lipids, for example, 1,2-di-O-octadecenyl-3-trimethylammonium-propane (DOTMA), the first synthetic cationic lipid reported [26], naturally complex with the negatively charged nucleic acids through electrostatic interactions. The resulting lipoplexes can be stabilized further with the use of neutral lipids, like cholesterol, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) [27], henceforth referred to as helper lipids. Rather than just providing better stability, the application of helper lipids, explicitly DOPE, offered higher transfection efficiency *in vivo* [27]. So, new synthetic methods to strengthen the helper lipid arsenal are noticeably important.

Self-assembled lipoplexes result in the formation of either a bilayer structure or an inverted non-bilayer structure. In a bilayer structure, the nucleic acids stand between the cationic membranes of liposomes, whereas in an inverted non-bilayer structure, they condense within the inverted lipids [28]. It is apprehended formulations containing DOPE and cholesterol are more inclined to favor the fusogenic inverted phase, providing better cargo delivery through the enhanced membrane destabilization [29].

The biodistribution of lipoplexes can be modified accordingly to their net surface charge. For instance, by varying the lipid to RNA ratio, positively charged lipoplexes tend to accumulate in the lungs, whereas lesser ones concentrate on the spleen. This particular property granted remarkable results in advancing the treatment of certain types of cancer [30], with potential applications for other therapies. Nevertheless, due to the net positive surface charge, these nanosystems are prone to trigger an immune response by activating both the innate and adaptative immune system mechanisms. Assuming the major role protein corona has on mediating immune responses [21, 31], studies on pre-coated lipoplexes with an artificial corona revealed it is possible to mitigate the interaction with immune cells [22].

Owing to the observed cytotoxicity, a DOTMA-homologue lipid possessing biodegradable ester bonds emerged, 1,2-dioleoyloxy-3-trimethylammonium-propane (DOTAP), and proved to cause lower toxicity [32]. Yet, significant levels of proinflammatory cytokines, such as IL-1β and TNF-α, were reported after systemic administrations of lipoplexes [33]. In an attempt to avoid immune responses and increase their half-life, the PEGylation of

cationic liposomes became a gold standard, being DOPE and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) the favored helper lipids to undergo that modification [9].

Although the PEGylation of the formulations improved the stability in the bloodstream [34], repeated administrations led to the reduction of lipoplexes' half-life, a phenomenon called accelerated blood clearance (ABC) effect [35, 36]. Right after the first administration of PEGylated lipoplexes, antigen-presenting cells (APCs) sense the PEG moieties and present them to naive T cells. In turn, naive T cells polarize into CD4+ and CD8+ T cells. At that point, CD4+ T cells activate B cells [37], which govern the humoral adaptative response, culminating in the formation of memory B cells. In succeeding administrations of PEGylated lipoplexes, memory B cells differentiate into antibody-producing plasma cells (Figure 3A). Their anti-PEG IgM antibodies target PEG moieties, opsonizing any PEGylated structures with complement proteins [38], accountable for directing PEGylated lipoplexes to the liver [35, 39]. To circumvent this effect, the replacement of PEG with a zwitterionic polymer, poly(carboxybetaine) (PCB), came about as a reliable alternative [35] (Figure 3B). PEG, an amphiphilic polymer, tends to dehydrate the polar head group region of lipoplexes, destabilizing those formulations lacking helper lipids. As a substitute, PCB still delivers the stealth-conferring hydrophilic layer to lipoplexes while further hydrating that same region due to its super-hydrophilic nature, even without the aid of helper lipids [40]. Acknowledging it does not instigate the ABC effect [35], the former property adds up an advantage. A closer look into PCB's structure denotes its separate positively and negatively charged groups as the main contributors to that nature (Figure 3C).

Lipoplexes' physicochemical properties are critical to their ability to overcome physiological barriers to nucleic acid delivery. As an example, less saturated hydrocarbon lipid chains impact the phase transition temperature, leading to an increase in membrane fluidity, which, in turn, results in higher transfection efficiency [41]. A recent study demonstrated it is possible to achieve superior cellular uptake and delivery with lipids possessing no linker molecules and asymmetric hydrocarbon chains, i.e., one saturated and one unsaturated when contrasted with symmetric ones [42].

All the above-mentioned approaches endeavor to tackle a vast number of problems with this type of non-viral vectors. Despite several successful attempts to minimize some issues, a search for different strategies endures.

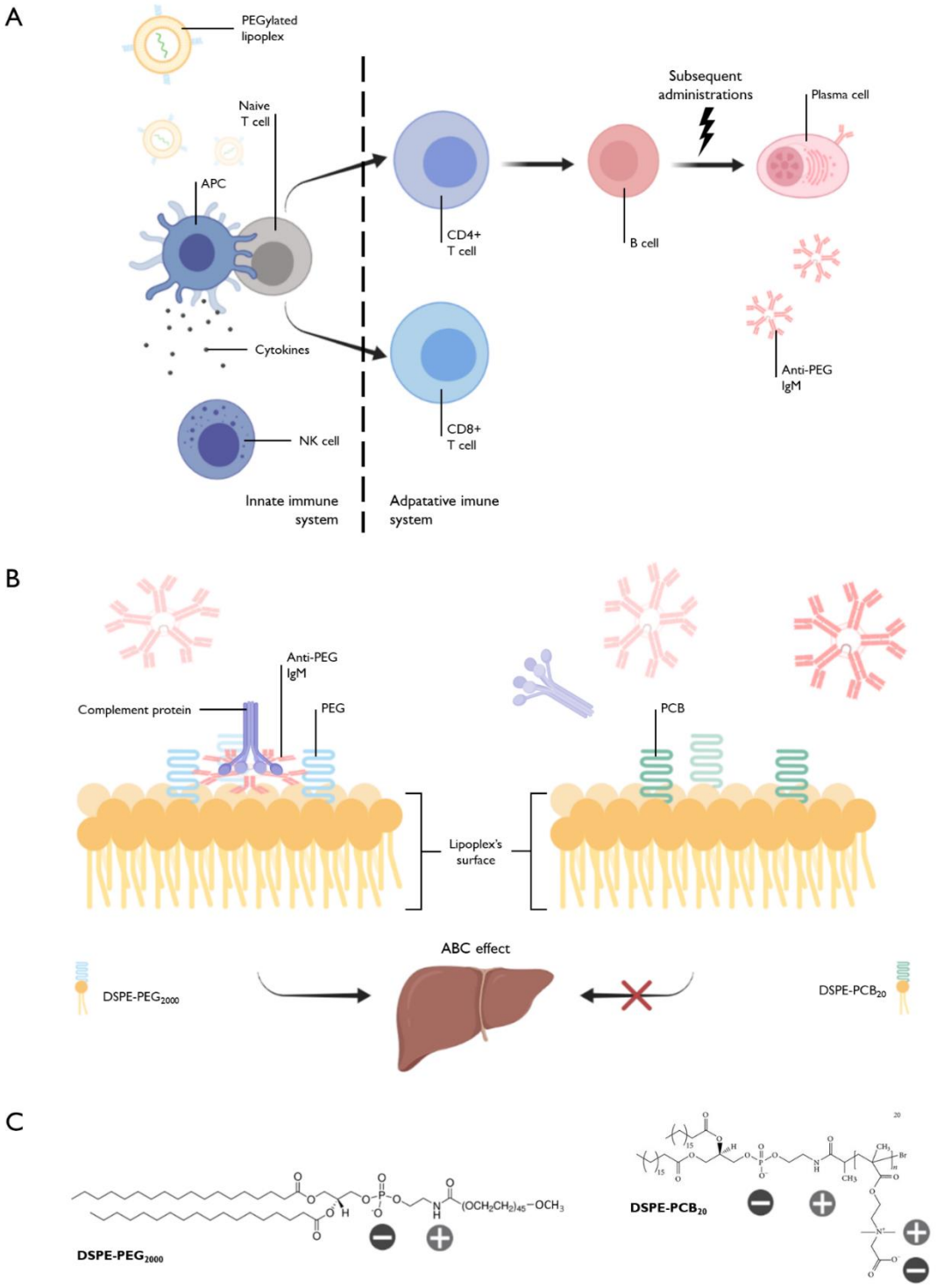

Figure 3. Visual concept of the accelerated blood clearance effect coordinated by the innate and adaptative immune response to PEGylated versus PCBylated lipoplexes. (A) The first in vivo systemic administration of PEGylated lipoplexes conducts to a series of innate immune responses, mediated by APCs, e.g., dendritic cells and macrophages. These cells process and present antigens for recognition by naive T cells. It ultimately sets off a sequence of adaptative immune responses leading to the release of anti-PEG IgMs. By norm, this is a slower response, justifying why the ABC effect only becomes apparent on subsequent administrations. In the meantime, cytokines regulate other immune cell's responses to foreign particles, for instance, NK cells. (B) Succeeding administered PEGylated lipoplexes end up with pentameric anti-PEG IgMs binding to their surface PEGs in a staple form, allowing for complement proteins to attach and enhance hepatic accumulation. In

contrast, that does not apply to PCBylated ones, as there are no reports on such effect taking place. (C) The main structural difference from PEG to PCB resides on the presence of positively and negatively charged groups on the latter, the reason for its greater hydrophilicity.

ABC: accelerated blood clearance; APC: antigen-presenting cell; DSPE: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; IgM: immunoglobulin M; NK: natural killer; PCB: poly(carboxybetaine); PEG: poly(ethylene glycol).

A Different Take on Conventional Lipoplexes: Lipid Nanoparticles

Lipid nanoparticles (LNPs) present an alternative to evade the use of exclusively cationic lipids. LNPs blend different lipids, which, in turn, enable improvements to their physicochemical properties, by modifying the lipid composition or the synthesis method, known to have a direct impact on the biodistribution and delivery efficiency. Commonly, LNPs have an ionizable lipid [43], cholesterol [44], another helper lipid [45], and also a PEGylated lipid. With the aid of cholesterol, their structural integrity is more robust, while the helper lipid provides support to the bilayer structure. On top of that, the PEGylated lipid diminishes aggregation occurrences and avoids nonspecific interactions [44-46].

The ionizable lipids serve the purpose of conferring a neutral charge at physiological pH (~7.4) and a cationic charge at acidic pH. The neutral charge of the LNPs potentiates its internalization into cells via apolipoprotein E (apoE)-mediated endocytosis. Thus, the absence of positive charges minimizes cytotoxicity and simultaneously induces apoE receptors-rich cells' uptake of LNPs [47].

Also, according to a recent study on ionizable lipids identified via combinatorial synthesis and high-throughput screening, tertiary amines as ionizable headgroups are active setting off a pH-triggered conformational switch of the lipids, and, therefore, are more liable to escape the endosomal/lysosomal compartment [48]. Under endosomal/lysosomal pH values (~5-6), the protonation of the lipid headgroup's pyridine modifies its hydrodynamic radius by allowing intramolecular hydrogen bonding (Figure 4A), ultimately inducing the rearrangement into an inverted non-bilayer structure. It is pivotal for the ionizable lipids to have an acid dissociation constant (pKa) value for the pyridine within the range of the endosomal/lysosomal pH one. To that end, in silico predictions lead to the selection of the headgroup structure that provided an optimal pKa value of 5.39, a 4-dialkylaminopyridine derived-cationic switchable lipid (Figure 4B). It is suggested the ionizable and endogenous anionic lipids adopt a cylindrical molecular shape, hence packing in a bilayer structure. But, at lower pH values, conformational changes to the ionizable ones make both likely to mix, producing ion pairs. The cross-sectional area of the combined headgroups is smaller than the one of each apart. Thus, the pairs manifest a conic molecular shape, promoting the formation of the inverted non-bilayer structure [49].

The acquired structure more promptly induces the displacement of lipids and membrane fusion with the endosome/lysosome compartment.

As previously demonstrated, LNPs portray the benefits of a rational approach when designing the non-viral vectors. The screening of a broad library of lipids enabled the selection of those with higher transfection rates and cell viability. This technique also permitted the establishment of structure-activity relationships that are intimate with LNPs' toxicity and biodistribution.

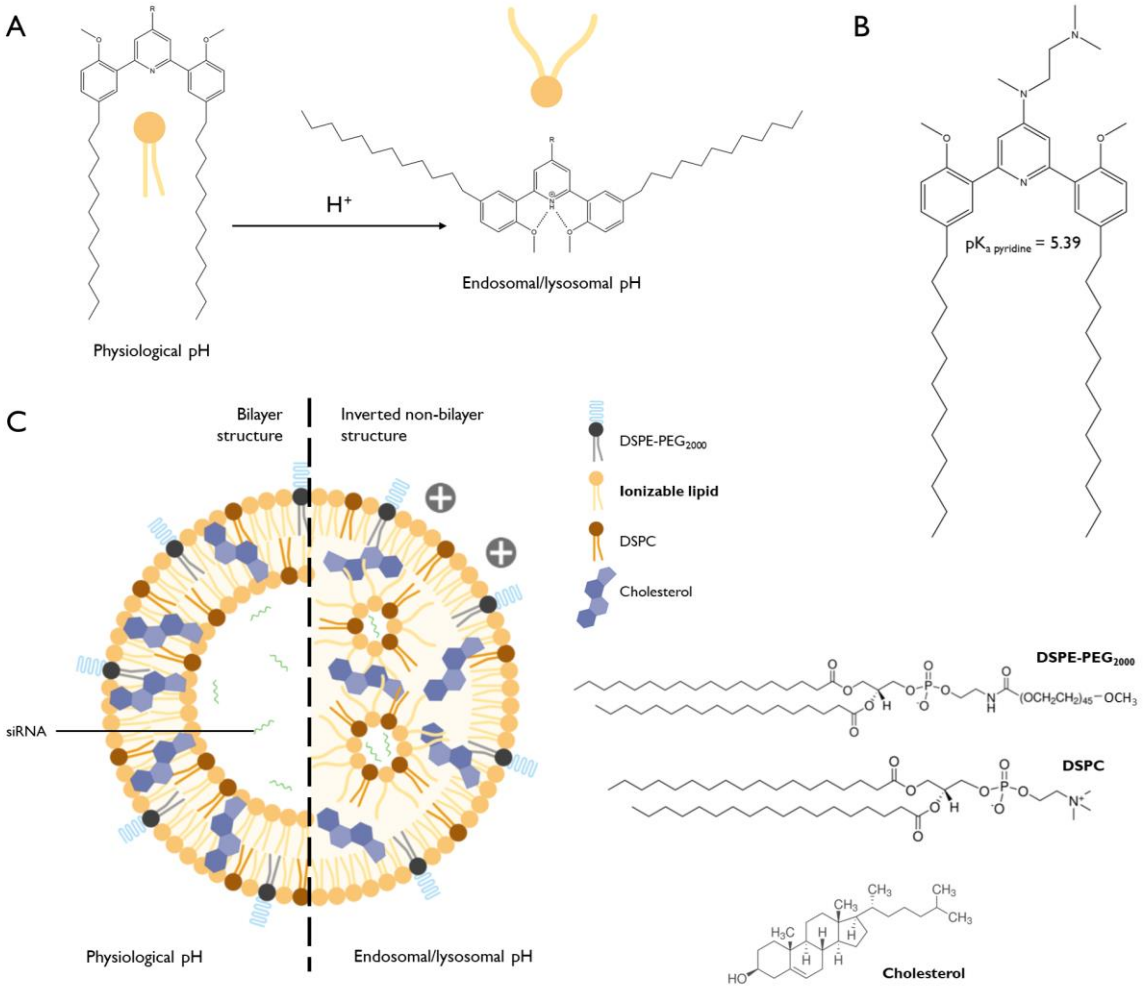

Figure 4. Lipid nanoparticle rational design for siRNA delivery. (A) Protonation-induced conformational change of the pH-sensitive ionizable lipids presumed to impact the LNP destabilization inside the endosomal/lysosomal compartment, resulting in endosomal escape and siRNA delivery. (B) Chemical structure of the lead rationally designed ionizable lipid. (C) Schematic representation of an LNP incorporating the ionizable lipid, a PEGylated lipid (DSPE-PEG_{2000}), DSPC as a helper lipid, and cholesterol. Towards the endosomal/lysosomal pH, the protonation-induced conformational change rearranges the LNP into an inverted non-bilayer structure, making it more prone to fuse with the endogenous anionic lipids from the cellular membrane.

DSPC: 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE-PEG_{2000}: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]; LNP: lipid nanoparticle; siRNA: small interfering ribonucleic acid.

POLYPLEXES AS DELIVERY NANOSYSTEMS

These particulate complexes originated from electrostatic interactions, linking cationic polymers and negatively charged nucleic acids, are known as polyplexes [50]. Typically containing a high density of amine groups, poly(ethylenimine) (PEI) and poly-L-lysine (PLL) are among the most thoroughly studied synthetic cationic polymers. Other carbohydrate-based polymers are used, such as chitosan [51], a semisynthetic natural-based one [52].

Usually, the synthetic cationic polymer's amine groups are mostly protonated below physiological pH values, meaning these nanosystems attain better colloidal stability in the bloodstream than lipoplexes [53]. Though, in terms of delivery efficiency and toxicity, distinct polymers have drastic differences, as discussed hereafter.

Chitosan-based Polyplexes

For instance, the chitosan structure includes repeating units of β-1,4-D-glucose-2-amine and N-acetyl-D-glucose-2-amine [51]. At acidic pH values, their primary amine groups (pKa = 6.5) are responsible for the polymer's positive charge, inadvertently allowing its application for nucleic acid delivery [54].

PLL-based Polyplexes

Historically, PLL was one of the first to be employed in nucleic acid delivery nanosystems [55]. Attributable to the PLL-based polyplexes positive zeta potential, these vectors enter host cells via endocytosis. But, despite the intrinsic properties favoring their cellular uptake, PLL's bottleneck resides in the low transfection efficiency [56, 57], hinting at a poor endosomal/lysosomal escape of polyplexes, something to be expected as the pKa of its amine groups falls well above the 5-6 interval (pKa = 10.5) [58, 59], the endosomal/lysosomal pH range. Because all its amines remain protonated in the bloodstream, some introduce endosomal lysis-inducing agents to PLL-based polyplexes, like chloroquine [60], to effectively ease the release of its cargo. Additionally, PLL-based polyplexes' safety profile closely correlates with PLL's molecular weight, where higher molecular weights result in higher cytotoxicity [61], associated with the greater PLL's charge density.

PEI-based Polyplexes

Another synthetic cationic polymer, PEI, under physiological pH values, only remains partly protonated, but when encountered with acidic conditions, it further increases its positive charge density, a process known as the proton sponge effect. It sets off an influx of chloride counter ions, raising the internal endosomal osmotic pressure, finally bursting its structure [62]. The described phenomenon explains how PEI can destabilize the endosomal vesicles to facilitate the delivery of the nucleic acids, although unaltered PEI-based polyplexes interaction with serum proteins often engulfs it, preventing the proton sponge effect of taking place [19]. Plus, PEI-based polyplexes raise concerns regarding their cytotoxicity, attributed to their high molecular weight forms and surface's positive charge density [63]. Nonetheless, the branched isomer, BPEI, remains the gold standard and most thoroughly studied compared to its linear counterpart, LPEI. The pKa of the latter is 7.4, while the overall pKa of the branched one is 8.4. So, according to the Handerson-Hasselbalch equation, at physiological pH, about 55% of LPEI secondary amines do protonate, contrasting with 90% of BPEI's [64]. The theoretical findings justify the superior BPEI complexation with nucleic acids experimentally observed.

PBAE-based Polyplexes as Lead Candidates

More recently, a different type of cationic polymer has emerged, the poly(β-amino ester) or PBAE. This polymer shares some properties of PEI while being less toxic, making it a promising candidate for gene therapy [3, 65]. PBAE-based polyplexes present reduced toxicity due to hydrolytically-cleavable ester bonds [66].

Similar to LNPs, this kind of polymers resulted from the screening of a broad library of polymers looking for a better-suited structure [67]. As a case study, highly branched PBAEs were designed and optimized. The optimized lipoplexes presented high transfection efficiency (77% in human multipotent adipose-derived stem cells and 52% in primary astrocytes) while preserving above 90% cell viability [65]. Further supporting their potential, PBAE-based polyplexes also provided superior transfection efficiency and low cytotoxicity in primary macrophages, compared to PEG-g-PEI [68]. Adding up to their safety profile, PBAE also presents a buffering capacity in the relevant pH range (pH 5.1-7.4) that, for a small library of modified linear PBAEs [69], varies from 34% up to 95% protonatable amines, deeming PBAE-based polyplexes capable of effective endosomal escape. On a per mass basis, the buffering capacity of the modified PBAEs reportedly goes up to 4.6 mmol H+/g, lower than that of 25

kDa BPEI (6.2 mmol H+/g). Despite buffering fewer protons per unit mass, the former polymers degrade faster, i.e., formulations with higher w/w ratios can surpass the buffering capacity of the current gold standard, BPEI, without necessarily being more toxic. That same study concluded uptake and transfection with these polyplexes are dependent on the polymer end-group structure, or more specifically, amine-terminated PBAEs significantly performed better than acrylate-terminated ones with minimal impact on particle size, zeta potential, and cytotoxicity. Another study demonstrated it is possible to tune the surface charge of the polyplexes by modifying the acrylate-terminated PBAE with differently charged oligopeptides [70]. These findings create an opportunity to explore this feature in future formulations.

Strategies to Enhance Polyplexes' Colloidal Stability

As previously reported, serum proteins can dramatically interfere with polyplexes' delivery capabilities [19]. By combining various types of polymers and changing surface properties, it is possible to enhance polyplexes' solubility and stability. The PEGylation of polyplexes, a well-established strategy, addresses this problem [71, 72]. But this strategy is flawed, either because of the inducing of the ABC effect or due to PEG's weak stability to lyophilization [73], a popular method to dehydrate and avoid degradation of polymers, such as poly(lactic-co-glycolic acid) (PLGA). During the lyophilization, PEG crystallizes [74], making it unlikely to prevent aggregation on resuspension. The better alternatives, zwitterionic polymers, such as PCB, stands out as it ensures colloidal stability after resuspension since it prevents crystallization thanks to its super-hydrophilic behavior, resolving the major drawbacks associated with PEG.

It was even hypothesized shear stress at magnitudes sensed in the bloodstream may impair the PEG outer layer of polyplexes, resulting in the degradation of its core by nucleases. Disulfide cross-linking was introduced to the core region to prevent the rapid clearance caused by shear stress, improving the *in vivo* blood circulation profile [75].

THE CURRENT STATE OF LIPOPLEXES AND POLYPLEXES DEVELOPMENT

As we enhance our prescreening tools to better predict *in vivo* outcomes of non-viral vectors, the portfolio of formulations described in the literature keeps growing. A quick analysis of the most recent formulations still reveals an unsystematic approach, an obstacle to successful translation into clinical practice.

Nowadays, most of the *in vitro* studies do not provide accurate and particularly comparable data for parameters like particle size, colloidal stability, and surface chemistry, some of the physicochemical properties known to be related to immunogenicity and toxicity [15]. Therefore, biocompatibility may stay unknown until later stages.

The lack of comparable and clinically relevant sets of data limits our know-how on how to optimally fine-tune the above-stated inherent properties of nanosystems to improve tolerability. Along with those, external elements, like the protein corona, require indirect control when engineering the nanosystems' properties [15]. As previously discussed, the protein corona formed right after an intravenous administration may result in unpredictable fluctuations in size and surface charge, and also affect the targeting capacities of the developed non-viral vectors. Altogether, those constitute some of the already debated reasons to concur blood compatibility analysis should be more thorough, possibly introducing a standardized *in vitro* serum interaction assay. As evidenced in Table 1, a vast heterogeneity of protocols is noticeable. First and foremost, *in vitro* assessments were carried out with different experimental methods, each yielding different types of information about the impact on the formulations' properties. While a gel electrophoresis assay can conclude if the tested formulation adequately protects its cargo from degradation by serum nucleases, it does not inform us whether the presence of serum interferes with its particle size, aggregates formation, or even its transfection efficiency. There are some, however, performing both dynamic light scattering (DLS) to quantify size variations and transfection studies in different cells.

Table 1. *In vitro* characterization results of distinct lipoplexes and polyplexes formulations and the impact on their properties after interacting with blood components.

Non-viral vector	Composition	Before			Serum interaction assay	Method	Output	Ref.
		Size (nm)	PDI	ζ potential (mV)				
Lipoplexes	Lec/Chol/DOTAP/DOPE	166	0.37	+30	10% FBS [a]	Gel electrophoresis w/ heparin displacement	This formulation provided adequate protection to its cargo, as there was minimal to no degradation of pDNA following a 4-hour serum incubation.	[76]
	HSPC/Chol/HODA/DOPE/ DSPE-PEG_{2000}	95	0.24	+30	10% FBS [b]	Gel electrophoresis w/ phenol-chloroform extraction	The lipid nanoparticles were able to retain above 90% pDNA activity for at least 8 hours in serum conditions.	[77]
	Chol/DOTAP	175	0.15	+30	10% FBS [b]	Gel electrophoresis w/ Triton X-100 displacement	Naked siRNA reportedly underwent serum degradation after 6 hours but proved to be stable up to 24 hours in serum conditions when complexed with the liposomes.	[78]
	M6/DOPE	172	0.26	-18	10% FBS [b]	Transfection studies in Hela and 293T cells	The transfection efficiency of lipoplexes with the added synthetic cholesterol-derived M6 lipids suffered little impact with the introduction of serum, even capable of surpassing other commercially available formulations.	[79]
Polyplexes	CD-OEI/PPD/PPS	232	0.26	-1.2	10% FBS [a]	Dynamic light scattering	Adding small amounts of PPS to the PPD-coated polyplexes proved to be effective in maintaining the particle sizes constant during incubation with 10% FBS.	[80]
					10 – 40% FBS [b]	Transfection studies in HeLa cells	At 40% serum concentration, the negative controls, PEI 25kDa and CD-OEI, kept only 2.5 and 0.5% of their serum-free transfection efficiencies, respectively. On the other hand, the coating mix of PPD and PPS maintained 48%.	

Non-viral vector	Composition	Before			Serum interaction assay	Method	Output	Ref.
		Size (nm)	PDI	ζ potential (mV)				
Polyplexes (cont.)	PUBAP/Dex-SS-BPEI-FA	265	0.15	+12	5 – 50% FBS [c]	Transfection studies in SKOV-3 cells	The transfection efficiency of the targeting polyplexes was not significantly reduced up to 10% FBS and provided similar expression levels to Lipofectamine® 2k after 24 hours after. Increasing to 50% FBS does not translate into a profound loss in transfection efficiency. By contrast, BPEI 25kDa polyplexes lost their transfection efficiency by ca 18 times.	[81]
	PEG-PCD-F	170	*NA*	+10	10% FBS [a]	Dynamic light scattering	Unlike PCD and PEG-PCD controls, after incubation with 10% FBS for 24 hours, the fluorinated polyplexes granted superior colloidal stability with negligible particle size increase.	[82]
					10 – 40% FBS [d]	Transfection studies in 4T1 and B16F10 cells	Even in the presence of 30% FBS, the polyplexes provided around 60% of gene expression, much higher than the one offered by PEI 25kDa polyplexes.	
	ε-PLL	194	*NA*	+11	10% FBS [a]	Gel electrophoresis w/ heparin displacement	The polyplexes' integrity was sustained to some extent for at least 3 hours, undergoing complete degradation by serum enzymes subsequently.	[83]

Non-viral vector	Composition	Before			Serum interaction assay	Method	Output	Ref.
		Size (nm)	PDI	ζ potential (mV)				
Polyplexes (cont.)	PFF-OEI	150	0.15	+50	10% FBS	Dynamic light scattering	After 2 hours, the polyplexes' particle size was not significantly altered with the addition of serum, hinting at high colloidal stability.	[84]
					10 – 50% FBS [e]	Transfection studies in HeLa cells	Even in the presence of 50% FBS, the formulation exhibited high transfection efficiency, cellular uptake efficacy along with well-maintained endosomal disruption capability.	
	PBAE/CM-PLH	174	*NA*	+25	5 – 50% FBS [e]	Transfection studies in HEK293 cells	At 50% FBS, polyplexes formed with only PBAE manifested a transfection efficiency ca 3-fold lower than the one obtained in serum-free media. However, the polyanionic shielding enhanced their serum stability, this way able to score 50% transfection efficiency at 50% FBS.	[85]
Lipopolyplexes	Chol/DOTAP/DSPE-PEG_{2000}/BPEI	99	*NA*	+43	50% FBS	Turbidity variation	No significant changes to the transmittance values reported after a 24-hour incubation period in serum conditions, i.e., no signs of aggregates formation.	[8]

[a] FBS was diluted in PBS

[b] FBS was diluted in DMEM cell culture medium

[c] FBS was diluted in McCoy's-5α cell culture medium

[d] FBS was diluted in RPMI-1640 cell culture medium

[e] FBS was diluted in H-DMEM cell culture medium

BPEI: Branched poly(ethylenimine); CD: Cyclodextrin; Chol: Cholesterol; CM-PLH: Carboxymethyl poly(L-histidine); Dex: Dextran; DOPE: 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTAP: 1,2-dioleoyloxy-3-trimethylammonium-propane; DSPE: 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; F: Fluorinated; FA: Folate; FBS: Fetal bovine serum; HODA: Histidine modified octadecylamine; HSPC: Hydrogenated soy phosphatidylcholine; Lec: Lecithin; *NA*: Not available; OEI: Oligoethylenimine; PBAE: Poly(β-amino ester); PCD: Poly(CBA-DMDPTA); pDNA: Plasmid DNA; PEG: Poly(ethylene glycol); PEI: Poly(ethylenimine); PFF: Perfluorooctanoyl fluoride; PLL: Poly-L-lysine; PPD: pH-responsive anionic polymer; PPS: pH-insensitive anionic polymer; PUBAP: Polyurethane containing 1,4-bis(3-aminopropyl)piperazine; SS: Disulfide link.

Furthermore, if we are to perform an analysis of the protein corona composition solely *in vitro*, it might fail to predict the fate of the non-viral vectors *in vivo* simply because it is now known shear stress in the bloodstream can significantly alter it both quantitatively and qualitatively [86]. Some studies on protein corona fingerprints started to correlate them with the cellular uptake efficacy in physiological milieus. It is noteworthy that LNPs dynamically incubated in the presence of fetal bovine serum (FBS) held an inferior content of inter-α-trypsin inhibitor and AMBP protein than those statically incubated. Subsequently, MCF7 cancer cells exhibited a smaller cellular uptake [86], like what a prior report described for other types of non-viral vectors on A549 human lung epithelial carcinoma cells [87]. These findings might justify working towards the introduction of a new dynamic serum interaction assay and reevaluate the structure-activity relationships established up until now exclusively based on *in vitro* studies.

Also supported by Table 1, the vast majority used FBS at 10% to simulate the *in vivo* conditions. It is still debatable whether this concentration of serum is physiologically representative for all formulations. Providing some insight into this, a study [19] conducted with a wide range of FBS concentrations evaluated its effect on the particle size, zeta potential, and transfection efficiency in HeLa cells of PEI/DNA polyplexes. It reports an increase in their hydrodynamic diameter preceding a significant decrease with further increments in the FBS:DNA weight ratio. When it is above 24, only non-significant decreases in size and zeta potential occur, hinting at a saturated state of protein adsorption. With the most commonly used DNA doses ranging between 1 and 5 μg/mL, 10% FBS returns weight ratios ca. 175 down to at least 35 times superior to 24, making the PEI polyplexes achieve full saturation with serum proteins. Further on this subject, the CD-OEI/PPD/PPS polyplexes' transfection studies also in HeLa cells described considerably better results at 40% FBS compared to its negative controls [80]. But, after examining the reported data, we conclude there are no significant differences between the values obtained with 10% and 40% FBS. It is necessary to notice that while this may be true for these, it cannot serve as a basis for all non-viral vectors. Take PBAE/CM-PLH polyplexes, for instance, where an increase from 10% to 50% FBS resulted in a ca. 20% reduction in their transfection efficiency in HEK293 cells [85].

In line with the non-viral vectors' thorough characterization, no single technique can fully characterize a disperse nanosystem sample. All size distribution assessment results presented in Table 1 stem from DLS measurements, providing the mean hydrodynamic diameter. Yet, it is advocated to resort to at least one other method, such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM), popular imaging techniques. Found to make use of TEM as a second method are just three among all reported vectors in

Table 1. In recent times, nanoparticle tracking analysis or NTA emerged as a better verification tool for DLS [88]. Even though none of Table 1's entries reported making use of it, NTA is arguably the best tool to verify DLS measurements since it relies on the same physical property, their diffusion coefficient. While the size reports of DLS rely on fluctuations of the scattered light, NTA ones depend on the number of single particles captured in a series of optical images. This way, we can account for DLS's inherent limitation of biased intensity-based detection when significantly larger aggregates than the main population offset the size distribution curve.

Table 2. Toxicity *in vitro* assay results of the interaction of different lipoplexes and polyplexes' materials with the biological environment.

Non-viral vector	Composition	Hematotoxicity		Immunotoxicity			References
		Hemolysis	Thrombogenicity	Complement activation	Cytokine induction	Cell viability	
Lipoplexes	DOTAP	•	•	*NA*	*NA*	•••	[89-91]
	DOTAP/DOPE	•	*NA*	*NA*	*NA*	•••	[77, 89]
	LNPs	-	-	-	•	••••	[33, 77, 91-93]
	Lipofectamine®	•	-	*NA*	*NA*	•••	[76, 77, 91]
Polyplexes	Chitosan	-	•	-	-	••••	[94-98]
	PLL	•	*NA*	••	•••	•••	[64, 83, 98, 99]
	LPEI	••	•	*NA*	••	•••	[64, 98, 100-102]
	BPEI	•	•	••	••	•••	[64, 98, 101-103]
	PBAE	-	*NA*	*NA*	-	••••	[69, 70, 98, 104, 105]

NA: Not available; Scale: -: Negligible; •: Low; ••: Moderate; •••: High; ••••: Very high.

BPEI: Branched polyethylenimine; DOPE: 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTAP: 1,2-dioleoyloxy-3-trimethylammonium-propane; LNPs: Lipid nanoparticles; LPEI: Linear polyethylenimine; PBAE: Poly(β-amino ester); PLL: Poly-L-lysine.

To better predict interactions in the bloodstream and non-viral vectors-caused immunogenicity, additional *in vitro* assays await implementation, especially when planning on using the systemic route of administration. In conformity with Table 2, cell viability alone does not directly translate to a safe *in vivo* profile. Proofs of that are some formulations that accounted for moderate to high cytokine induction while demonstrating high levels of cell viability. Interestingly, among those are BPEI-based polyplexes, often regarded as the gold standard, showcasing moderate complement activation and some degree of hemolysis as well as potential thrombogenicity. Apart from LPEI lacking detail on its potential to activate the complement system, compared to BPEI, the former causes a higher degree of hemolysis [98]. More on that matter, molecular dynamics simulations suggest linear polymers with solely

charged backbones, e.g., LPEI, electrostatically interact with cell surfaces more actively than those with backbones and charged side chains. The formed hypothesis is that the positively charged portions of the linear ones are sterically more accessible to the lipid head groups, lysing the red blood cells. So, aside from larger molecular weights and higher charge densities, the hemolysis events depend on the structure of the polymers.

Paving the way for a reality where the published research papers seize comparable and, more importantly, clinically relevant data to evaluate the risk of these non-viral vectors inducing toxicities in humans is the Nanotechnology Characterization Laboratory (NCL) of the Frederick National Laboratory for Cancer Research. When it comes to assessing the common markers halting clinical translation of the nanosystems, like hemolysis, thrombogenicity, cytokine induction, opsonization, and so on, there are too few reference standards for these [106]. Visibly enough, in Table 2, it is possible to conclude there are many preclinical works not addressing pivotal end-points indicative of potential immunotoxicity. Although, for DOTAP and DOTAP/DOPE-based lipoplexes, the missing information appears to result from the loss of interest by the scientific community. Stating that papers making use of DOTAP/DOPE liposomes are utilizing them as performance comparators [77, 89] holds truth. By comparison, presented as the better choice, LNPs do promise improved safety profiles for clinical translation. This finding comes as no surprise given DOTAP mixed with DOPE, a fusogenic lipid, results in a loose complexation. Such complexes may release the nucleic acids sooner, setting off a chain of immune responses while lowering its therapeutic activity. And DOTAP alone would make the excess positive charge density of the liposomes inadequate for therapeutical use [89]. Note that, perhaps, LNPs might also outperform Lipofectamine® transfection reagents with lower hemolysis propensity.

Now, looking back at polyplexes' results, it is clear PBAE-based ones may fare better in terms of biocompatibility than BPEI, but similar to the semisynthetic natural-based polymer, chitosan. Chitosan formulations point to a low level of thrombogenicity, which cannot, however, be compared with PBAE's results because the assessment of further interactions with the latter is thus far unavailable. Plus, there is a need to point out the information on the other assays was retrieved from different sources, therefore, making it possibly misleading. Regardless, PBAE polymers provide a platform for the development of less toxic non-viral vectors. They can be easily synthesized from a variety of molecular precursors, endowing them with different properties. Beware PBAE polymers frequently have narrower buffering capacity intervals than BPEI, the reason why determining their effective pKa value is a crucial indispensable step like it is for ionizable LNPs [107]. With the promising preliminary results

concerning their safety, more thorough *in vitro* assessments with PBAE-based polyplexes are needed, especially tackling their thrombogenicity and cytokine induction tendencies.

To solve the issue of no good or fair *in vitro-in vivo* correlations, the harmonization of methods to support toxicity studies seems at first glance the answer, but current efforts to get them still miss the point of tackling the broad spectrum of end-points. Reportedly, there is an overall lack of harmonization on the dose metrics, assay protocols, and cell species [106]. What is more, the reviewed articles here corroborate this claim. Of course, efforts, as the NCL's *in vitro* assay cascade, to evaluate nanosystems quickly and systematically need to be commended as they can also avoid unnecessary, more costly, and labor-intensive *in vivo* studies. Whenever standardized operating procedures can be adequately employed, invaluable details on how most of these non-viral vectors perform when in contact with blood components, for instance, could be known.

CONCLUSIONS AND FUTURE CONSIDERATIONS

First, the formulations holding great potential *in vitro* as nucleic acids delivery nanosystems are LNPs and PBAE-based polyplexes, although, within a complex biological organism, their interactions must remain adequate to drive the introduction of these innovative delivery systems to our therapeutical arsenal. New to our toolbox, better alternatives to the current standard PEGylation emerged with zwitterionic polymers, such as PCB, which will be beneficial to enhance both lipoplexes and polyplexes' colloidal stability. Just a hint of what the future holds to engineer these non-viral vectors with the inevitable physiological barriers to *in vivo* systemic delivery in mind.

Currently, no standardized operating procedures capable of providing a reproducible and thorough *in vitro* characterization are widely implemented, a fact already verified by other researchers [108]. Since the publication of a review on this exact topic was published back in 2014 [109], little has changed regarding the early adoption of a more exhaustive prescreening before moving onto *in vivo* studies. Non-viral vectors subjected to rigorous safety and efficacy assessments that raise no red flags along the way may hold promise to ultimately enable the translation of new nucleic acid-based therapies from the bench to the bedside. While *in vitro* tests can sometimes fail to predict adverse reactions, the literature supports that many of these have a good enough correlation to *in vivo* responses to justify doing them, even if those events only get detected at later stages of the development. By not skipping steps, not only time and funding can be better focused, but also, the risk of toxicity caused by either lipoplexes or polyplexes will not surpass the benefit to humans.

Finally, here are some considerations for future works on these non-viral vectors: 1) Physicochemical attributes, e.g., size, key factor impacting both lipoplexes and polyplexes *in vivo* behavior and tolerability, should be carried out knowing for a fact no single technique can fully characterize a sample; 2) Characterization of stored samples should become standard since it is known non-viral vectors commonly suffer changes while stored; 3) Studies should include at least two other formulations as comparators, preferably those known to perform well; 4) *In vivo* studies should only be pursued after a rigorous physicochemical characterization, microbial and endotoxin contamination check, and thorough investigations on hematological and immunological responses. One of the outcomes of these, if applied, might very well be seeing a growing number of these products reaching the market in the future.

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