UNIVERSIDADE D
COIMBRA

Marta Baptista Sequeira

Relatórios de Estágio e Monografia intitulada "Cell Membrane-Coated Nanosystems for Gene Delivery Applications" referentes à Unidade Curricular "Estágio", sob a orientação do Doutor Cláudio Cruz e da Professora Doutora Ana Cláudia Santos apresentados à Faculdade de Farmácia da Universidade de Coimbra, para apreciação na prestação de provas públicas de Mestrado Integrado em Ciências Farmacêuticas.

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Coimbra, 30 de Outubro de 2020.

Marta Baptista Sequeira

(Marta Baptista Sequeira)

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"Quero ignorado, e calmo
Por ignorado, e próprio
Por calmo, encher meus dias
De não querer mais deles.

Aos que a riqueza toca
O ouro irrita a pele.
Aos que a fama bafeja
Embacia-se a vida.

Aos que a felicidade
É sol, virá a noite.
Mas ao que nada espera
Tudo que vem é grato."

- Fernando Pessoa

Obrigada!

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Parte I – Relatório de Estágio em Farmácia Comunitária

Abreviaturas

DGS - Direção Geral de Saúde

FFUC - Faculdade de Farmácia da Universidade de Coimbra

MICF - Mestrado Integrado em Ciências Farmacêuticas

MNSRM - Medicamentos Não Sujeitos a Receita Médica

MSRM - Medicamentos Sujeitos a Receita Médica

PVP - Preço de Venda ao Público

1. Introdução

A Faculdade de Farmácia da Universidade de Coimbra (FFUC) é um lugar cheio de oportunidades e conhecimento, que tem muito para dar e ensinar aos alunos que a frequentam.

Como estudante de Mestrado Integrado em Ciências Farmacêuticas (MICF), foram inúmeras as oportunidades propostas: as unidades curriculares opcionais, os congressos, simpósios, workshops e os estágios extracurriculares disponibilizados anualmente. Efetivamente, a FFUC fornece-nos todas as ferramentas para estarmos dentro do que é a realidade atual e o conhecimento aprofundado sobre todos os setores de farmácia.

Ciências Farmacêuticas é um curso bastante completo que despertou em mim o "bichinho" da ciência, o gosto pela descoberta do que é o mundo farmacêutico e as áreas que dele fazem parte.

Neste âmbito, culmino a minha jornada com 810h de estágio curricular em farmácia comunitária, a parte prática que completa o conhecimento teórico que fui adquirindo ao longo destes anos e me permitiu contactar com uma realidade mais próxima do meu futuro.

O meu estágio teve início em setembro de 2019, na Farmácia Sanal, em Oliveira do Bairro - Aveiro.

O relatório de estágio que apresento de seguida através de uma análise SWOT, visa destacar as competências adquiridas ao longo do estágio, os desafios, o que está por melhorar e como pode o farmacêutico, como profissional de saúde, ter impacto e poder efetivamente promover a adesão à terapêutica e a um estilo de vida mais saudável dos utentes.

2. Farmácia Sanal

A Farmácia Sanal localiza-se em Oliveira do Bairro, Águeda, pertencente ao distrito de Aveiro.

A equipa é formada por dois técnicos auxiliares de farmácia e três farmacêuticos, entre os quais o Diretor Técnico.

Oliveira do Bairro é uma localidade situada entre Aveiro e Coimbra, os maiores centros urbanos da região centro de Portugal. Devido a essa estratégica localização, é bastante interessante e diversificada a quantidade de utentes que visitam a farmácia, bem como os aconselhamentos terapêuticos requeridos.

O horário da farmácia é das 9h às 21h, com turnos noturnos até às 00h em dias alternados da semana, uma vez que, a localidade possui apenas duas farmácias e um centro de saúde com consultas até às 23h.

Aos sábados está aberta das 9h às 13h, excepto nos fins de semana em que está de serviço, em que se encontra aberta das 9h às 00h (serviço alternado com a outra farmácia).

Esta farmácia pertence a um grupo de farmácias, First Pharma, com quase 250 farmácias envolvidas e encontra-se devidamente organizada segundo as regras de boas práticas farmacêuticas para a farmácia comunitária (1).

Os proprietários da Farmácia Sanal possuem, para além desta, a Farmácia São José em Sangalhos, localidade vizinha de Oliveira do Bairro.

Figura 1 – *Front office* da Farmácia Sanal

3. Análise SWOT

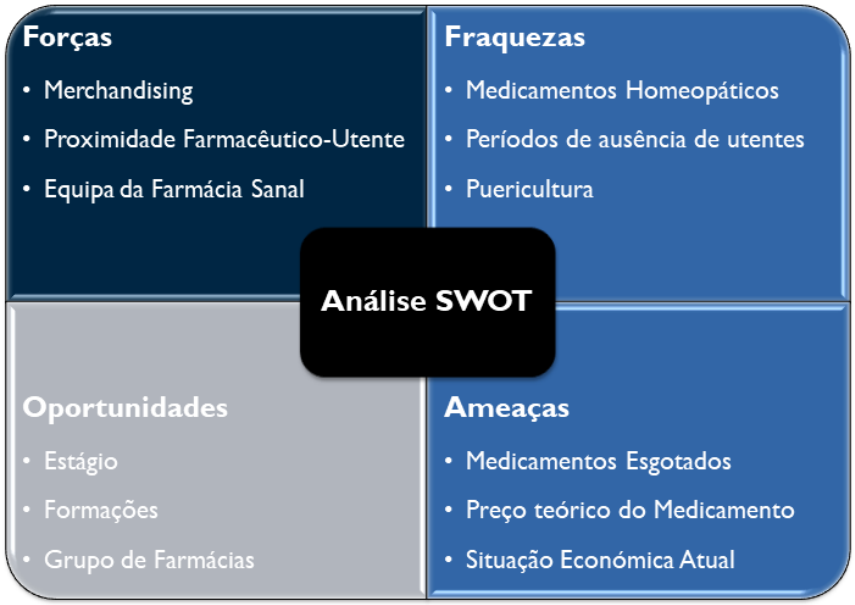

Figura 2 – Esquema da Análise SWOT relativa ao período de estágio em farmácia comunitária.

A apresentação do meu relatório de estágio, sob a forma de análise SWOT um acrónimo para *Strenghts*, *Weaknesses*, *Opportunities* and *Threats*, que se traduz em Forças, Fraquezas, Oportunidades e Ameaças e visa uma compreensão sintética e esquemática da minha análise pessoal correspondente a este período.

3.1 Forças

3.1.1 *Merchandising*

A equipa da Farmácia Sanal integra um técnico auxiliar de farmácia responsável pela disposição dos produtos com formação em *merchandising*, uma ferramenta de *marketing* para potenciar vendas.

Ao longo do estágio pude constatar que a disposição dos lineares tem muita influência no utente e apresenta-se como uma estratégia forte e inteligente para a potencialização das vendas.

A presença de alguns conceitos lecionados no MICF como "*cross selling*", que abrange produtos medicamentosos ou não medicamentosos de alguma forma relacionados dispostos perto uns dos outros estrategicamente, como por exemplo, a exposição de biberões junto dos respetivos produtos de limpeza e esterilização aconselhados; "*First in, first out*", conceito que se aplica não só em relação à disposição dos medicamentos não sujeitos a receita médica (MNSRM) nos lineares, mas também aos medicamentos sujeitos a receita médica (MSRM) nas gavetas, considerando sempre a validade dos medicamentos, de forma a que os de validade mais curta estejam posicionados à frente dos medicamentos com validade mais

longa e incluir a verificação regular dos prazos de validade dos mesmos; disposição por marca, este método facilita a escolha do consumidor e a autonomia na seleção do produto em relação ao preço-qualidade pretendida e preferência, para além disso, transparece organização e variedade de escolha; disposição por indicações farmacêuticas ou por sistemas do corpo humano, também é uma organização auxiliadora tanto para a equipa da farmácia como para o consumidor na identificação daquilo que necessita ou possa identificar por necessidade ou impulso.

3.1.2 Proximidade Farmacêutico-Utente

A Farmácia Sanal apresenta-se como um meio pequeno que transparece uma sensação de acolhimento familiar, não só pelo espaço, mas também pela boa disposição da equipa.

Este ambiente acolhedor e de proximidade proporcionam o estabelecimento de relações de maior confiança entre o farmacêutico e o utente, o que se traduz num melhor acompanhamento dos utentes.

Como futura farmacêutica, é gratificante entender o impacto que temos na vida dos utentes e como a podemos melhorar. O facto de termos a sua confiança permite uma recolha de informação maior acerca do que os preocupa, o que por sua vez permite um melhor atendimento e aconselhamento farmacoterapêutico individual mais personalizado.

Na minha opinião, como primeiro estágio em farmácia comunitária, este foi um ponto bastante forte. Não tinha noção da frequência com que recorrem à nossa profissão para conselhos e dúvidas, quer pessoalmente, quer por chamada, o que destaca o nosso papel e a sua importância para o utente.

3.1.3 Equipa da Farmácia Sanal

Um bom serviço e produtibilidade de trabalho é o que destaca um bom funcionamento e gestão de uma farmácia. As pessoas que integram a equipa não se limitam apenas a fazer o seu trabalho, mas também têm iniciativa para todo o tipo de tarefas, uma base sólida de entreajuda transparente e um bom ambiente de trabalho. Cada um era responsável pela organização e manutenção da limpeza do espaço que utilizava durante o dia e este, mesmo sendo pequeno, estava distribuído de maneira a que fosse possível desempenhar todas as funções necessárias que eram devidamente distribuídas.

No início foram partilhadas muitas dicas para fazer um atendimento mais eficaz e ágil, de forma a encurtar o tempo de espera dos utentes, como ir ao cerne da questão, as mini-formações dadas pelos membros da equipa quando chegavam produtos novos e a disponibilidade prestada em caso de dúvidas no atendimento. A forma evolutiva como foi

planeado o meu programa de estágio permitiu uma melhor integração no espaço, uma melhor relação com os membros da equipa e uma aquisição gradual de conhecimento prático.

3.2 Fraquezas

3.2.1 Medicamentos Homeopáticos

Os medicamentos homeopáticos, apesar de serem mencionados, não são uma vertente muito explorada durante o MICF.

A homeopatia baseia-se no princípio da semelhança e inclui a utilização de substâncias homeopáticas de origem animal, vegetal ou mineral, que são diluídas e dinamizadas para a produção de medicamentos homeopáticos, segundo métodos que estão presentes na Farmacopei Europeia ou de um modo oficial num Estado-Membro (2).

Em alguns atendimentos, senti dificuldade no aconselhamento deste tipo de medicamentos, devido à existência de alguma controvérsia e ceticismo na sua utilização por parte de alguns profissionais de saúde.

Enquanto estagiária, apresentei algumas reservas em aconselhar este tipo de produtos, mas a realidade constatada foi que a homeopatia apresenta uma aderência ainda significativa da parte de alguns utentes. Neste sentido, acho adequado que este tema seja mais explorado, assim como as suas vantagens e desvantagens, uma vez que a sua comercialização é aprovada pelo INFARMED e há um interesse evidente no consumo deste tipo de produtos pelos utentes.

3.2.2 Períodos de Ausência de Utentes

A Farmácia Sanal é um espaço pequeno, adequado às necessidades de uma localidade, com a desvantagem de possuir muitos períodos calmos, com uma maior concentração de utentes da parte da manhã e ao fim da tarde, períodos coincidentes com horários pós-laborais e horários de uma população idosa que frequentava a farmácia maioritariamente da parte da manhã.

Apesar de esta desvantagem limitar o meu tempo de atendimento, este era rentabilizado com pesquisa de informação sobre alguns medicamentos e doenças, exposição de dúvidas que ia apontando, troca de ideias com os membros da equipa em relação a alguns casos práticos e conselhos de forma a melhorar o meu atendimento. Tarefas como alteração de lineares e reposição de stocks, também eram realizadas nestes períodos.

3.2.3 Puericultura

Segundo a Direção Geral de Saúde (DGS), a taxa de natalidade no primeiro trimestre de 2019 foi de 21.348 recém-nascidos, o valor mais alto nos últimos 7 anos (2).

Podemos então deduzir, que a área da puericultura está a entrar numa fase ascendente.

Como farmacêuticos, o nosso papel no acompanhamento do crescimento de uma criança é fundamental, por isso devemos estar munidos de toda a informação possível sobre estes seres com tantas peculiaridades que não podem ter acesso à grande maioria dos medicamentos que a farmácia disponibiliza.

Durante o meu período de estágio detetei muitas lacunas de conhecimento e informação nesta área, apesar de a abordarem parcialmente na maioria das cadeiras leccionadas ao longo dos cinco anos do MICF. Com o tempo, tornou-se evidente que a puericultura é muito vasta em termos de produtos e informação. Muitas dúvidas que pré-mamãs e mamãs expunham eram difíceis de responder com as bases que tínhamos. A possibilidade de existência de uma cadeira opcional sobre este tema ou um maior aprofundamento de conteúdos no MICF seria uma alternativa para um aconselhamento e acompanhamento mais adequado na prática.

3.3 Oportunidades

3.3.1 Estágio

Como única estagiária da Farmácia Sanal, o meu acompanhamento e o espírito de entreajuda dos membros da equipa foram notáveis.

A minha inexperiência foi perspetivada como um incentivo, no sentido de me desafiar, colocar mais empenho nas tarefas propostas e usufruir o tempo de estágio para adquirir o maior conhecimento possível.

No primeiro mês e meio, foquei-me na familiarização com o espaço, disposição dos medicamentos, revisão sobre aconselhamento terapêutico, uso racional do medicamento, a melhor forma de abordar o utente, ferramentas do Sifarma 2000®, técnicas de "*cross selling*", gestão do medicamento, gestão de reservas e devoluções, receção de encomendas e observação de atendimentos. Após esta parte de trabalho "*back office*" necessária, iniciei os atendimentos ao balcão com aconselhamento terapêutico aos utentes, desempenhei um papel mais ativo na disposição da farmácia e realizei todo o tipo de tarefas inerentes ao seu bom funcionamento, sempre devidamente orientada e acompanhada pela equipa que a incorpora.

Este período foi uma oportunidade para melhorar e adquirir novas capacidades como futura profissional, através da solidificação de conhecimentos e perceção de outros que faltavam colmatar.

3.3.2 Formações

Durante este período de estágio tivemos diversas visitas de delegados de informação médica para a apresentação de produtos e formações que tive a oportunidade de assistir.

Estas formações são complementares ao conhecimento teórico adquirido e recordam pormenores em relação a determinadas patologias que se encontram menos presentes. Também considero as sessões de dúvidas no final de cada formação um extra muito importante, porque permite a partilha de experiências, informação e brainstorming, que podem ser úteis no futuro, com farmacêuticos recém-graduados e outros com mais experiência.

Durante o estágio pude realizar algumas formações muito interessantes sobre determinados temas como cefaleias, obstipação e diarreia, tratamentos capilares e suplementação alimentar, entre outras disponibilizadas gratuitamente em plataformas online de empresas multinacionais farmacêuticas.

3.3.3 Grupo de Farmácias

Pertencer a um grupo de farmácias tem as suas vantagens no que diz respeito às visitas frequentes dos responsáveis para confirmar se esta se encontra devidamente integrada nas atividades e propostas do grupo.

Para além das visitas, a First Pharma também providencia diversas formações para os membros da equipa e propostas de produtos com a melhor relação preço/qualidade para venda, proporcionando uma melhor gestão da farmácia.

A Farmácia São José de Sangalhos, como farmácia mais próxima pertencente ao grupo e dos mesmos proprietários, desempenhou um papel muito importante no bom funcionamento da farmácia, com trocas de medicamentos necessários e na realização de encomendas que, posteriormente, dividíamos.

3.4 Ameaças

3.4.1 Medicamentos Esgotados

Os medicamentos esgotados são um problema para tanto para os utentes como para os farmacêuticos.

Por um lado, podem ser vistos como uma oportunidade para colocar o nosso conhecimento em prática e capacidade para aconselhar alternativas igualmente eficazes e

viáveis em relação ao medicamento esgotado. Por outro lado, pode apresentar-se como ameaça, comprometendo a saúde do utente, devido à existência de medicamentos sem alternativa de aconselhamento, como o Eliquis®, correspondente ao fármaco apixabano, um anticoagulante e antitrombótico, inibidor direto do fator Xa, para o tratamento e/ou prevenção de trombose venosa profunda e embolia pulmonar em adultos com idade superior a 75 anos. Este medicamento esteve esgotado durante quase duas semanas durante o meu período de estágio e foi difícil gerir o conflito e a indignação que se gerou perante os utentes (3).

3.4.2 Preço teórico do Medicamento

Por vezes, nas receitas eletrónicas, anexado a cada fármaco prescrito vem referenciado o preço de venda ao público (PVP) do medicamento genérico mais baixo. Apesar de ser um ponto positivo de informação e referência, se apresentarmos outro genérico ligeiramente mais caro ou até um medicamento de marca, a pedido do utente, com uma grande discrepância do preço anexado, os utentes apreendem como um erro de faturação porque o preço do medicamento não corresponde ao que está referenciado na receita.

Posteriormente, mesmo que expliquemos esta situação, é frequentemente requerida a alteração dos medicamentos aviados pelos correspondentes mais baratos, o que implica todo um processo de eliminação da venda, justificação da eliminação e refazer todo o processo novamente, o que desperdiça bastante tempo de atendimento.

Na minha opinião, esta situação acaba por direcionar e limitar as vendas de certo modo, com a possibilidade de uma interpretação errada como venda tendenciosa da parte dos farmacêuticos, comprometendo a nossa ética profissional.

3.4.3 Situação Económica Atual

Oliveira do Bairro é uma localidade que apresenta todo o tipo de classes sociais, no entanto, foi notória a predominância de classes sociais mais desfavorecidas. Esta discrepância de classes sociais, para além de não facilitar o crescimento económico da farmácia, impede que a maioria dos utentes tenha acesso aos medicamentos que necessitam.

Durante o estágio deparei-me com pessoas com extremas dificuldades económicas, que a Farmácia Sanal tinha a amabilidade de auxiliar dentro do possível.

O acesso a cuidados de saúde e a medicação é um direito de todos, independentemente da classe social a que pertençam. Fiquei muito sensibilizada com todas as situações que testemunhei e espero que, no futuro, existam mais movimentos de

solidariedade e sensibilização, como o Programa Abem, para que estas dificuldades deixem de ser uma limitação na saúde e qualidade de vida da população.

4. Conclusão

Com este estágio adquiri uma maior consciencialização que o papel do farmacêutico engloba muito mais do que a troca e venda de medicamentos e produtos não medicamentosos.

Como futuros profissionais de saúde, de forma a exercer eficazmente as nossas funções, realço a importância de unirmos forças com todos os profissionais de saúde das diversas áreas para estabelecermos uma relação interdisciplinar e possibilitarmos o desenvolvimento de um aconselhamento farmacoterapêutico mais personalizado com foco no utente e não no medicamento dispensado.

Muitas vezes somos o primeiro e único contacto dos utentes em estado de emergência, daí a preponderância da existência de uma relação de confiança, que nos responsabiliza a ter um bom desempenho profissional, uma boa formação, uma capacidade de diálogo acessível, transparente e esclarecedora, para podermos dar o melhor aconselhamento farmacoterapeûtico e o mais adequado possível.

Este período de estágio foi fundamental para sedimentar o conhecimento teórico adquirido ao longo do MICF e colocá-lo em prática, ganhar experiência profissional e de vida, que irei certamente aplicar no meu futuro como farmacêutica.

Acrescento ainda que concluí o estágio de forma muito mais elucidada em relação ao acompanhamento farmacêutico, conceito multitarefa e consciente da responsabilidade desta profissão. Esta etapa não correspondeu ao último passo para a conclusão do curso, mas sim ao primeiro passo impulsionador de muitos anos de formação contínua que é imperativa para um bom desempenho profissional.

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Anexos

Casos Práticos

a) Senhora de cerca de 30 anos refere que apareceram umas borbulhinhas vermelhas na zona da fralda ao seu bébé de quase 12 meses. Entre o Mitosyl® e o Halibut®, questiona-me qual seria o melhor produto.

Esclareci que se tratava de um eritema da fralda e questionei melhor sobre o seu aspeto, se apresentava vermelhidão e/ou descamação mais na zona do rabo ou nas pregas e há quanto tempo tinha surgido. A senhora esclareceu que o bebé apresentava vermelhidão concentrada na zona das pregas com aspeto de pele seca e que tinha aparecido há cerca de dois dias.

Perguntei se já tinha utilizado alguma das pomadas que referiu e acrescentei ainda que, em relação à composição, tanto o Mitosyl® como o Halibut® possuem componentes com propriedades reparadoras. Referi ainda que existem outras marcas como a Barral Babyprotect® e a Bepanthene® em *stock*, igualmente bem referenciadas para tratar este tipo de problema e que era uma questão de preferência.

Acabei por sugerir o Mitosyl®, que, para além da presença de óxido de zinco em maior percentagem com funções protetora, calmante, adstringente e antisséptica, possuía também óleo de fígado de bacalhau com ação cicatrizante (5). Aconselhei a administração de uma camada fina de produto sobre a zona lesada após cada muda de fralda e que esta fosse mais frequente (de duas em duas horas), entre cinco a sete mudas por dia. Se nos próximos três dias não existissem melhorias, que se dirigissem ao pediatra, pois poderia tratar-se de uma dermatite mais severa de origem bacteriana ou fúngica.

Como medidas não farmacológicas aconselhei que evitassem o uso de toalhetes e os substituíssem pela aplicação de gazes com uma água de limpeza própria, sugeri a água de limpeza da Barral Babyprotect® (6), adverti também para que depois do banho ou da limpeza secassem bem a zona afetada com uma toalha sem esfregar. Depois do tratamento, poderia recorrer a uma pomada de hidratação como prevenção do reaparecimento deste tipo de dermatite.

b) Um senhor de 60 anos dirigiu-se à farmácia com sintomas gripais e rinorreia visíveis.

Perguntei-lhe quando tinham aparecido aqueles sintomas e se tinha febre, dores de cabeça ou garganta. O senhor respondeu que no dia anterior tinha apanhado um resfriado e acordou com aqueles sintomas, confirmando que tinha febre alta e fortes dores de cabeça,

mas que não tinha dores de garganta. Questionei se tinha Ben-u-ron® ou paracetamol em casa para aliviar a febre sintomática, mas a resposta foi negativa.

Aconselhei, então, a administração de dois comprimidos Ilvico® de 8/8h durante o máximo de três dias, que corresponde a um complexo de 250 mg de paracetamol, 36 mg de ascorbato de cálcio dihidrato (equivalente a 30 mg de vitamina C), 10 mg de cafeína e 3 mg de hidrogenomaleato de bromofeniramina sob a forma de comprimido, para a febre e dores de cabeça e a lavagem dos seios nasais com água do mar para aliviar os sintomas de rinorreia (7).

Como medidas não farmacológicas recomendei repouso, evitar exposição a diferenças de temperatura abruptas, aumentar a ingestão de líquidos e, em caso de ausência de melhorias, dirigir-se ao hospital.

c) Senhor de cerca de 40 anos entra na farmácia com ar excruciante queixando-se que tinha sido picado por uma vespa asiática no braço.

Questionei como se sentia, se costumava ter alergia a picadas de insetos, se tinha falta de ar ou tonturas. O senhor respondeu-me que não, referindo que apenas tinha muita dor e comichão no local.

A zona da picada não apresentava nenhum vestígio de ferrão e possuía um edema exuberante de cerca de 5-7cm. Recomendei-lhe a administração de 1 comprimido por dia de cetirizina, um antihistamínico H1 não sedativo, a aplicação de Pandermil® pomada, que corresponde a um corticosteróide de aplicação tópica indicado para este tipo de casos, três vezes ao dia, apenas no local da inflamação, por um período máximo de 7 dias e fazer gelo ou colocar compressas frias no local.

Além disso, sugeri também que evitasse ao máximo coçar a região infetada e, se os sintomas não aliviassem nos próximos três dias, que se dirigisse ao médico.

d) Menina de cerca de 20 anos dirigiu-se à farmácia muito aflita e sussurrou que tinha tido relações sexuais desprotegidas com o seu parceiro na noite anterior. Apesar de tomar a pílula, estava preocupada porque tinha feito um tratamento com Cotrimoxazol e a médica tinha recomendado que, após a suspensão do tratamento, utilizasse proteção barreira nos 7 dias seguintes, período em que ainda se encontrava. Questionou-me se aconselhava ou não a administração da pílula do dia seguinte.

Perguntei qual era a pílula que tomava e em que semana do ciclo menstrual se encontrava. Ela respondeu que utilizava a Minigeste® e que se encontrava na última semana

do blister. Referiu ainda que a administração de Cotrimoxazol tinha origem numa toxoplasmose ocular congénita adquirida.

Depois de um pouco de pesquisa, no resumo das caraterísticas do medicamento (RCM) Minigeste®, encontrava-se referenciada a tal recomendação da utilização de proteção barreira nos próximos sete dias após suspensão de um antibiótico (8). Tendo em conta que havia a hipótese da proteção contracetiva estar comprometida, depois de confirmar com a farmacêutica Margarida, aconselhei a administração de contraceção de emergência.

Forneci-lhe uma embalagem de Postinor®, que corresponde a 1500 µg de Levernogestrel, visto que a relação tinha ocorrido há menos de 48h. Adverti ainda que, no caso de aparecimento de vómitos nas 3h após a administração deste fármaco, teria que proceder novamente à sua administração e que deveria continuar com a pílula normalmente em simultâneo com a utilização de proteção barreira, pelo menos, até ao aparecimento da próxima menstruação.

Part II – Monografia

"Cell Membrane-Coated Nanosystems for Gene Delivery Applications"

Resumo

O crescimento exponencial da terapia génica tem proporcionado o sucesso de várias estratégias laboratoriais aplicadas à entrega de ácidos nucleicos para a terapia de várias doenças inatas e adquiridas. Contudo, devido à existência de barreiras extracelulares e intracelulares, que dificultam a eficiência e a integridade da entrega de material genético, tem-se verificado limitações nas aplicações clínicas desta terapia.

Inicialmente, a utilização de estratégias como, por exemplo, a modificação química de ácidos nucleicos resultou, apenas, em aplicações clínicas para administrações locais e tópicas. Posteriormente, a segurança, custo reduzido e a elevada capacidade de transfecção de genes através de vetores não virais avaliada em ensaios para a distribuição sistémica de genes in vivo, promoveu o desenvolvimento de nanopartículas com estruturas inorgânicas e orgânicas para o transporte de material genético. No entanto, a falta de complementaridade biológica na totalidade e de evasão imunológica das nanopartículas icentivou o desenvolvimento de novas estratégias inspiradas em mecanismos e componentes celulares biológicos.

Ultimamente, experiências com abordagens inovadoras de nanossistemas biomiméticos têm reportado um aumento da capacidade de direcionamento, interação com mecanismos, internalização celular e farmacocinética das nanopartículas. Os contemporâneos e multifuncionais nanossistemas biomiméticos de nanopartículas revestidas por membranas celulares emergentes consistem no encapsulamento de nanopartículas sintéticas enriquecidas pelo revestimento de membranas celulares, por métodos "*top-down*". Este método de revestimento de nanopartículas possui a capacidade de preservar as biofuncionalidades das membranas utilizadas incluindo a complexidade de componentes presentes na sua superfície. Existe uma grande variedade de tipos de membrana utilizados de acordo com os tipos de células disponíveis, como por exemplo, glóbulos vermelhos, células cancerosas, glóbulos brancos, plaquetas e células estaminais com capacidade de revestimento de nanopartículas diferentes.

O revestimento de nanoparticulas com membranas celulares tem potenciado a estabilidade e a proteção de ácidos nucleicos, prolongado a sua circulação sanguínea, promovido a utilização de nanopartículas transportadoras de ácidos nucleicos providas das biofuncionalidades da membrana utilizada para o seu revestimento e permitido a entrega de ácidos nucleicos e fármacos sinergicamente num único sistema com várias aplicações no diagnóstico, terapia e teranóstico de várias doenças oncológicas, infeciosas e cardiovasculares.

Esta monografia descreve detalhadamente as nanopartículas revestidas por membranas celulares utilizadas na entrega eficiente e inteligente de material genético, os

desafios inerentes e as perspetivas futuras desta tecnologia para promover a entrega direcionada de ácidos nucleicos e ampliar as suas aplicações terapêuticas.

Palavras-Chave: terapia génica; entrega de ácidos nucleicos; modificações químicas; vectores não virais; nanossistemas; biomimetismo; revestimento por membrana celular; vesículas derivadas de células; revestimento por membranas celulares híbridas.

Abstract

The exponential growth of gene therapy has propelled various successful laboratory strategies focussed on nucleic acid (NA)-based therapies applied in several innate and acquired diseases. However, hurdles in the efficient and integral delivery of the genetic material ascribed to distinct human body extracellular and intracellular barriers have been broadly hindered the successful clinical translation of NA-based therapies.

Initially, attempts with NAs chemical modifications achieved clinical translations status in local and topical administrations. Posteriorly, studies with non-viral vectors for systemic NA delivery *in vivo* demonstrated safety, reduced cost, and high transfection capacity, induced inorganic and organic nanoparticles (NPs) improvements as gene carriers. Nevertheless, their lack of complete biological complementarity and immune evasion capacity hurdles have led to the development of innovative biointerfacing strategies.

Recently, several studies have reported an emerging era of novel biomimetic nanosystems endowed with not only increased targeting and biointerfacing features, but also enhanced cellular internalization and improved pharmacokinetics. Cell membrane-coated NPs are multifunctional and innovative biomimetic nanosystems consisting of synthetic NPs cores coated with cell membranes by a top-down approach. Such cell membrane coatings are able to preserve the natural biofunctionality of parent cell membranes and inherit the vast complex surface repertoire present on the surface cells' membranes, including a diversity of membrane types on account of the different cell types available, such as red blood cells, cancer cells, white blood cells, platelet cells, and stem cells with the ability to coat distinct nanoparticle cores.

Overall, cell membrane-coated NPs for gene delivery have displayed enhanced NA protection and stability, prolonged NA blood circulation half-life, improved NA-loaded cores provided with cell membranes biofunctionalities and allowed both versatile and multivalent drug and NA delivery toward synergistic approaches, evidencing diverse applications in the diagnostic, therapeutic, and theranostic of a myriad of diseases including cancer, infectious and cardiovascular diseases.

This review integrates a detailed compilation of the recent cell membrane-coated nanosystems as efficient, safe and smart NA nanocarriers, critically addressing the challenges and future perspectives toward enhanced cell-targeted NA delivery and improved NA therapeutics.

Keywords: Gene therapy; nucleic acid delivery; chemical modifications; non-viral vectors; nanosystems; biomimicry; cell membrane-coated; cell-derived vesicles; hybrid cell membrane-coated.

Abbreviations

ABCT - ATP-binding cassette transporter

antimiRNA - Antisense miRNA

APC - Antigen-Presenting Cell

ASO - Antisense Oligonucleotide

cBSA - cationic bovine serum albumin

CCM - Cancer Cell Membrane

CO-SLN - Cholesteryl Oleate-containing Solid-Lipid Nanoparticle

CRISPR/Cas9 - Clustered Regularly Interspaced Short Palindromic Repeats associated nuclease Cas9

CSC - Cancer Stem Cell

DC-Chol - DC-Chol3β[N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol

DNA - Deoxyribonucleic acid

DOPE - dioleoyl phophotidylethanolamine

DOTAP - 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane

DOTMA - N-[1-(2,3-dioleyloxy) propyl]-N, N, N-trimethylammonium chloride

DOX - Doxorubicin

DSB - DNA double-strand break

dsDNA - Double-stranded DNA

EGFR - Endothelial Growth Factor Receptor

GalNAc - N-acetylgalactosamine

HDR - Homology-Directed Repair

HNSCC - Head and Neck Squamous Cell Carcinoma

ILsi - interleukin-1α silencing siRNA

KSP - Kinesin Spindle Protein

mcDNA - Minicircle DNA

miRNA - microRNA

MOF - Metal-Organic Framework

mRNA - Messenger RNA

MSC - Mesenchymal Stem Cell

N/P - Nitrogen and nucleic acid Phosphorus ratio

NA - Nucleic acid

NHEJ - NonHomologous End-Joining

NKC - Natural Killer Cell

NLS - Nuclear Localization Signal

NP - Nanoparticle

PA - Photoacoustic

PAA - polyamidoamine

PAE - polyaminoester

PAMAM - polyamidoamine

PD-L1- Programmed death-ligand 1

PDMAEMA - polydimethylaminoethylmethacrylate

pDNA - plasmid DNA

PEGylation - Polyethylene glycol coating

PEGylation - Polyethylene glycol coating

PEI - polyethyleneimine

PL - Platelet

PLGA - Poly(Lacticco-Glycolic Acid)

PLL - poly-L-lysine

pre-miRNA - Precursor miRNA

pri-miRNA - Primary miRNA

PTX - Paclitaxel

QD - Quantum Dot

RBC - Red Blood Cell

RISC - RNA-induced silencing complex

RNAi - RNA interference

SELEX - Systematic Evolution of Ligands by Exponential Enrichment

shRNA - Short hairpin RNA

siRNA - Short interfering RNA

TALEN - Transcription Activator-Like Effector Nuclease

TEV - Tumor cell-derived Extracellular Vesicle

TLR - Toll-Like Receptor

WBC - White Blood Cells

YSA - ephrin-A2 receptor-specific peptide

ZFN - Zinc Finger Nuclease

1. Introduction

In the last ten years, the evolution of gene therapy with gene transfer efficiency and safe delivery improvements evoked clinical progress, including several gene-modified drugs approval around the world [1].

NAs are a diversified class of DNA or RNA with different molecular weights, composition, and geometry utilized for NA-based therapies development, which can be subdivided into Watson-Crick base pairing target sequence complementarity including plasmid DNA, Antisense Oligonucleotides, microRNA, short interfering RNA, short hairpin RNA, gene-editing single guide RNA and no complementary target sequence NAs as messenger RNA, aptamers, among others, with the ability to up- or down-regulate gene expression and immune response modulation, presenting versatile functionalities, high specificity, high reproducibility, and tunable immunogenicity applied in several diseases treatments [2-4].

The earliest focus of NA-based therapies was only on monogenetic failings like primary immunodeficiencies; however, the trials increment accomplishments in overcoming NAs challenges like biological environment limited stability and intracellular compartment access necessity to exert action, expanded gene therapy applications to cancer and chronic diseases, comprehending heart failure diseases, Parkinson Disease, diabetes, among others [5, 6].

Hurdles to the efficient delivery of NAs involve degradative serum nucleases, off-target effects, and immunogenicity, leading to chemistry advancements and fundamental principles knowledge of oligonucleotides *in vivo* behavior applied in biologically active oligonucleotides development to overcome those hurdles using NAs chemical modifications, such as sugars, bases, and phosphate backbones, among others, however, NAs short blood circulation time, lack of targeting abilities, and difficult cellular internalization can only be overcome by vectors utilization as gene delivery systems [7-9].

Vector-based delivery systems comprehend viral and non-viral vectors. Viral vectors may be described as efficient gene deliverers, however, limited cargo loading, cytotoxicity, immunogenicity, and the possible mutagenesis insertion can compromise their safety and effectiveness [10, 11]. In contrast, non-viral vectors are safer, less expensive, easy manufacturing, and seize bigger genetic loadings [11].

Over the last few years an interdisciplinary field, Nanomedicine, has been developing better devices, means of diagnosis, drugs specificity, efficiency and personalized disease treatments, whose key elements are nanoparticles (NPs) with several structures types and replication capabilities of globular biological macromolecules presenting four distinct main

applications: *in vivo* diagnosis, *in vitro* diagnosis, *in vivo* therapeutics, and implantable nanomaterials [12].

NPs have innumerous advantages including, enhanced biodistribution and pharmacokinetics, therapeutic stabilization, hydrophobic drugs solubilization, and off-target tissues toxicity mitigation [13]. Their adjustable surface functionalities and biocompatibility generated a myriad of studied possibilities for biomedical application, including inorganic, lipid, and polymer compositions, which sometimes are combined to improve their performance and safety [14].

The main concerns in the design of nanosystems are safety, NPs aggregation, long-term accumulation, haemolytic effects, and immunogenic behavior, therefore, it is crucial to optimize NPs drug loading capacity, their sustained release and specific targeting *in vivo* [15].

Whereas most of the developed NPs remain in the research stage with good results *in vitro*, *in vivo* results presented limited clinical transitions. The notion that some nature complexities can only be solved by nature leads to the development of biomimetic designs as an optimal alternative [14].

The NPs majority enter the cell via endocytosis towards lysosomal degradation, depending on the used nanomaterials and biological factors like cell type, thus, as they presented limited endosomal escape, strategies utilizing endolysosomal functions have been explored especially for NA delivery [16]. These investigations enable intended NPs efficient and targeted uptake by studying how NPs size, shape, elasticity, and surface modifications feature influence cellular uptake. After NPs cellular uptake, they undergo several intracellular trafficking destinations such as NPs endosome entrapment, leading to lysosomal degradation, which could be overcome by surface charge and surface ligand display of NPs, or intracellular organelles and compartments like the nucleus, mitochondria, endoplasmic reticulum, and Golgi apparatus by engineering targeted NPs with surface modifications and surface ligands [17].

Recently, advances in NP-based therapeutics led to a novel paradigm of biomimetic properties insertion into these therapies by biological systems and surfaces features replication to increase their biointerface [18]. These next-generation biomimetic particles have the ability to mimic cellular or viral aspects, including size and shape or chemical and biological features of biological surfaces as chemical composition, 3D surface protein exhibition and membrane fluidity [18].

Particularly, a major inspiring source that has been motivating the development of biomimetic nanostructures is the diverse existing membranes cells structures and functions, which inspired researchers to design biomimetic membranes nanoplatforms endowed with

multifunctional properties such as immune response activity, vascular system clearance escape and the cells membranes specific cell-cell interactions, recognition, adhesion and communication [19].

Depending on the origin of the cell membranes used, such as red blood cell, cancer cell, white blood cell, platelet, and stem cell, they may be responsible for extending systemic circulation, immune clearance escape, tissue targeting, moreover, the fusion of different cell-membranes called hybrid cell membrane-coated NPs are capable of assembling multiple cell membranes functionalities [15].

The main challenges related to cell membrane-coated NPs are its complex production processes, different cell membranes type heterogeneous efficacy, and stability issues; however, their tremendous promising to revolutionize nanomedicine in a myriad of diseases treatments and diagnosis prompt improvements for these nanosystems [20].

In this review, it will be covered the recent advances on single and hybrid cell membrane-coated NPs for gene delivery, their challenges and future prospects in nanomedicine field.

2. Gene therapy

2.1 Types and therapeutic applications of nucleic acids

Gene therapy includes high molecular weighted and negatively charged NAs introduced to cells, which interact with transcriptional and translational machinery and produce delivered NAs or proteins with localization capacity and continuous production of therapeutic molecules, demonstrating a notorious therapeutic prospective for tissue regeneration and degenerative diseases [6, 21].

There are three types of gene therapy objectives: gene augmentation (manipulation of deoxyribonucleic acid (DNA) or messenger RNA (mRNA)), silencing (Antisense Oligonucleotides, Aptamers, RNA Interference (RNAi)), or gene editing approaches such as meganucleases, zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR)-associated nuclease Cas9 (CRISPR/Cas9)) [6].

2.1.1 Plasmid DNA, Minicircle DNA and Messenger RNA - Gene Augmentation

Gene Augmentation comprehends double-stranded DNA (dsDNA) with transgenes inserted into plasmids (pDNA) or the more recently discovered episomal minicircle DNA (mcDNA) for both safer and longer gene expression duration of a higher number of proteins [6].

pDNA integrates a double-stranded NA of several kilo bp size with anionic phosphodiester backbones, DNA deoxyribose stability, and target cell nucleus reach necessity to exert the desired therapeutic action [6, 10]. This gene augmentation episomal entity current worldwide promising progress has a bacterial replication origin, an antibiotic resistance gene to avoid bacteria propagation, a transcription unit with a eukaryotic promoter (GOI) and a polyadenylation sequence to potentiate GOI expression and nuclease resistance [22]. The negative charge of pDNA enables electrostatic interactions to condensate the NA into lipoplexes or polyplexes with nano sizes, which in case of tumor potentialize passive targeting accumulation with enhanced permeability and retention effect [10, 23].

mcDNA is a non-viral, episomal, and covalently closed circular vector with minimalistic backbones and singular characteristics such as the lack of bacterial replication origin, antibiotic resistance absence, a therapeutic transgene controlled expression by a mammalian promoter, and a recombination site-specific sequence for the precursor parental plasmid yield the minicircle [22]. Comparatively with pDNA, mcDNA benefit from bacterial unit removal by preventing prokaryotic resistance marker genes insertion into the human genome or its transference into human microbiota, reducing the vector size and successfully influence gene treatments effectiveness [22].

Gene augmentation also integrates artificial mRNA translated in the cytoplasm with low risk of mutagenesis, a simple and rapid production process, high reproducing and transfection properties, relatively small amounts utilization to strong efficacy signals and when combined with pDNA can bring more efficient co-delivery [6, 24].

The simple and flexible production of synthetic mRNA facilitates the response to epidemic outbreaks of infectious diseases more than current vaccines and includes *in vitro* transcription (IVTmRNA) with a linearized pDNA or a PCR template origin, a bacteriophage promoter and a bacteriophage RNA polymerase for recognition, an open reading frame (ORF), 5´and 3´untranslated regions (UTR), and an optional poly[d(A/T)] sequence [25].

The smaller size of 300-5000 kDa and efficiency in nondividing cells protein expression induction without genomic insertion risks of mRNA allowed its development as an alternative to pDNA therapeutics, although mRNA shorter protein expression duration [26, 27]. As gene-editing tool deliverer in ZFNs and CRISPR-Cas nucleases, mRNA has been mitigating difficulties associated with Cas9 protein such as the manufacture, the protein activity preservation, the *in vivo* protein delivery, and the ability to modify mRNA sequences to encode regulatory elements, thus providing a specific control expression and paving the way for non-viral gene-editing therapy [26].

Current clinical trials studied examples of mRNA drugs are vaccines for influenza and Zica viruses, cancer immunotherapy, such as myeloma, leukaemia, and glioblastoma, haemophilia B, myocardial infarction, and human immunodeficiency virus (HIV) [28]. The most recent mRNA clinical trial application was a potential lipid NP mediated vaccine for SARS-CoV-2, which reveals the importance of this nanomaterial as a delivery system for human application [29].

2.1.2 Antisense Oligonucleotides, Aptamers and RNA interference - Gene Silencing

Oligonucleotides are an emerging therapy class utilizing NAs for downregulation of a specific gene, which includes antisense oligonucleotides (ASOs), aptamers, and RNAi NAs [30].

Antisense Oligonucleotides (ASO) are small DNA or RNA sequences that can be Rnase H activators to specific downregulation of the target or pre-mRNA splicing modulators to alter protein functions [6]. This specific gene therapy is approved for cytomegalovirus retinitis (fomivirsen), hypercholesterolemia (mipomersen), a muscular dystrophy disease Duchenne (eteplirsen) and spinal muscular atrophy (nusinersen) treatment. It also has a promising diagnostic function, immunotherapy, and other genetic disorders therapeutic studies [3, 6].

ASOs share some similarities with siRNA such as standard Watson-Crick base-pairing binding to target RNA, biodistribution, potency, maximal effects, specificity, and the activity duration; nevertheless, they have different mechanisms. While ASOs function by RNase H endonuclease activity induction to cleave mRNA-ASO duplex, thus participating in multiple RNA molecules destruction, siRNAs are complementary associated and stabilized with mRNA until guide strand selection, which posteriorly associates to a complex leading the guide strand to the target, this latter mechanism confers to siRNA more structural stability and targeting efficiency than ASOs [30].

Aptamers are a gene silencing 15-18 oligonucleotides single-stranded sequence of DNA or RNA with a stable and flexible capacity to be modulated into a 3D structures [6]. They are selected through a systematic evolution of ligands by exponential enrichment (SELEX) and named as chemical antibodies working as antagonists to knockdown protein-protein or receptor–ligands interactions[31]. The advantages of this gene silencing strategy are efficient *in vitro* selectivity of any target with high binding affinity due to aptamers-target noncovalent interactions, and manufacture reproducibility, thus presenting a promising alternative to antibody- or peptide-based target ligands [32]. Aptamers-target binding

capabilities provide an option to gene therapeutics lack of specificity, by working as targeting ligands of RNAi-based therapeutics, which inspired aptamers and miRNA, siRNA, or shRNA conjugates formation currently in clinical trials for cancer, infectious and bone diseases applications [32].

To date, the only approved clinical utilization is Macugen® (pegaptanib) for macular aging degeneration treatment [6]. Moreover, the aptamers advantages as targeting ligands due to their low molar mass, immunogenicity, and high specificity for cellular antigens, present an excellent strategy for polymeric NPs cell binding and internalization [13].

RNAi is a natural mechanism of gene silencing by chromatin remodeling, protein translation inhibition, or direct mRNA degradation, including NAs, such as a) MicroRNA (miRNA); b) Short interfering RNA (siRNA); c) Short hairpin RNA (shRNA) (Figure 1) [6]. It has the advantage of relying on a catalytic mechanism that requires less NAs sent to the cell and, in contrast with CRISPR-Cas9, it focuses only in RNA rather than DNA, thus avoiding situations related to genetic safety [33].

a) miRNA

miRNA are small (18-25 nucleotide in length), polycistronic nature and noncoding RNA molecules well suitable to different vectors, simple engineering, high variability and multiple target regulators that tend to cluster and regulate gene expression at the posttranscriptional level[6, 34]. miRNA origin relies on transcribed primary miRNA (pri-miRNA) from DNA, which is processed by Pasha and Drosha proteins into a precursor miRNA (pre-miRNA). In the cytoplasm, pre-miRNA is processed in miRNA by the enzyme Dicer and introduced into RNA-induced silencing complex (RISC) where is unwind by a helicase, resulting in the formed antisense strand partial binding to the complementary mRNA and their afterwards cleavage [6].

The only partial complementarity of miRNA to their target mRNAs has two distinct ends: in one hand, it can result in miRNA degradation or translational inhibition; on the other hand, can result in miRNA binding to different targeted mRNAs and influence the expression of multiple genes, besides it has been verified miRNA specific expression profiles to different cancer types, thus allowing clinical utilities to cancer discrimination and identification [35].

For overexpressed oncogenic miRNAs, there is an antagomir strategy composed by miRNA antagonists (anti-miRNAs), which utilize single-stranded oligonucleotides with partial or full complementarity to target endogenous miRNA, this way preventing miRNA

processing by RISC. Therefore, it may be asserted that miRNAs have the ability to play two different roles as therapeutic agents or as therapeutic targets [35].

Since miRNA linking with gene dysregulation and human disease, biopharmaceutical researches led to compound SPC3649, miravirsen, the first miRNA therapeutic approach by Santaris Pharma from Denmark for hepatitis C virus infection treatment, a short locked NA mir-122 currently in phase II of clinical trials [36].

miRNAs critical role in mRNA disruption and consequently disease development including cancer, promote their clinical utility as biomarkers, diagnostic and therapeutic agents [37].

b) siRNA

siRNA is a small 21-23 nucleotides double-stranded RNA fragment utilized for gene silencing therapies. The miRNA similar mechanism of siRNA includes dsRNA processing into small dsRNA by a ribonuclease (RNase) III-like present in cytoplasm, Dicer, forming siRNA that posteriorly interacts with RISC by argonaute 2, resulting in siRNA sense strand cleavage and, subsequently, the antisense strand will guide RISC to the full-length complementary target mRNA with specific gene silencing performance and conventional small drug molecules target limitations surpassing [37]. It has a wide variety of investigations and uses including an already FDA and EMA approved siRNA-based delivery therapy, patisiran (Onpattro®), with lipid NPs for hereditary transthyretin amyloidosis treatment [3, 6], several viral infections, hepatic diseases, carcinomas and inclisiran a final stage experimental clinical trials treatment for hypercholesterolemia [6].

siRNA-based therapeutics applications also have been indicating ease target site with local or topical delivery of naked siRNA for ocular diseases with no toxicity, and non-viral vector local administration for lung diseases with safer and lower cost-effectiveness than viral vectors and successfully delivery systems investigations for cancer therapy [37].

Both miRNA and siRNA are single-stranded forms presenting post-transcriptional gene silencing mechanisms, however, miRNAs, known as endogenous gene regulators, regulate gene expression through combination with Argonaute proteins, usually utilizing eight nucleotides from their 5`-end to target mRNA identification [36]. By contrast, siRNAs contribute to genome stability utilizing their full-lengths to target mRNA identification and cleavage mediation [36].

Although the overlapping possibility of siRNAs and miRNAs similarities in mRNA downregulation, there are differences between them related to siRNA one specific targeting and miRNA multiple mRNAs expression with a more complex target recognition presenting

multiple different complementarities and target sites binding, due to miRNA partial target mRNA complementarity, which can be translated in mRNA degradation by deadenylation, decapping or exonucleases making miRNA-based therapeutics development suitable for multiple targets, instead of RISC activation process utilized in siRNA, which is more suitable for target identification and validated drug developments, except for rare cases of miRNA high complementarity that evoke mRNA endonucleolytic cleavage mechanism similar to siRNA mechanism process [37].

c) shRNA

Although shRNA, a 4-11 nucleotides length NA, and other RNAi therapeutic present similar outcomes, shRNA formation has its place in the cell nucleus and is, posteriorly, transferred to the cytoplasm and processed by RISC activity. The design of this oligonucleotide tries to mimicry miRNA maturation pathways and consists in the expression cassette transcription by either RNA polymerase II or III promoters, containing a hairpin stem-loop structure appearance processed by Drosha enzyme and DGCR8 dsRNA-binding domain protein and its posterior transport for cytoplasm into RISC complex [6, 38].

Chemical modifications of shRNA, when compared to the siRNA ones, are harder to execute, though cheaper and can be achieved by shRNA redesigning or varying promoter regulation. siRNA and shRNA have the same off-target effects independently of the delivery method, which could be overcome easily by sequence-based modifications; however, non-specific off-target endosomal immune responses triggered by naked siRNA are less likely on shRNA because of its endogenous splicing mechanism [38].

The shRNA encapsulation into vectors, preferentially plasmids or viral vectors, enhance gene silencing transfection efficiency and its modulation for effective cancer therapy and the already FDA clinical trial approval hepatitis B applications *in vivo* [38].

The main advantages of this type of gene silencing are their synthesis in the target cell, long-lasting and high specificity, when compared with other RNAi-based therapeutics. It wasn´t officially approved yet, but shRNA has been showing optimal research approaches for viral mRNA delivery nanosystems, among others studied for infections and gene therapy applications, such as familial adenomatous polyposis, ovarian cancer and HIV infection inhibition [39].

2.1.3 CRISPR/Cas system - Gene Editing

Gene editing is a wide technique field based on the targeted repairing of DNA double-strand breaks (DSBs) by homology-directed repair (HDR), which consists on DNA

template targeted mutations insertion at DNA break site or nonhomologous end-joining (NHEJ) with randomly inserted or deleted nucleotides into DNA break site [40, 41].

It was discovered that the CRISPR system reduced into a single RNA guide (sgRNA)/Cas complex binds to its complementary target gene sequence identified due to a sgRNA guided protospacer adjacent motif (PAM) presence, which posteriorly specifically cut the DNA at specific sites [40, 42]. This DSB gene editing is performed by HDR or NHEJ mechanisms like ZFNs and TALENs, with the particular aspect of not requiring new protein production for each target site [40].

The CRISPR/Cas system is simpler to manufacture and more convenient to utilize, affordable, and time-saving comparatively to other previously reported gene-editing tools, resulting from a partial gene insertion of an infected source into the bacteria genome to enhance resistance against infectious diseases and viruses, which involves multiple target sequences and areas such as gene regulation, chromatin modification, imaging, and other high standard future technologies [6, 42, 43].

Cas9 is a 4 to 7 kB nuclease comprehending two lobes with the capacity of negatively charged sgRNA accommodation: the nuclease lobe with RuvC endonuclease site and HNH endonuclease site for exogenous dsDNA cleavage, and PAM; the target recognition lobe corresponds to a long α helix subdivided into two domains [42, 44]. Whereas Streptococcus pyogenes Cas9, the first workable Cas nuclease demonstrated in 2013, won the merit as an efficient gene-editing tool with a wide targeting range; however, it presented defects such as high molecular weight and potential off-target effects, which difficult its delivery by the current existent vehicles. Therefore, during the past few years, various engineered Cas9 variants from other bacteria and amino acid specified modifications were discovered to enhance CRISPR/Cas9 systems [42].

There are only three existing forms for Cas9 and sgRNA delivery, which could overcome the Cas9 large size protein and sgRNA long phosphate backbone delivery challenges, accordingly to their applications with absent mutagenesis insertion, low immunogenicity and negligible off-target effects: pDNA delivery form with higher stability and cost-effectiveness; mRNA with the lack of nuclear entry requirement advantage and relative instability, due to sgRNA apart combination; and ribonucleoprotein (RNP) that promotes Cas9 and sgRNA self-assembly into one single payload, thus circumventing mRNA instability hurdles and representing the most suitable format for this gene editing type delivery because it combined beneficial properties as transient delivery, no insertional mutagenesis, low immunogenicity and low off-target effect [42, 44]. The prominent advantages of RNP delivery form, such as temporary Cas9 delivery, no insertional

mutagenesis, weak immunogenicity and low off-target effects, perform superior CRISPR/Cas9 delivery [41].

The combination of viral vectors for sgRNA transport with controlled synthetic nanomaterials or physical methods for Cas9 delivery approach circumvents viral vectors genotoxicity and take advantage of its efficient transduction followed by the packaging of DNA templates, including successfully applied examples with electroporation and adenovirus vectors for haematopoietic stem cells mutations. Additionally, the CRISPR-Cas9 system off-target effects can be minimized by a limited dose or duration of Cas9 activity [45].

Non-viral methods for CRISPR/Cas system delivery such as NP-based therapeutics, due to its high cargo loading capacity without the risk of genomic insertion, can integrate modified Cas9-sgRNA RNP complexes with application examples such as human osteosarcoma cell lines and Duchenne muscular dystrophy, thus alleviating the majorly *in vivo* gene editing delivery hurdles, especially the off-target effects [46]. Furthermore, strategies such as Cas13 and Cas12a enzymes engineering demonstrated important and rapid DNA tumor and tumor-related virus detection, with the optimal detection ability of multiple DNA targets, even though the further required investigations and comparisons to the already existent detection methods [46].

Although the optimal results with polymeric and lipid-based NPs for CRISPR/Cas9 delivery, they need further investigations as cleavage site control to reduce side effects and the fundamental understanding of cellular uptake, vasculature permeability, and deep tissue penetration mechanisms before clinical translation [47].

Although the NA vector relevance, the release of NAs in cytosol or nucleus is a determinant factor for non-viral gene delivery systems clinical translations [29]. The correct NA selection depends on the treatment goal, therefore, pDNA could be considered a better tool for enhanced gene expression in the lack or loss function of the cell, however, it presents hurdles *in vivo* applications, nevertheless, rather than pDNA, the fact that mRNA does not need to overcome nuclear envelope and the success of IVTmRNA in immunotherapy applications benefits its utilization [29, 48]. Oligonucleotide-based therapies differ from gene therapy classic definition of defected genes replacement or new gene insertion, because it relies on antisense technology with unrequired cell transcriptional or translational mechanisms, downregulating a specific gene, which bestows the capacity to treat or cure almost any disease [29, 49]. Gene editing CRISPR/Cas9 for sgRNA delivery provides a powerful, precise, and targeted gene modification tool for cell genomic sequences

manipulation with favorable Cas9 ribonucleoprotein as the delivery cargo for therapeutic applications, due to their safety and low potential off-target effects [42].

2.2 Hurdles to Efficient Delivery of Nucleic Acids

Naked NA characteristics could difficult its delivery, such as intracellular action, which implies barriers crossing; limited stability in a biological environment, which increases their susceptibility to serum endonucleases enzymatic degradation, resulting in a short plasmatic lifetime and decreased cellular uptake; negative charge and a high molecular weight that cause diminished transfection efficacy; hydrophilic properties with consequent rapid clearance and renal excretion; immunogenicity due to exogenous NA introduction; off-target effects; and low tissue penetration [3, 6, 50].

For example, as a novel non-viral vector with absence of the usually pDNA bacterium backbone, mcDNA presents limited bioavailability by rapid clearance *in vivo* and lysosome nuclease degradation [51]. Whereas mRNA has been studied as an option to DNA-based therapy with minor immunogenicity and does not require cell nucleus entry, however, it presents less stability [52].

Posteriorly, despite the promising findings with RNA-based therapies, they also showed some hindrances such as off-targeting, minor serum stability, and innate immune responses, which corresponds, consequently, to significant challenges in clinical applications, highlighting the delivery of therapeutic agents into targeted cells. Gene therapy with RNAi-based therapeutics has better bioavailability when locally or topically applied than in non-targeted tissues [36].

CRISPR/Cas9 system, when compared with RNAi-based therapeutics transient and partial gene downregulation, includes extra gene insertion, mutation, and activation effects with the additional permanent and complete gene deletion of Cas9, resulting in unnecessary regular dose administrations, however, viral and non-viral vector delivery of CRISPR/Cas9 system has been reporting issues related to large molecules (Cas9 protein simultaneous delivery with sgRNA into pDNA, mRNA or protein delivery forms) and nucleus entry requirements, the engineered sgRNAs varying activity for specific targeting and the possibility of off-target effects associated to DNA cleavage, with conclusive riskier features of CRISPR/Cas9 system utilization comparatively with RNAi-based therapeutics [53]. Although the CRISPR/Cas9 gene-editing tool referred hindrances, the researchers biggest concerns are ethical issues with misuse gene-editing techniques in germline genetics [54, 55].

NAs resumed obstacles are degradation by nucleolytic enzymes, immune system cells uptake, and low tissue penetration, thus for effective gene delivery fulfilling requirements as payload protection, targeting and release are mandatory [56].

The design of NPs for gene delivery applications represents a current nanotechnology research area of interest, however, the nanotechnology-biology interactions are complex, dynamic, and multiparametric, exhibiting obstacles to effective nanomedicine engineering. The NPs physicochemical properties that contribute to its complexity are the particle size, shape, surface chemistry, composition, architecture, density and modulus, the biological microenvironments like organ/tissue, biomolecular composition and biochemical factors, and nano-bio kinetics interactions [17].

Bare NPs have been demonstrating limited clinical applications because the human body recognizes them as foreign substances and, consequently, stimulates unwanted immune responses and toxic effects [14]. Following the NPs contact with blood, serum proteins are adsorbed into their surface forming the so-called protein corona, which provides unintentional biological identity to NPs by altering its physicochemical properties and posteriorly controlling their *in vivo* transport and biodistribution by affecting their interactions with target cell membranes and their intracellular fate [17, 57].

The endocytic pathway is a cellular uptake mechanism formed by endosomes, vesicles with a pH of 5, which, in the mature state, fuses with lysosomes which are intracellular organelles containing digestive enzymes responsible for particles enzymatic degradation processes, causing *in vivo* limited intracellular delivery of targeted therapeutic agents [58]. Non-viral vectors lack of endosomal escape ability, a hurdle in gene delivery, requires the improvement of synthetic transfection designs *in vivo* [58]. The entry of NPs into the cell through endocytosis and the NP size, shape, and surface modifications designed performance requires careful consideration of the target cell microenvironment also. The NP endocytosis process comprises the particle engulfment into membrane invaginations, followed by the formation of endocytic vesicles posteriorly transported for intracellular compartments, which can be classified into five main mechanisms: phagocytosis, with NPs surface opsonization by blood proteins, which may influence NP toxicity; clathrin-mediated endocytosis, occurs in the plasma membrane area rich in clathrin and is responsible for the acquisition of nutrients and plasma membrane components of cells like cholesterol and iron by non-specific or specific adsorptive uptake; caveolin-mediated endocytosis includes caveolae invaginations in epithelial and non-epithelial cells, due to caveolin and other proteins presence, that anchor to the cytoskeleton and influences many biological processes involving cell signaling, transcytosis, regulation of membrane structure and diseases like

cancer, diabetes, and viral infections, whose pathway allows lysosomal escape, thus providing a useful route for gene delivery; clathrin/caveolae-independent endocytosis corresponds to cells without clathrin or caveolin proteins, which has the ability to accomodate different payloads; and macropinocytosis [57]. It has been demonstrated that ensuing the endocytic recycling pathways or autophagy contributes to the limited dose of therapeutic agents delivered, which require further studies in cargo delivery nanosystems [13].

NPs can be designed with positive, negative or neutral surface charge estimated by zeta potential, a colloidal dispersion NP electrokinetic potential quantified in aqueous or buffer environment, which indicates the average NP surface charge and changes in response to environmental conditions, thus influencing the NP adhesion to plasma membranes, their cellular uptake and cytotoxicity. The entry of positively charged NPs into the cells can cause lysosome and organelles damage or reactive oxygen species (ROS) formation, leading to apoptosis [17].

Essentially, the general NA containing NP trajectory *in vivo* embraces initial interaction with blood components by NPs entry into the bloodstream, their transport across vasculature, and their microcirculation entry, posteriorly, near the blood vessel wall and accordingly to the size, charge, and shape of NPs, they accumulate and interact with microvasculature endothelium wall followed by adhesion and cellular uptake, thus the design of NPs for gene delivery must provide: stability in body fluids; serum nuclease degradation, clearance, renal excretion and RES escape; passive or targeting ligand active targeting cell abilities; efficient cellular uptake and internalization; endolysosomal pathway escape; efficient cargo release in the cytoplasm for gene editing and RNAi therapeutics or in the nucleus for pDNA, mcDNA and mRNA therapeutics; non-toxicity; and biodegradability [50].

2.3 Strategies for Nucleic Acids Delivery

The development of an efficient and safe NA vector delivery system is key to overcome gene therapy delivery hurdles, by filling some requirements for vectors success in extracellular and intracellular trafficking pathways like accurate size, surface charge, flexibility and morphology, which impacts vectors pharmacokinetics, biodistribution, and cellular internalization rates [11, 13].

Chemical modifications of NAs positively influence their metabolism, interfering in blood and tissues NAs degradation by RNase activity, kidney filtration loss of small molecules by urine secretion, and intracellular degradation in RES and targeted cells [8]. They can be conjugated with noncationic inorganic NPs through metal-ligand interactions, lipids, polymers, cell-penetrating peptides, performing efficient and high stable NA-

noncationic conjugates for NA delivery. However, due to the lack of established electrostatic interactions with cell membranes, noncationic materials may present limited cell internalization [59]. Although chemical modifications are a strong contour manner of the NA delivery main concerns, the other inherent weaknesses can only be solved by gene carriers [9].

Non-viral vectors for gene delivery engineering tries to mimic viruses properties as gene packaging, degrading enzymes protection and high specific transfer to target cells in a safer way and promote NAs cellular entry and release inside the cells, through NAs condensation and protection from degrading factors. A successful non-viral delivery system should support three crucial factors: 1) beneficial circulation time to allow the system penetration into the targeted tissues, 2) accurate system unpackaging with stable complexes and high transfection efficiency, and 3) a 50-100 nm size and a ± 10 mV zeta potential values for effective results, especially in tumor microenvironments for enhanced access and reduced RES uptake [29]. Non-viral vectors embrace both inorganic particles and organic particles. The organic particles can be subdivided in polymer-based, lipid-based systems and peptides, that will be further described [6].

Extracellular biological barriers like blood substances, serum proteins, and enzyme nucleases contribute to the degradation of some polymers. Another extracellular barrier is the tissue that, due to the polymer size, could block its flow [60]. At the intracellular level, there are physical methods (microinjection, gene gun, electroporation, sonoporation, and laser irradiation) for direct NPs transfection, with no systemic delivery administration in humans and endocytosis ways by chemical carriers utilization for genetic material delivery. The non-viral gene delivery hurdle relies on the endosomes that bind to the lysosomes and lead to the transported material degradation [60]. The last obstacle, the cell nuclear envelope, requires a limited mass of 50kDa and a short nanometers diameter difficult to achieve for passive transport, that can be overcome by an active transport complex and a peptide nuclear localization signal (NLS) [61].

The achievable noncationic materials could present neutral or negative charges, high biocompatibility, flexible material dosage, and wide range applicability like neutrally charged poly(ethylene glycol) (PEG) derivatives increasing nanomaterial hydrophilicity and increasing NP nonspecific interaction with the surrounded environment, or covalently conjugated anionic polysaccharides to NPs as hyaluronic acid with reduced nanomaterial toxicity, or NAs themselves attached to NPs through electrostatic interactions with also reduced cytotoxicity of the nanomaterial [59]. Polyethylene glycol coating (PEGylation) example to mitigate degradation by blood elements, decreases the reticuloendothelial system (RES) and

mononuclear phagocyte system uptake, and increases vector time blood circulation, presenting a great solution, although PEG-modified NPs have also been showing immune system activation and efficacy lost over repeated administrations [14, 61].

The discovery of NPs physicochemical properties alterations in biological fluids and cell-culture media such as NP surface modifications by protein and biomolecule adsorption the named "protein corona", led to a better understanding of NPs cellular uptake and trafficking mechanisms that may be the key to design optimized nanosystems with increased cellular targeting, uptake, and trafficking properties [57]. For example, in order to repel the opsonization of NPs in phagocytic pathways, solutions like pegylated NPs prevent the particle surface protein adsorption, forming a repulsive thin barrier around the NP and increasing NPs half-life by avoiding RES uptake [57].

Once inside the cell, enzymes and bioactive molecules trigger the NPs content release, for example, glutathione (GSH) biomolecule present in cytosol at higher concentrations than blood, which reduce disulfide bonds, a strategy utilized in disulfide crosslinked polymeric NPs coating siRNA. Other applied cytosol enzymes comprehend adenosine triphosphate (ATP) or Dicer, which has been reported as more successful than non-dicer substrates for siRNA release. However, tumor tissues have demonstrated lower Dicer expression, which highlights the importance of choosing the target tissue accordingly with the applied strategies and the selected NAs-based therapies [13].

The NP surface targeting ligands modification strategy utilizing small molecules, proteins, antibodies, antibody fragments, and NAs have recognition capabilities of overexpressed malignant cells, therefore targeting ligands can increase cellular interactions with NPs, stimulate cell signalling pathways to intended biological responses, or enhance the cellular uptake for payload delivery of therapeutic or diagnostic agents, considering target ligand length, target ligand density, hydrophobicity, and avidity [17].

2.3.1 Inorganic Nanosystems

Inorganic vectors for gene delivery comprise calcium phosphate NPs, silica, quantum dots (QDs), gold, and iron compounds [6, 61]. This delivery system establishes electrostatic interactions with genetic material, is simple to produce, biocompatible, stable, non-toxic and inert against the activity of microorganisms [6, 62].

It all started with calcium phosphate, a biological tissue component with already inherent biocompatibility and affinity with gene material phosphates [62].

Silica NPs silanol groups allowed chemical changes such as combination with other inorganic particles to efficient transfection, tracking, and specific targeting [62]. Mesoporous

silica NPs are constituted by a network of pores within a silicon oxide framework, acting as a payload abduct similar to a rigid sponge with larger surface area compared to other nanosystems with excellent *in vivo* gene therapy effects; however, it requires cationic condensers due to its anionic surface and careful design size and structure due to relative lack of biostability with administration-dependent toxicity [63]. For example, Pinese *et al.* utilized the biocompatibility of mesoporous silica NPs and its easy pore modulation to open a new door for gene delivery applications. By electrospun scaffolds utilization, mesoporous silica NPs surface pegylation and siRNA incorporation, they created a complex nanofiber. *In vitro*, the utilized scaffold increased siRNA sustained delivery for at least 30 days and showed gene silencing activity efficiency. *In vivo*, they used collagen type I as a target to regulate fibrous capsule evolution, verifying a successful capsule reduction with this nanosystems [64].

QDs are described as luminescent NPs that have both tracing NA and transfection activity [62]. To demonstrate QDs delivery abilities, Wu *et al.* used a QD NP modified with ZnS shell and PEG coating to diminished its cytotoxicity. This QD nanosystem incorporated siRNA targeting SOX9 gene, which differentiates chondrogenic mesenchymal cells. *In vivo*, siRNA gene silencing activity reduced chondrogenic cells differentiation and retarded cartilage repair with high-efficiency transfection and endosomal escape in living miceby efficient siRNA escape from the endosomes[65].

Gold NPs are the most studied delivery system because of its easy modulation into multifunctional monolayers and present great siRNA loading capacity [62, 66].

Iron oxide magnetic characteristics are particularly interesting for cancer therapy because of its selectivity to tumor tissues by external magnetic orientation[62].

Metallic systems as gold and iron oxide have the advantage of presenting longer-shelf life, surface chemistry with physical stability and tunability, endosomal escape, and cargo sustained release. Although their multi-modality for imaging and phototherapy, it has been reported a relatively low silencing effect comparatively to other therapeutics, that is why they are generally coated with other polymer/lipid nanosystems to protect RNAi therapeutics [63].

Inorganic particles have the ability to combine between each other or with other non-viral delivery vectors for safer and efficient transfection of genetic material [62].

2.3.2 Organic Nanosystems

During the past few years, polymers demonstrated a high average of gene silencing efficacies [63].

Cationic polymers are a positively charged structures due to the presence of several amine groups, which influence their electrostatic interactions with negatively charged NAs, forming polyelectrolyte complexes also called polyplexes that facilitate NAs cell entry via adsorptive endocytic pathways [29].

Among all the existent polymers, the most utilized for gene delivery are poly(lacticco-glycolic acid) (PLGA), polyethyleneimine (PEI), poly-L-lysine (PLL), and chitosan [63].

FDA-approved PLGA polymer represents high stability and biocompatibility, requiring cationic condensers such as PEI for efficient gene loading [63]. Since naked NAs are eventually degraded in endolysosomal pathway acidic environment, endosomal escape agents are required for delivery formulations, performing functions as "proton sponge" effect with protonated groups at acidic pH as PEI, which promote the chloride anions influx, osmotic swelling and posterior endosomal lysis, leading to NP content expulsion in the cytosol; pore formation responsible for destabilizing and disrupt the membrane; or membrane destabilization by pH-switchable groups like endosomolytic peptides [13]. PEI, the most utilized polymer, requires a nitrogen and NA phosphorus ratio (N/P) higher than ten for stronger NA incorporation, taking into account that an overage N/P ratio invoke NP clearance by RES and higher cytotoxicity [63]. The length of the PEI chain also influences cellular uptake and endosomal release, thus, the usually utilized PEI corresponds to the low molecular weight form (<2 kDa) [10, 13, 67].

In contrast, PLL does not require any condensers, however, high vasculature serum content could limit its transfection, by serum protein competition [63].

Despite cationic polysaccharide, chitosan, minor solubility *in vivo*, PEGylation and hyaluronan-conjugation are reasonable solutions for enhanced gene delivery. However, it needs further researches for reliable performance [63].

The polymers emerging properties also included dendrimers, such as polyamidoamine (PAMAM) dendrimer, polyamidoamine (PAA), polyaminoester (PAE), polydimethylaminoethylmethacrylate (PDMAEMA), etc. [68]. Detaching PAMAM, the most workable and studied cationic dendrimer, due to its transfection capability and commercial availability [69], it can also be improved by chemical modifications, including the addition of fluorescent compounds to earn both monitoring and transfection efficiency properties [68].

Polymers are an appealing class for gene editing delivery, especially for the CRISPR/Cas9 system with stabilized plasmid delivery forms in serum environment, avoiding hindrances related to aggregates formation. The outstanding polymeric NPs applications in

CRISPR/Cas9 delivery have been maximizing Cas9 therapeutic efficiency, by Cas9 improved stability and minimized side effects with successful results in brain cancer, for example [42].

Polymeric NPs can create nuclease resistance by the steric bulk of polymeric corona, thus preventing the nuclease reach to, for example, siRNA [13]. The internalization pathways utilized by polymeric NPs to carry siRNA include cationic charge, cell-penetrating peptides (CPPs) usually more efficient *in vitro*, antibodies, and aptamers, due to their interaction with cell membrane anionic proteoglycans, enabling endocytosis [13].

Regarding lipid-based vectors, they can be designed into lipoplexes and liposomes, solid lipid-based NPs or nano lipid-based emulsions [61]. While lipoplexes correspond to cationic lipids (non-vesicular structure), liposomes correspond to a liposome structure (vesicular structure) [61]. Among the first utilized cationic lipids, there is N-[1-(2,3-dioleyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTMA) that gave rise to 1,2-bis(oleoyloxy)-3-(trimethylammonio)propane (DOTAP), whose difference relies on DOTAP ester bonds, lowering the cationic lipid cytotoxicity [61]. DOTMA cytotoxicity and DOTAP high positive charged surface required modifications with other cationic lipids, such as dioleoyl phophotidylethanolamine (DOPE), which displays fusogenic properties to enhance lipoplex endosomal escape, or DC-Chol3β[N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) to potentiate NA condensation to stable structures for transfection efficacy [10, 61, 69]. Already commercialized application examples are Lipofectamine®, a DOTMA derivative with a spermine group addition, (2,3-dioleyloxy-N-[2(sperminecarboxamido) ethyl]-N, N-dimethyl-l propanaminium trifluoroacetate) (DOSPA), with DOPE and Lipofectin®, a DOTAP combination with DOPE [61]. Di-octadecyl-amido-glycyl-spermine (DOGS), structurally similar to DOSPA, is commercialized as Transfectam® protecting the carried content against pH-sensitive nucleases elimination [61].

Liposomes are a natural amphipathic phospholipid bi-layer delivery systems that, depending on the size and bi-layers amount, form uni-lamellar or multi-lamellar vesicles [70].

Following the liposomes, in the 1990s, emerged the solid lipid-based NPs. They are spherical structures with lipophilic core domains that shaped solid stable complexes with DNA by eletrostatic interactions. Unfortunately, their lipophilic core presented hurdles in gene material encapsulation, which limit its applications as a gene delivery system [61, 71].

Zwitterionic amino lipids correspond to non-viral delivery nanosystems tailored for *in vivo* gene editing delivery and composed by zwitterionic sulfobetaine head groups, amine-rich linker regions and several hydrophobic tails, an easy modified and prepared nanosystem to enhanced stability, reproducibility, low immunogenicity, and low toxicity with reduced protein corona formation with applications in aptamers-based biosensors, diagnosis and drug

delivery [4, 17, 42]. This lipid-based system can promote permanent CRISPR/Cas9-mediated DNA editing *in vitro* and *in vivo*, resulting in increased levels of Cas9 protein expression [42].

Lipid-based nanoemulsions are O/W NPs, equilibrated thermodynamically by surfactant cationic lipids [61, 71]. Although, gene material integration into the oil phase protects it from enzyme degradation [61, 71], negatively charged substances of nanoemulsions create an unstable gene encapsulation and incapability of nano-size maintenance [61]. Researches of modified lipid-based nanoemulsions with other non-viral vectors can exceed these limitations [61, 71].

Lipid-based nanosystems have essentially been explored for mRNA delivery to dendritic cells for the development of novel vaccines and siRNA with surface conjugated aptamers [66]. They also have revealed higher biocompatibility and, consequently, lower toxicity than polymer-based NPs; however, lipid-based materials transfection efficiency is lower than polymeric compounds and may induce inflammatory responses after systemic administration [29].

Peptides are amino acid chains with a small size, stable and easy to produce [72]. CPPs, also categorized as cationic peptides, are positively charged amino acid sequences, such as trans-activator of transcription, penetratin, oligoarginines, amphipathic and hydrophobic peptides [72]. CPPs have been studied for cago delivery into cells; however, they have the ability to cross any cell membrane due to their lack of specificity. There are some strategies to contour this issue such as triggered deprotection of CPPs at tumor sites, local delivery, or conjugation to cell-targeting ligands [13]. Surface receptors to specifically cross cell membranes, as platelet-derived growth factor receptor or vascular endothelial growth factor receptor (EGFR), among others, are often utilized [72].

Zhang *et al.* reported the first polycation-mediated CRISPR/Cas9 application in aorta gene editing [73]. The gene-editing nanosystem was designed with cholesterol-terminated ethanolamine-aminated poly(glycidyl methacrylate) (CHO-PGEA) rich in amino and hydroxyl groups NP, carrying plasmid Cas9 and sgRNA-Fbn1 expression cassettes. This engineered complex *in vitro* studies demonstrated high stability and, posteriorly, *in vivo* studies revealed transfection efficiency with absent toxicity for Marfan syndrome treatment, a connective tissue autosomal dominant and lethal disorder with origin in fibrillin-1 mutations, with enriched aortic tissue accumulation due to peptide angiotensin II addition, increasing the vasculature pressure and enhancing vascular permeability [73]. Other CPP transfection efficacy example was reported by Khalil *et al.*, utilizing octaarginine, a cationic peptide coated with a pH-sensitive cationic lipid NP, which is neutral at physiological pH and positively charged at acidic conditions, posteriorly, loaded with pDNA, creating a synergistic non-

positively-charged system with high serum resistance and active targeting abilities, when compared with other non-viral delivery systems [74].

Non-viral delivery systems progress emerged a new generation of NA delivery, lipolyplexes. Lipolyplexes composed by both polymer-based and lipid-based nanomaterials combined all of their properties advantages for enhanced drug delivery, covering NAs, polymers and lipids individual limitations with *in vivo* multigenic diseases as cancer and neurodegenerative disorders applications [37]. For example, Jung *et al.* created a flexible nanocarrier by percolating tumor tissue capability and siRNA delivery. The flexible nanocarrier based on AT1002 peptide and PEG surface modifications of siRNA/PLL polyplex (N/P ratio of 0.8) coated with fused DOTAP/DOPE cationic liposomes gathered all the flexible nanocarrier made modifications, creating a unique lipolyplex concept of intercellular penetration with improved permeability and retention effect (EPR) *in vitro*; however, PEG-to-AT1002 ratio was difficult to optimize *in vivo* [75].

For siRNA and DNA-based therapeutics delivery success, cationic polymers have been played effective delivery roles; however, the inherent disadvantages of positively charged polymers may induce adverse effects through excessive interaction with cell membranes and intravenous administration with posterior serum protein-induced aggregation. Therefore, in order to overcome these issues and optimize these vectors, micelle-like NPs were developed, defined as self-assembled half micelles and half polymeric NPs vectors, which are capable of diverse diblock and triblock copolymers conformations with stimuli-responsive and targeting functions and simultaneously delivery small molecules, NAs and imaging therapeutic agents [76].

In summary, the "non-self" characteristic of synthetic nanosystems utilized for gene delivery *in vivo* can lead to some adverse effects. Some hydrophilic polymer solutions applied to circumvent these hurdles also presented issues, such as low cell adhesion and decreased cell internalization. Lipids were considered ideal materials due to their inherent biocompatibility; however, its lack of structural integrity caused leakage content problems [77].

Although chemical carriers right systemic and transfection features for gene delivery, the main associated hindrances are the brief-lasting systemic circulation, low target capacity in the lack of passive and active directions, the weak monitorization capacity of gene delivery and therapy efficiency, and the incapacity for synergically and simultaneously deliver multiple therapies and mechanisms agents [60].

Compared with bare NPs, the emerging biomimetic NPs approaches present better biocompatibility, photostability, environmental sustainability, prolonged blood circulation

time, tumor cell selectivity, and targeted delivery with unnecessary organic solvents utilization, complex ligands or strong reducing agents [78].

2.3.3 Biomimetic Nanosystems

The already studied NPs efficacy, safety, flexibility and multiple functionality advantages in payload incorporation with a specific therapeutic or diagnosis function *in vitro* have an ultimate goal: efficient bio-interfacing ability for *in vivo* translation [19, 79].

Therefore, nanomaterials including both inorganic NPs and organic NPs have been improved with biomoieties, such as chemical modifications with targeting ligands conjugation attempts to promote nanomedicine *in vivo* performance. These improved hybridized nanosystems via bottom-up approaches shall display immune response, phagocytosis and vascular clearance escape by mimicking inherent host cells membranes functions, such as specific cell-cell interactions, intercellular recognition and adhesion [19, 79]. It was, posteriorly, demonstrated that chemical conjugations were unsuitable for modifications of biological membranes due to membrane surface components susceptibility for denaturation, ever since, nondisruptive strategies have been developed to preserve membrane biological activity [80].

Studies with NP design demonstrated that non-spherical geometries mimicking the shape and deformability of RBCs had prolonged blood circulation and lower accumulation in tissues, which usually prone more rigid particles to faster clearance from circulation. Accordingly, these findings triggered the development of artificial delivery vehicles to achieve RBCs properties, minimizing vascular collisions, avoiding stacking capillaries and bypassing RES uptake with enhanced delivery properties, such as tissue targeting, cellular uptake, and payload transfection efficiency. The mechanical deformation of polymeric particles to mimic RBC shape is a feasible strategy, including the amphiphilic assembly of lipids, surfactants, and block copolymers in buffer solutions, with layer-by-layer assembly of biconcave polymeric templates [81], promoting these NPs mimicking strategies for other cell types. For example, Anselmo *et al.* developed a platelet-like NP utilizing the layer-by-layer technique to form flexible capsules morphologically similar to natural platelets, mimicking platelet innate ability to interact with vascular wall due to its shape, flexibility and complex surface. The designed NPs demonstrated *in vitro* specific surface binding capabilities, target sites adhesion, and platelet-aggregatory properties and *in vivo* revealed platelet mimicking efficacy to target injured vascular sites, a promising treatment of vascular disorders as cancer, inflammation, thrombosis and haemorrhage [82].

In this essay, the most recent and further detailed biomimetic nanosystem example will be cell membrane-coated NPs.

3. Cell membrane-coated nanosystems for gene delivery

Biomimetic design principles applied in nanomaterials created a novel class of cell membrane-coated NPs composed by a synthetic NP core cloaked by a layer of a natural cell membrane, thus combining both natural cells and artificial NPs properties for effective biointerfacing, such as long blood circulation time, specific targeting and nonspecific uptake, bypassing chemical modifications hindrances and including easy preparation steps like membranes extraction of source cells, NPs construction, and the fusion of both previously prepared materials and their inherent properties via top-down approaches [79, 83].

In 2011, researchers attempted the first reported cell membrane-coated NP concept utilizing red blood cells (RBCs) membranes extracted by hypotonic lysing of RBCs and posteriorly molded into vesicles, as the natural material source, due to its extensive circulation periods mediated by surface markers, such as cluster of differentiation 47 (CD47) and complement regulatory proteins, as the nanoparticulate core, they selected a polymeric PLGA NP, resulting in a final nanosystem with a significant extended period half-life elimination of ≈40h [84].

There are several different methods for cell membrane-coated NPs manufacture: initially, only physical extrusion methods were applied, adapted from liposomes synthesis and mechanical disruptive extrusion force with NP cores and purified membranes coextruded by porous membranes; recently, sonication approaches with component ultrasonic disruptive energy forces have been applied to the spontaneous formation of the core-shell nanostructure with the advantage of less material loss, the semistable nature combination of NPs and cell membrane-derived vesicles and charge asymmetry of biological membranes provides a core-shell configuration with right-side-out membrane orientation; other recent techniques, such as electroporation in RBC membrane-coated magnetic NPs forming a microfluidic system which demonstrated high stability and complete NPs coating, *in situ* nanomaterials coating technique using live cells through inorganic NPs incubation in serum-free environments that secrets vesicles containing exogenous NPs, among others, have been optimizing cell membrane-coated NPs production methods [79].

Since the first reported cell membrane-coated NP application, researchers have applied and expanded the use of this nanosystems with several different NPs combined with various cells types-derived membranes, embracing different cell types and associated

applications, including RBCs, cancer cells, white blood cells, platelets, stem cells, and others [79].

Regardless of NP core material, the essential NPs negative zeta potential facilitates the membrane orientation around the NP, due to electrostatic interactions between negatively charged NPs surface and negative extracellular components. To date, the utilized NP cores for cell-derived membranes coating included inorganic nanocrystals, mesoporous silica NPs, metal frameworks, gold-based or magnetic NPs, and organic polymeric cores like PLGA NPs or protein cores, which dictates the cargo release, efficacy, biocompatibility, and targeting capacity of NPs, moreover, the NPs membrane coatings extra benefit of decreased premature content release, allowed NPs slow diffusion and accumulation in targeted tissues with substantially less cargo lost, thus maximizing the nanosystem therapeutic effects and safety [19, 85].

Critical steps in cell membrane-coated NPs production include a complete analysis of the manufactured nanosystem pathway and final product features. A successful membrane coating must confirm a 10-20 nm particle size increased difference through dynamic light scattering, transmission electron microscopy, or NP tracking analysis techniques, and a complementary zeta potential measurement with a similar charge to that of the cell membrane-derived vesicle. Besides, by performed Western blotting and SDS-PAGE analysis on the manufactured cell membranes, inherent surface biological properties must be ensured [85, 86].

The diversity of cell membrane-coated NPs development encloses a wide range of applications such as, gene and drug delivery, imaging and phototherapy, detoxification which corresponds to an exclusive feature of cell membrane-coated NPs that safely neutralizes toxins, immune modulation with potential to address bacterial infections and cancer by substrate neutralization, enabling toxins safe delivery, and as a source of multiantigenic material, promoting immunity against specific targets [79].

3.1 Red blood cell membrane-coated nanosystems

RBCs are the most abundant type of blood cells in the human body responsible for oxygen transportation with a diameter of 7-8 μm, anucleated and organelles absence, expanded surface, and large surface-to-volume ratio, representing a biocompatible, low-cost, and easily modulated cell with tissue and plasma redistribution ability, long time systemic circulation along with blood elements, endothelium and reticuloendothelial system constituents as accessible targets, and vasculature passive exit in pathological situations, all cell type 51isulphide characteristics as a promising delivery system [80, 87].

The self-markers of RBCs surface that allows the human body 120 days blood circulation period are CD47 protein which regulated macrophage phagocytic uptake, complement receptor 1 (CR1) and decay-accelerating factor (DAF), which prevents improper self-recognition by C3 convertase inhibition, CD59 and C8 binding protein, preventing full membrane attack complex assembly, all biodegradable elements without toxic bi-product formation, conferring high biocompatibility and immunogenicity escape ability to RBCs [80].

Although the micrometer size of RBCs limits their extravascular diffusion, the nanometer size of synthesized RBC membrane-coated NPs could penetrate tissues and achieve intracellular drug delivery [80]. Moreover, the RBC surface negatively charged polysaccharide, called glycocalyx, is important for the stability and as immune escape factor of the nanosystem, which ensures the monolayer film coating of NPs and plays an interfacial interaction role between RBCs membranes and NPs [88].

The engineering of RBC membrane-coated NPs mimics RBCs by fusing NPs cores with naturally derived RBC membranes, providing smart delivery carriers, due to RBC membrane semi permeability, which prevents the rapid clearance of the NP payload, thus achieving cargo sustained release, and the lack of genetic materials in mature RBCs, which enables more safety when compared to other gene therapies [14, 80].

Therefore, RBC cloaking protects therapeutic or gene molecules from inactivation pathways and immunogenicity [87].

RBC membrane applications are mostly used for oncologic, inflammatory, and neural treatment purposes [87].

Huang *et al.* utilized mcDNA, due to its advantages comparatively to pDNA, incorporating it into an already PEG and biodegradable chemical groups modified mixture of commercialized cationic polymers reagents XtremeGENE and ε-caprolactone modified PEI, to avoid mononuclear phagocytosis rapid clearance and enhance the biodegradability of the nanomaterial, posteriorly cloaked with an RBC membrane prepared by hypotonic-osmotic methods, which removed the 52isulphide52 inside the RBC, thus preventing cargo oxidation [51]. *In vitro* studies revealed an optimal 8 μg μL−1 mcDNA concentration with loading efficiency in cationic polymer complex, an RBC membrane-coated cationic polymer-mcDNA complex with maintained membrane integrity and origin morphology, no significant size or structure alterations, significantly neutralized zeta potentials with a final slightly positive charge with consequent better biocompatibility and lower cytotoxicity than membrane-uncoated cationic polymers, and enhanced gene transfection efficiency, demonstrating RBC membranes ability to protect nanomaterials from lysosomes nuclease degradation and

enhance the nanocomplex biocompatibility through RBC complete membrane biostructure elements utilization. The *in vivo* nanosystem transfection efficiency is currently being further investigated [51].

The severity of vascular diseases and the research on vascular tissue engineering with biomimetic RBC membranes nanocoating for immune-evasive properties and prolonged-time blood circulation via CD47 protein self-marker, this way reducing the susceptibility to macrophage uptake inspired Hao *et al.* to create a simple method for manufacturing an amphiphilic co-polymer PLGA-PEI complexed with pZNF580, a plasmid that promotes endothelial cells proliferation and migration, with a particle size of 106 ± 3 nm, PDI of 0.26, and zeta potential of -13.5 ± 0.7 mV, then cloaked it via electrostatic interactions with two different N/P ratio types of RBC membranes, type I with N/P ratio of 1 and type II N/P ratio of 10 with a final particle size of 178 ± 3 nm, PDI of 0.32 and 27.5 ± 0.8 mV zeta potential [77]. *In vitro* and *in vivo* studies demonstrated that the created RBC membrane-coated polymeric micelle nanosystem presented reduced phagocytic uptake, increased circulation time, low cytotoxicity, strong immune-escaping capability and biocompatibility, gene delivery efficiency with intracellular and extracellular barriers overcoming ability suitable for *in vivo* applications, RBC membrane-type II comparatively to RBC membrane-type I had more successful gene migration inhibition and enhanced endothelial cell transfection, the immunomodulatory proteins on RBC membrane were also successfully translocated to the RBC membrane-coated NPs [77]. This strategy opened a new avenue for developing gene delivery systems camouflaged by RBC membranes for the treatment of vascular diseases [77].

Taking into account that the ideal siRNA carrier should present neutral or negative surface charge to achieve longer time blood circulation, intracellular site turning capacity for a positive surface charge to endosome disruption, high transfection efficiency and low toxicity, Wang *et al.* designed the first worm-like biomimetic RBC membrane-coated nanosystem, in order to solve the inconsistency between positive surface charge and prolonged-time blood circulation of gene delivery systems, with charge-reversible targeted siRNA vector of cationic bovine serum albumin (cBSA) with a final size value of 235.7 nm × 75.5 nm [89]. *In vitro* studies revealed that, by adjusting the proton buffering properties of siRNA/cBSA ratio value for 2, it accomplished negative charges at the neutral pH value that turned for a positive charge at lysosomal pH values, releasing the siRNA into cytoplasm with enhanced stability, long circulating time, absent toxicity, and no immunogenicity in autologous applications, when compared with multiple doses PEGylated nanocoating. The worm-like cloaked nanosystems also enhanced tumor site accumulation and provided longer

circulating lifetime comparatively to spherical micelles. *In vivo* studies supported the *in vitro* results of the worm-like RBC membrane-coated nanoplexes, demonstrating stability, low cytotoxicity, biocompatibility, lysosomal escape, enhanced cellular uptake, protection against Rnase A degradation, gene downregulation of luciferase genes in mouse melanoma cancer B16F10 cell line *in vivo*, high transfection efficiency, and multifunctionality as gene vector for cancer therapy and other diseases [89].

These biomimetic nanosystems required specific characteristics to overcome obstacles of cell internalization, especially in cancer. Thus, NPs surface modifications methods by targeting ligands can increase targeted tissue permeability and minimize target effects [88].

The current cancer therapies limitations required novel therapeutics and alternative combinations for safer and more effective cancer treatment. Ou *et al.* developed a novel nanostructure composed by an inner core of tailored black phosphorus (BP) of 60 nm, a biodegradable and near-infrared range (NIR)-stimulus core more morphologically uniform and biocompatible than untailored BP to address issues related to carbon-induced oxidative stress and inflammation and metal-associated cytotoxicities, grafted with poly-L-histidine (pH-responsive polypeptide), and loaded with interleukin-1α silencing siRNA (Ilsi) and paclitaxel (PTX) (a chemotherapeutic agent) [90]. *In vitro* studies demonstrated that the BP/poly-L-histidine/Ilsi complex had a zeta potential value of 6.08 mV showing strong electrostatic interactions. Posteriorly, they coated the inner core with ephrin-A2 receptor-specific peptide (YSA) anchored to an RBC membrane as the outer shell resulting in a final 174 nm nanosystem size, which was smaller than native RBCs with increased encapsulation efficiency, high content loading capacity, and preserved RBC membrane proteins. *In vitro* studies revealed that the YSA-RBC membrane-coating had no significant effect on BP crystallinity, maintained BP singlet oxygen generation properties, enabled sustained release of PTX comparatively to the noncoated NPs, presented increased tumor targetability, silencing effect, lower kidney cells internalization, amplified photodynamic effects after NIR irradiation and triggered Ilsi endosomal escape by NIR. *In vivo* studies demonstrated the nanosystem biocompatibility, suitable biodegradability, enhanced EPR effect, prolonged blood circulation, suppressed toxic effects, showed MC-38 cancer cells inhibition of C-C motif chemokine 22 secretion (macrophage and dendritic cell product stimulated by cancer cell resulting interleukin) and, consequently, reduced regulatory Treg cell migration, decreased interleukin-1a expression, and suppressed tumor growth by NIR irradiation with negligible toxicities, showing increased anti-tumor activity, thus, it was developed a promising and successful combination of phototherapy, chemotherapy and immunotherapy as a novel

cancer chemophotoimmunotherapy nanosystem, which could be further used for smarter nanostructures constructions with other cancer therapies combinations [90].

In order to accomplish successful covering, some factors have to be considered like the encapsulation of agents with considerable water solubility, RBC activity, and absent physical and chemical interactions with RBCs membranes to prevent RBCs vesicles leakage contents that can promote toxicological issues [88]. RBCs classification is subdivided into different types as A, B, O, Rh+ and Rh-, a vital guide application in clinical transfusions and diagnosis, thus RBC-derived carriers should coincide with patients' blood type and present compatible Rh, with solutions as the patient himself blood collection or type O and Rh-negative RBCs; however, this may origin blood-supply hindrances [14, 80].

In summary, RBC membrane-coated NPs main advantages include 1) mature RBCs lack of nucleus that facilitates membrane extraction and purification processes, 2) the "marker of self" protein, CD47, density and orientation, 3) absent immune responses induction after repeated administrations, 4) reduced RES uptake, 5) absent toxicity *in vivo*; 6) inherent biocompatibility and biodegradability, 7) high loading capacity, 8) NPs stability enhancement, agglomerates creation discouragement, and tumor tissue stimulation entry via EPR effect, 9) translation potential, 10) and promising application in drug delivery and functions as photosensitizers, probes, vaccines and antidotes (Figure 1) [14, 88].

Currently, the mature stage of RBCs membrane-coated NPs has relatively increased flexibility, with the possibility of NPs preparation independently of the RBC membranes-derived vesicles, which allows their large-scale production [88].

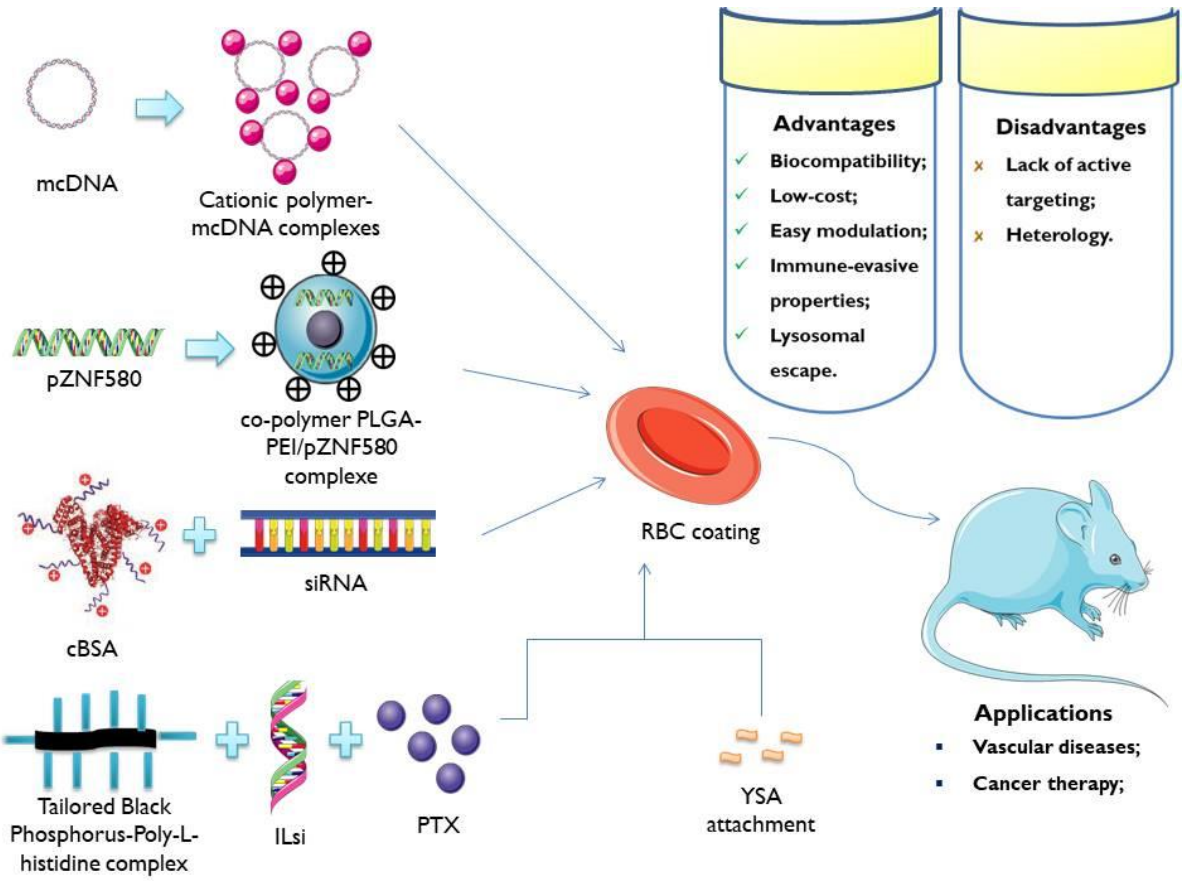

Figure 1 - Scheme of all the examples of RBC membrane-coated nanosystems described with the inherent advantages, disadvantages, and future *in vivo* applications based on the followed sources: Huang *et al.* [51]; Hao *et al.* [77]; Wang *et al.* [90]; Ou *et al.* [92].

3.2 Cancer cell membrane-coated nanosystems

The conventional methods for cancer therapy generally encompass cancer surgical resection, chemotherapy and radiotherapy, however, when unresectable or metastasized tumors occurred, chemotherapy is the only available alternative to control tumor size and spread with incapable targeting abilities, thus, limiting the administered free drugs dosage and, consequently, reducing treatment efficacy. Besides, the cancer heterogeneity demonstrated single therapeutic agent treatments ineffectiveness for complete tumor elimination [85].

The unique features of tumor microenvironment included a highly aggressive replicative nature responsible for poor lymphatic drainage, extensive fibrosis, and dense extracellular matrix leading to increased interstitial fluid pressures, which would, in turn, restrict the interactions of NPs with tumor cells [57].

Although the success of RBCs membrane coating, they revealed lack of active targeting characteristics and heterology, potentiating cancer cell membranes (CCMs) occurrence with a higher specific homologous binding [91]. On the one hand, smart

designed NPs offer the chance to carry both single therapeutic cargos and contrast agents with enhanced permeability and retention effect by passive accumulation in poor lymphatic drainage tumor tissues; on the other hand, biomimetic approaches with CCMs harnesses the unique biological CCM complexity with reduced nonspecific uptake, avoiding immune recognition and higher specific targeting [85].

Zhu *et al.* understood how to "lurker" the initially negatively charged NPs and then transform them into "attackers" by conversion into positively charged NPs [92]. They created a CCM-coated nanosystem with a mean hydrodynamic diameter of 200 nm and a zeta potential value close to zero that *in vitro* and *in vivo* revealed a positively charged nanoplex disguised by the inner domains of the NP composed by oligoethylenimine and DNA with an optimal 2.6:1 ratio as inner core[92]. The HeLa CCM-coating imparted strong adhesion and accumulation in tumor sites with adsorption resistance against the negatively charged serum components, thus conferring DNA protection, after, undisguising the positively charged nanoplex through the Warburg effect-induced in tumor acidic microenvironments. They provided a strong cellular uptake by homotypic HeLa cells and high transfection efficiency, this way covering the limitations of positively charged NPs *in vivo* applications [92].

Based on the recent photoacoustic (PA) imaging technique, that combines optical excitation with ultrasonic waves signal tracking, Zhang *et al.* developed ratiometric PA activatable probes with a desirable catalytic ratiometric PA of low-abundance miRNA *in vivo*. This new development presented greater penetration and higher resolution than the classical fluorescence imaging techniques[93]. In summary, they utilized DNA strands modified by a near-infrared fluorophore/quencher adding DNA linker to form a PA probe with contact-mediated quenching (Linker/F/Q DNA structure), recognized by target miRNA-21 binding, the selected miRNA with higher PA signal, and stimulated by glutathione-responsive DNA fuel strands. After that, both modified DNA strands and DNA fuel strands were simultaneously attached to the surface of a mesoporous silica NP by 57isulphide bonds. Finally, this nanosized system was successfully cloaked with a cancer cell MCF-7 membrane type, resulting in an imaging product with a hydrodynamic size of 293 nm and a zeta potential value of −28.3 mV[93]. *In vitro* and *in vivo* studies demonstrated the potential of miRNA-21 toehold-mediated strand-displacement hybridization function that separates fluorophore-labelled DNA strand from the quencher-labelled DNA strand, inhibiting the contact-mediated quenching and accomplishing miRNA-21 detection. The DNA fuel released in the acidic and glutathione-rich tumor environment showed recycling functions by miRNA-21 displacement, in order to promote a stronger and easier detected PA signal ratio in low-

abundance miRNA *in vivo*. Plus, the optimized Pa probes and DNA fuel strand (1:5) ratio allowed the maximum amplification and NP loading efficacy. The MCF-7 CCM-coating provided biocompatibility, low cytotoxicity and homologous binding capability to avoid immune clearance, prolonged circulation time, and enhanced tumor accumulation[93]. This cloaked nanosystem enabled the tracking of miRNA expression changes, which potentiated the discrimination of normal and cancer cells by miRNA imaging, a new feasible product to advanced molecular imaging *in vivo* [93].

Programmed death-ligand 1 (PD-L1) surfeit expression mitigate T cell response in tumor cells, therefore, Chen *et al.* assumed that PD-L1 could represent an optimal target for cancer therapy. Bearing that in mind and taking advantage of doxorubicin (DOX) chemotherapy properties, they jointed targeted siRNA PD-L1 (siPD-L1) with DOX into a PLGA nanoparticle and cloaked it with a HeLa CCM, leading to a final biomimetic nanosystem with an optimal average size of 110 nm [91]. *In vitro* studies showed steric stabilization of polysaccharides, cargo loading capacity, immune-evasive activities, and homologous-targeting of CCMs, all combined properties in a single nanosystem with highly evasive abilities [91].The projected CCMNP was a hit and emerged a future translation possibility of personalized cancer therapy[91].

PTX has limited treatment for cervical cancer heterogeneity, mainly caused by HPV infection. To overcome this limitation, Xu *et al.* exploited E6 and E7 oncoproteins involved in HPV carcinogenicity and the possible synergistic co-delivery with PTX[94]. In order to do that, the suitable studied PLGA nanoparticle, modified with PEG to prevent RES clearance and the lack of specific targeting, was the core of a HeLa cancer cell membrane cloaked homologous nanosystem, co-delivering PTX and siRNA targeting HPV18-E7 (Si/PNPs@HeLa), thus taking advantage of the CCMs immune escape and "self-homing" abilities [94], that were extracted following a pre-studied protocol[95]. *In vitro* studies demonstrated high Si/PNPs@HeLa accumulation in the tumor area and cellular uptake, restoration of the Rb protein anti-cancer effect and increased PTX intracellular concentration by E7 knockdown that suppressed the AKT pathway and MDR1 expression, solving PTX resistance issue. Plus, the co-encapsulation efficiency of siRNA and PTX was 90.2 ± 0.43% (diameter, zeta potential and PDI values non-reported)[94]. The CCMNP achieved synergistic anticancer effects and, due to the presence of CD47, an anti-phagocytic receptor, and cellular adhesion molecules, demonstrated homologous targeting and immune tolerance. It also showed an optimized biodistribution profile and sustained release of the transported content, delaying nanoparticle macrophage-mediated clearance and, consequently prolonging systemic circulation time[94]. The *in vivo* studies supported the *in*

vitro ones, proving that this HeLa membrane cloaking nanosystem can comprise a larger necrotic area, diminish the tumor size, enhance gene silencing with a successful HPV18-E7 downregulation, shows biocompatibility with no significant immunogenicity or hepatotoxicity, finally resulting in cervical cancer suppression. It also showed co-delivery ability with other drugs and other gene therapy Nas such as miRNA or ASO, potentiating a wide range of tumor treatments and personalized tumor therapies feasibility [94].

As Xu *et al.*, inspired by multidrug resistance (MDR) of cervical cancer, Zhao *et al.* built a cancer cell membrane nanosystem with a modified silica nanoparticle co-delivering Ca2+ channel targeting siRNA with DOX cloaked with a HeLa CCM[96]. Although P-gp downregulation or inhibition, an important member of ATP-binding cassette transporter (ABCT) well-known pathway, exerts effective MDR reversing, they demonstrated an alternative T-type Ca2+ channel strategy, that includes two subtypes 3.1 and 3.2, responsible for the increase of cytosolic Ca2+ and stimulation of pump independent MDR overexpressed in multiple cancer types[96]. *In vitro* studies supported the *in vivo*, showing CCM cloaking homologous transport and internalization of the designed nanosystem to HeLa cells, Ca2+ targeting siRNA with MDR reversing and synergetic anti-tumor effect with DOX, the electrostatic adsorption fabricated modified silica NP and siRNA with a w/w ratio of 15 with excellent transfection efficiency, and the perfect final w/w ratio of 7.5 after CCM cloaking, forming a desirable particle size of 122.39 ± 4.69 nm with an optimal zeta potential of -27.76 ± 3.12 mV. DOX solubility is greater in acidic environments, so cancer cells acidic matrix facilitated DOX releasing, moreover, the prepared nanosystem dilution in blood circulation with negligible hemolysis, proved its safety and biocompatibility when administrated *in vivo*, even at the highest concentration[96]. The cell cycle variations rely on Ca2+ signalling pathways thus, the regulation of Ca2+ at the intracellular level, inhibited the abnormal tumor cell proliferation, retarding the cell cycle in the G0/G1 phase by efficient knockdown of the Ca2+ subtype channels expression in HeLa/DOX cells[96]. The CCMNP also showed higher tumor targetability, tumor tissue accumulation and distribution, lower capture in RES, with much more anticancer benefits and synergistic properties than free DOX or other mono-delivery nanosystems [96].

CCM-coated NPs presented many advantages as 1) multiple cargoes encapsulation with synergistic actions, 2) reduced immune clearance after systemic administration, 3) homotypic targeting with reduced accumulation in healthy tissues and higher specific accumulation in tumor tissues, 4) biointerfacing capabilities with targeting capabilities and enhanced drug delivery, 5) localized phototherapy including dynamic and thermal phototherapies with exploited stimuli-responsive NPs drug release by specific enzymes in

low pH tumor microenvironment or inactive NPs external triggered stimuli for high precision therapy, 6) intensified imaging with high contrast agents , 7) immunotherapy functions as a cancer treatment or as a preventative cancer vaccination with higher specificity and lower cytotoxicity, 8) robustness and easy culture *in vitro* for mass membrane collection, 9) improved anti-tumor effects with loading capacity of more than one therapeutic agents as, photothermal therapy and chemotherapy combinations, providing treatments for both primary tumors and metastatic lesions and triggering anti-tumor immune responses, which maximizes therapy duration, or photodynamic therapy with chemotherapy combinations, with drug release activity in the presence of ROS with hypoxic tumor environments limitations and by starvation glucose therapy with glucose oxidase that transforms glucose into gluconic acid and hydrogen peroxide, starving tumor cells from a vital nutrient for tumor growth, 10) personalized therapies that induced specific cancer immune responses due to CCMs coated NPs biocompatibility and targeting abilities [85, 91-94, 96].

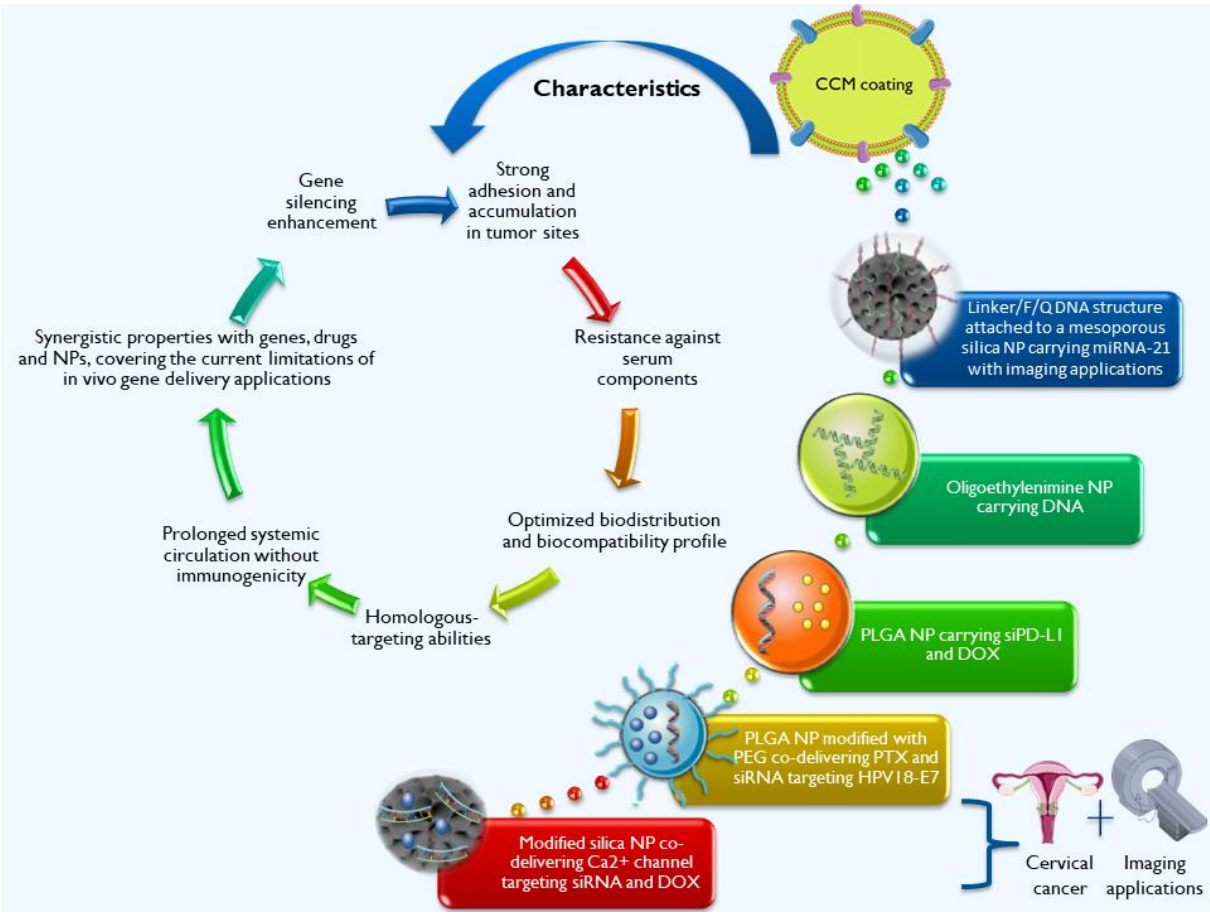

Figure 2 - Schematic presentation of all CCM coating previously described examples with the respectives applications and main properties enumeration, based on: Zhu *et al.* [93]; Chen *et al.* [92]; Xu *et al.* [96] ; Zhang *et al.* [94]; Zhao *et al.* [97]

3.3 Immune cell membrane-coated nanosystems

The ability of white blood cells (WBCs) to recognize, capture, and destroy strange body targets appealed the attention for inflammatory diseases and tumors treatments. WBCs or leukocytes are produced from hematopoietic stem cells located in the bone marrow. They defend the body against pathological conditions by pathological sites recruitment from the bloodstream and blood vessels crossing by the diapedesis process [97].

The advantages of WBCs application in drug delivery are its biocompatibility, extended lifetime, and inflamed/tumor tissues specific-targeting, providing a good example strategy for chemotherapeutic agents delivery [97].

WBCs include two main groups: granulocytes and agranulocytes. Granulocytes with cytoplasmatic granules are sub-segmented into neutrophils, basophils, and eosinophils. Agranulocytes comprehend lymphocytes (natural killer, T, and B cells) and monocytes (macrophages and dendritic cells) [98].

The highlighted most studied WBCs coating examples are neutrophils, lymphocytes, and monocytes, which will be further described [97].

Neutrophils are the most abundant WBCs and play the first-arrival immune response by LFA-1 and β1-integrin activation, migrating by transcellular pathways to inflamed and tumour sites, suggesting this way a promising delivery strategy [97].

The main neutrophils membranes features that can possibly make them suitable delivery systems are their fast transmigration ability to inflammatory sites mediated by LFA-1and β1-integrin surface adhesion molecules in both neutrophil and endothelium and recognition of blood vessel epithelium selectins during the inflammation that beneficiates their contact with endothelium surface chemokines responsible for initiating neutrophil activity. Besides, in tumor microenvironments, neutrophils have the ability to differentiate into divergent phenotypes that depend on tumor-derived factors and could affect tumor growth and metastasis [97].

A giving example of a recently applied neutrophil coating method with high biocompatibility was realized by Wang *et al.* to treat lung inflammation. They developed a core-shell of 5 mg sparfloxacin integrated into a polycaprolactone-poly(ethylene glycol) (PCL-PEG) NP and added a neutrophil membrane, resulting in a biomimetic coated nanostructure for drug delivery, which *in vivo* studies improved mice induced lung inflammation by methicillin-resistant *Staphylococcus aureus*, demonstrating that this is a promising treatment for lung inflammation with lower cytotoxicity and sustained drug release [99].

After neutrophils, lymphocytes are the second most abundant leukocytes (25% of WBC population) [98].

Lymphocyte subtype T cells belong to the cytotoxic adaptative immune system against pathological cells and include T helper cells (CD4+), that alert immune system by cytokine production; Cytotoxic T cells (CD8+) responsible for generating perforin-containing toxic granules and killing pathogen-infected cells; Memory T cells, that corresponds to the remaining legacy of antigens after T cell activation, increasing its targeting specificity and efficiency [97, 98]. Lymphocytes T cells subtype represents the most explored for biomimetic membrane coating of NPs, because of their unique specific targeting site properties, especially its tumour-targeting affinity, making them ideal drug delivery systems for cancer therapy. However, it requires dual targeting strategies for enhanced NP accumulation when compared to the insufficient single targeting ones due to tumors elevated heterogeneity [86].

Inspired by T cells membrane potential biomimetic properties, Wei *et al.* created a T-cell-membrane-coated PLGA NP, mimicking the surface of CD4+ T cells, maintaining the human T cell membrane native conformation of CD4 receptor and CCR5 or CXCR4 coreceptors, coated onto polymeric PLGA cores, which played a successful decoy role in HIV infection X4 and R5 strains inhibition, by selective binding to HIV envelop glycoprotein gp120, thus, inhibiting viral CD4+ T cells induced apoptosis and, consequently, the viral infection [100].

Finally, natural killer cells (NKCs) are associated with the innate immune system, defending the body against pathogens and cancer cells. They establish an immunologic synapse with target cells, releasing granules carrying perforin and granzyme, evoking cell lysis. Moreover, NKCs release tumor necrosis factor molecules, that bind to the receptor of target cells and trigger an enzymatic cascade, causing cells apoptosis and interferon release that affects adaptive immune response by dendritic cell and macrophage activation [101].

Considering NKCs properties, and unlike T cells, the spontaneous target cell elimination, along with cytokine secretion and antigen-presenting cells (APCs) maturation, stimulates T cells activity. Deng *et al.* discovered that NKCs-membranes could produce tumor-specific immunity by targeting and promoting the polarization of M1 macrophages. Therefore, they coated a PEG-PLGA polymeric NP loaded with 4,4′,4″,4‴-(porphine-5,10,15,20-tetrayl) tetrakis (benzoic acid) core with an NKC membrane. *In vitro* and *in vivo* studies suggested selective tumor site accumulation of the constructed biomimetic nanosystem, primary tumor cell destruction and phototherapy induced APCs activation,

presenting an efficient anti-tumor immunity and a future inspiring anti-tumor therapy for vaccines-like applications without exogenous immunologic adjuvants delivery [102].

Monocytes generate macrophages that are innate immune system regulators, whose migration to pathogenic and tumor sites are guided by chemoattractant gradients, like colony-stimulating factor-1 and chemokine ligand 2. Moreover, the transformation capacity of monocytes and macrophages into tumor-associated macrophages that have a hypoxic area tumor infiltration abilities, makes them a promising drug delivery and diagnostic applications system [97]. Despite all the macrophages promising features, intracellular degradation of drugs directly loaded into macrophages trigger premature drug release and inactivation, therefore, the incorporation capacity of NPs could contour this hurdle and benefit drug delivery [97]. For example, Sun *et al.* picked saikosaponin D, a Bupleurum extracted triterpene saponin breast cancer therapeutic attributes, a PLGA NP targeting and vectoring properties, and a hybridized membrane of macrophage with the attachment of a T7 peptide overexpressed in tumor cells, creating a biomimetic nanostructure of macrophage membrane-coated PLGA NP as drug delivery system to maximize therapeutic efficiency and contour saikosaponin D side effects, which resulted in selective inhibition of cancer growth and trivial cytotoxicity, exhibiting a potential strategy for cancer therapy [103].

Dendritic cells have origin in the hematopoietic bone marrow and accumulate in tissues like skin, nose, lungs, stomach and intestines. They are classified into mature and immature dendritic cells subdivided into many subtypes with innate and adaptive immune responses connection mainly applied in vaccines, performing potent surface antigen-presenting cells functions along with major histocompatibility complexes, which interacts with lymphocytes and NKCs. The capacity of dendritic cells to control immune system by immature dendritic cell pathogen recognition, mature dendritic cell migration to lymph nodes and, posterior, naïve T cells differentiation into cytotoxic T cells leads to the elimination of the infected cells. Whereas natural dendritic cells present amount does not allow robust immune responses, *ex vivo* antigen pulse modifications are required. Based on these properties, the accurate engineering of NPs could positively modulate the immunological functions of dendritic cells [97].

3.4 Platelet cell membrane-coated nanosystems

Platelets (PLs) are megakaryocyte derived nuclear fragments with 1-3 nm diameter that maintain homeostasis and potentiate clot production through immune responses for several infectious diseases and cancer. Akin to the other cell membranes types giving examples, PLs functionalities are related to its surface proteins and antigens specific

targeting, prolonged lifetime (8-10 days), with the extra hemostasis functions to vascular damage with high vascular adhesion and PL granules wound healing, and cancer cell affinity [86, 104].

Besides, PLs, due to their P-selectin receptor, modulate inflammatory responses by interaction with WBCs, exhibiting an important role in atherosclerosis and cancer [105]. Giving an application example, Song *et al.* used the already known anti-atherosclerosis therapeutic drug, rapamycin, to incorporate it into a PLGA core, which was posteriorly coated by a PL membrane. The final created biomimetic nanosystem performed the PLs inherent plaques affinity and their natural atherosclerotic sites accumulation; with preserved PLs surface adhesion properties and targeting abilities. Besides, this nanosystem allowed rapamycin systemic toxicities reduction and enhanced its therapeutic effect by diminishing atherosclerotic plaques with macrophage autophagy stimulation [106].

In vitro studies with PLs revealed its capacity to promote Fas-receptor-expressing cancer cells death, when activated, evidencing the potential of cancer cells-PLs complex relationship. The increasingly supported PLs recognition as innate immune system members with both stimulation or inhibition of cancer roles and their possible combination with NPs as controlled drug delivery systems has been investigated, exhibiting more powerful biomimetic PLs membranes coatings than other cell-derived membranes types for cancer therapy, due to their high tumor affinity [107].

Zhuang *et al.* reported a biomimetic platelet membrane-coated zeolitic imidazolate framework-8 (ZIF-8) porous metal-organic framework (MOF) NP loading siRNA whose particle size of 175 nm and negatively charged zeta potential pointed successful coating [108]. *In vitro* studies showed that MOF pH-dependent properties influenced siRNA traffic and protection into the cytosol and siRNA release triggering in endosome acidic environment [108]. *In vitro* and *in vivo* results demonstrated PL membrane coating improved affinity towards CD24 expressing cells when compared to RBC membrane-coated NPs, higher cellular uptake, tumor site accumulation capacity, and induced mice cancer cell apoptosis by gene silencing of overexpressed survivin mRNA in breast carcinoma [108]. The reported biomimetic nanosystem is a promising delivery and immune modulation platform to targeted and effective gene silencing in several therapeutic applications. As universal donor membrane, PL membrane-coated MOFs could mitigate long-term immunogenicity and facilitate their downstream clinical translation [108].

Other PL membrane-coated NPs applications include thrombus, infection and thrombocytopenia, taking advantage of PL cells dynamic actions as shape deformation,

contraction, and granule release, which provides sensitive cargo release and highly targeted adhesive properties to biomimetic nanosystems [104].

In summary, the biomimetic approach of PL membrane-coated NPs has been studied with inner cores as PLGA, nanogel, magnetic NPs, dextran and gold nanorods, revealing immunocompatibility, binding ability to damaged vasculature, and pathogen adhesion with interesting immunosuppression and drug resistance interventions, which require further studies for large-scale production and molecular mechanisms elucidation [109].

3.5 Stem cell membrane-coated nanosystems

MSCs are multipotent cells originated in bone marrow, umbilical cord or adipose tissue, that have been studied for solid tumors intrusion, lesioned tissues migration and targeted cells interaction, presenting intrinsic properties that could be potentiated for drug and gene delivery [110].

When compared to embryonic stem cells or induced pluripotent stem cells limited clinical utilization, MSCs are free from ethical concerns, besides, the easy MSCs isolation and proliferation *in vitro*, the already established gene carriers systems for MSCs recombination, and innate targeting ability, due to the potential dislocation for inflammation and lesion sites, are features that increase clinical attractions [110].

MSCs dissemination capacity into solid tumors, migration to lesion vicinity, direct interaction with target cells, MSCs modulation capacity to express specific therapeutic genes and to uptake drug-loaded and diagnostic NPs to specific target sites, highlighting the extensively studied inorganic NPs as photothermal agents for cancer therapy, attract even more this cell type utilization for drug and gene delivery; however, after intravenous injection, the MSCs lung entrapment induced microembolism. Therefore, the development of new platforms to seize MSCs features and eliminate possible risks led to cell membrane coated NPs drug delivery good application prospects [110]. MSCs membrane coatings are mostly designed for anti-tumor drugs delivery, due to MSCs membranes tumor tropism and RES clearance avoidance by surface biomarkers as CD47 [110].

The unsatisfactory attempts to *in vivo* delivery the polyanionic and instable character of siRNA through liposomes, polymers, and peptide conjugates raised the initial application of cell membrane-derived vesicles for drug delivery, which, in turn, encouraged Mu *et al.* to construct a new natural biomimetic delivery nanoplatform inspired in stem cell membrane-derived vesicles long circulating time and high tumor targeting features. As the inner core, they utilized the intensively studied iron oxide NPs with magnetic resonance imaging abilities, biocompatibility and low toxicity, coating it with polydopamine, which have been presented

both high NIR optical performance and photothermal conversion efficiency, after, they attached siRNA for gene therapy and, finally, as the outer shell, they coated the inner core with a stem cell membrane, revealing a final successful hydrodynamic diameter of 109 nm and zeta potential of -30.28±1.32 mV, a close value to the natural existent stem cells vesicles. *In vitro* results demonstrated the siRNA binding to the surface of the modified NP, due to polydopamine numerous functional groups, which performed π-π stacking interactions between the polydopamine aromatic groups and the nucleobases of siRNA, posterior coating with retained MSCs membrane proteins, performing polydopamine photothermal effects and iron oxide NP magnetic resonance imaging contrast, moreover, it also showed stimuli-responsive cytotoxicity under laser irradiation in normal 293t cells with good biocompatibility. *In vivo* studies showed Plk1 proto-oncogene overexpressed in tumor cells which was exploitated for anti-tumor strategies by siRNA targeting with consequent apoptotic pathways induction and, subconsequent tumor cells growth inhibition, a remarkably successful application strategy for the constructed nanosystem, performing higher tumor-targeting abilities, biocompatibility, low toxicity, and, in combination with laser radiation, exhibiting dramatic tumor regression by 60% after 15 days of treatment. Thus, it was created a new biomimetic nanoplatform for cancer therapy and a promising alternative to synthetic NPs with both imaging-guided photothermal therapy and gene therapy functions [111].

To overcome the clinical utilization hurdles of exosomes, Yao *et al.* engineered a semisynthetic exosome mimicking nanomaterial via self-assembly for myocardial infarction treatment, one of the most alarming causes of morbidity and mortality in the world, composed by miRNA-21 loaded into a mesoporous silica NP core, a NP with high miRNA loading capacity and effective miRNA protection from degradation, posteriorly, coated with a MSC membrane, presenting a final successful coating technique with negative zeta potential. miRNA, a key component of exosomes, is a promising NA-based therapy alternative for the current heart disease treatments. Therefore, they selected miRNA-21 type because of its critical role in cardiogenesis and cardiac regeneration, also considering miRNA-21 side-effects on fibroblasts, which can lead to myocardial fibrosis and remodelling [112]. *In vitro* studies suggested targeted delivery of the developed nanosystem through the ITGB2 antibody presence in MSC membrane that specifically binds to ICAM-1 on ischemic injured cardiomyocytes, confirming the well-retained source cell membrane proteins; sustained release of miRNA-21 in cardiomyocytes, suppressing the translation of apoptosis-promoting proteins programmed cell death 4 (PDCD4) and phosphate and tension homology deleted on chromosome ten (PTEN) and, consequently, inhibiting the apoptosis of

cardiomyocytes; reduced side-effect on fibroblasts due to the miRNA-21 specific targeting to cardiomyocytes, preventing myocardium fibrosis and remodelling; significant MSC membrane camouflaged effect with enhanced circulation time, endosomal and lysosomal escape, and decreased RES uptake; reduced toxicity and cardiomyocyte proliferation; and high therapeutic effect with a decreased number of dead cardiomyocytes. *In vivo* results supported the *in vitro* demonstrating the exosome-mimicking nanocomplex accumulation in the infarcted area of mouse models, long blood circulation time, biocompatibility with absent organs tissue toxicity, and remarkable anti-apoptosis therapeutic efficiency with improved infarcted myocardium functions, providing a feasible strategy to inspire other exosome-like nanosystems and an optimal solution for myocardial infarction treatment [112].

4. Challenges and Future Prospects

The heterogeneity and the lack of standardization in non-viral gene delivery nanosystems characterization create variability in the experimental results, exhibiting difficulties in results reproducibility among researchers and data extrapolation [61].

The cell membrane-coated NPs abilities to overcome the majorly of synthetic NPs hurdles also present associated challenges as reproducibility, complexity, stability, heterogeneity efficiency of the different existing type of cell membrane-coated NPs, large scale production, cost, and immunogenicity related to the use of multiple source cells for mass production, and although there is the possibility of utilize autologous sources, a more practical method is required, such as a bank material from type-matched donors [20, 79, 83].

For membrane preparation, freeze-thaw techniques seemed more suitable for organelles free cells as RBCs and PLs, however, due to ice crystals breakage cell membranes could loss structural integrity and reduce protein stability, also disruptive electroporation techniques can cause irreversible deterioration of membrane structural integrity, therefore, osmosis-based cell lysis method by mechanical membrane disruption with further sonication or extrusion presented better results, although sonication-based methods showed less material loss than physical extrusion, presenting the most popular methods utilized in CCMs manufacture. Lastly, microfluidics approaches combining the rapid mixing of NPs and membrane vesicles has been applied successfully on RBCs membranes production, despite the potential manufacturing difficulties related to practical unfamiliarity because of the optimized process requirements comprehending pulse voltage, duration, and flow velocity [85]. For example, RBC membrane-coated NP has longer retention than bare NPs; however, it is significantly shorter than RBCs yet [80]. Besides, RBCs membrane-coated nanosystems exhibited possible engineering challenges, including the optimization of fusion processes,

quality control with no biological or chemical contaminations, the required aseptic and standardized manufacturing steps, and the recent association of blood cell types with certain diseases risks. In the near future, efforts for mature biomimetic strategies should stimulate the success of RBCs membrane-coated nanosystems from bench to bedside [80].

In regards to CCM-coated NPs, future perspectives may certainly evaluate the acceptable mismatch utilization of distinct cancer cell sources-derived CCMs with maintained homotypic binding. There are still many clinical translational hurdles to overcome for this cell type-derived nanosystems, such as the development of testing procedures to ensure CCMs purity with absent contamination or molecules that could promote cancer growth, and patient-specific CCMs-coated NPs preparation with donor cells as preventive vaccination, which will require stricter quality control and regulatory methods to prevent immunostimulatory issues. Accordingly, the potential of researchers to address the mentioned hurdles will strongly impact personalized cancer therapy for a wide range of different tumor types, therefore, despite all the difficulties, CCMs coated NPs researches are worth to chase, even for a single cancer type eradication [85].

The nucleated WBCs intracellular components are more complex and, consequently, requires more difficult workflows for membranes extraction when compared to RBCs or PLs; however, their site-specific targeting to tumors and vascular disorders are worth to be explored [79].

Although PLs-tumor cells advantageous relationship for cancer therapy, the construction of biomimetic PLs membrane-coated nanosystems still exhibited clearance by the liver and off-target drug delivery effects by non-specific PL attraction to both damaged vasculature and cancer cells, limiting its clinical translation [107].

Another challenging issue is gene therapy cost-effectiveness that involves patient tailoring, only one administration, a cure for a life-time, patients monitoring for years and total repayment in case of therapy failure, which implicates a higher price tag, and that is why in an emerging field as gene therapy, the focus relies on a largest target consumer market, such as cancer therapy, to increase price tag acceptance and consumerism [61].

Nanostructures that match both diagnostic and therapeutic properties attract more interest because they provide both specific detection and targeted image-guided treatments with a potentially safer single clinical procedure [113].

Although the previously discussed single cell-membrane coating bestows promising effectiveness, their possibility of increased performance in more difficult tasks among certain biological contexts, promoted an emerging new approach, the hybrid cell membranes-coated NPs, comprehending the fusion of two different cell membranes sources, to form new bio-

coatings [88, 114]. Dehaini *et al.* engineered a new class of nanosystems based on an RBC membrane fusion with a PL cell membrane marked with distinguishable fluorescent dyes cloaking a pre-prepared PLGA NP core. *In vitro* and *in vivo* studies evidenced the success of the coating procedure with diminished macrophage uptake and strong metastatic binding to MDA-MB-231receptor of human breast cancer cells [114]. RBC-PL membrane fusion conveyed both RBC and PL membranes immunomodulatory properties and natural functionalities with prolonged systemic circulation [114].

Source cells membranes selection depends on the biological cells functions and the disease therapy requirements. Therefore, different source cell membranes confer different properties, for example, RBCs, PLs, and WBCs membrane-coated NPs tended to perform immune evasion and transverse endothelium properties, CCMs plays homotypic binding, different outputs display depending on the membranes biomarkers and surface adhesion molecules [115]. For example, Rao *et al.* designed PL-WBC hybrid membrane-coated immunomagnetic beads, modifying the complex posteriorly with circulating tumor cells targeting antibodies. This hybrid system enhanced PLs cancer binding abilities with homologous WBC interactions and prevented cell clusters formation by WBCs non-specific binding, thus, drawing a near-future possibility of personalized cancer therapy [116]. Taking advantage of both RBC membrane immune evasion capacity, by inhibiting macrophage uptake via membrane-exposed CD47 surface protein, and CCM homologous targeting and adhesion, Wang *et al.* reported a hybrid biomimetic fusion of a RBC membrane with a B16-F10 CCM, which coated a hollow copper sulfide NP loading DOX presenting both photothermal and melanoma therapies [117]. *In vitro* and *in vivo* studies showed successful hybrid membrane fusion, biocompatibility, photostability and potential PA imaging. It also demonstrated that B16-F10 CCM enabled melanoma homologous targeting and adhesion on account of surface expression of gp100 tumor antigen cells, long lifetime blood circulation, the B16-F10 cells destruction upon NIR radiation, high DOX loading efficacy, and improved drug release, providing personalized nanomedicine advances in cancer therapy [117].

Hybrid cell membranes-coated nanosystems biological moieties and functions are a promising future possibility of a synergistic delivery with enhanced therapeutic *in vivo* performance and a possible substitute of single cell membrane-based therapies and NP-based theranostics in clinical fields [86]; however, the therapeutic efficacy of these hybrid cell membranes-coated NPs is attributed inherent limitations related to specific targeting which can be overcome by active targeting solutions, mature production techniques, protocol strategies and sophisticated procedures to obtain sufficient cell membranes; issues

associated with purity of final products and long-term storage, requiring aseptic conditions [115].

Future hypothetic utilities of cell membrane-coated NPs encompass opposite immunomodulation, by stem autoimmunity in an antigenic-specific way, strong diagnostics performance, bioactive membrane ion channels and enzymes moieties, individual membrane components clarification, and many others to be discovered [79].

The future directions of this innovative approach are its continuous monitoring and analysis for increased biocompatibility and specificity for several therapeutic applications [83].

5. Conclusion

NA therapy has assumed so far huge interest regarding the biopharmaceutical market development with gene expression up- or down-regulation and immune modulation capacities to insert, silencing or editing genes, which have been benefiting from NPs studies as non-viral gene delivery systems in several infectious diseases and cancer treatments [6]. Cell membrane-coated NPs have been studied to increased NPs biointerfacing properties, evidencing enhanced transfection efficiency of NAs, prolonged NPs blood circulation time, improved specific targeting and nonspecific uptake [79].

Biomimetic nanotechnology is at the forefront of nanomedicine, a powerful approach for novel NPs development endowed with biomoieties designated biomimetic NPs or the direct utilization of naturally occurring biological materials like cell membranes to form cell-derived membrane-coated NPs [78]. Recently, the biomimetic approach of cell membrane-coated NPs have been intensively studied as a novel nanosystem template provided with biocompatibility, immune escape, ligand recognition and specific targeting, and prolonged blood circulation abilities by mimicking the antigenic diversity and biological functions of natural existing cells membranes [78, 118]. This emerged biomimetic cellular membrane-coated NPs concept comprehends the well-known and optimized bottom-up approaches utilized for synthetic NPs cores production combined with top-down assembly approaches by coating NPs with natural existent cells complexity decreasing the gap between synthetic nanomaterials and biological systems with *in vitro* and *in vivo* potential applications as drug delivery, gene therapy, imaging, vaccination, detoxification, anti-tumor, immunomodulation, and phototherapy applications, resulting essentially in immune elimination escape and prolonged-time circulation [83, 91, 119, 120].

For cell membrane–coated NPs production, the utilized synthetic NPs cores are ranging from organic to inorganic NPs as silica, gold nanorods and NPs, polymer, nanogel,

protein and metal organic frameworks, all negatively charged cores for enhanced membrane-coating [110].

At first, RBCs membranes-coated NPs effective studied NAs carrier capacity and long circulation half-life astonished scientists, inspiring the development and utilization research of other cell membrane sources, including PLs cells, immune cells, cancer cells, and MSCs, taking into consideration the diseases features [79, 88].

RBCs membranes are currently the most well-studied cell membrane type in the field, mainly due to their lack of intracellular organelles, which provides simpler membranes collection and large scale manufacture applied in drug and gene delivery, detoxification, diagnostic, imaging, phototherapy and immune modulation [51, 77, 79, 89, 90]. CCMs-coated NPs have been shown potential for prophylactic vaccines overcoming tumor cell challenges or therapeutic agents by triggering anti-cancer immune responses [85]. WBCs reported researches showed promises in their site-specific targeting capacities for tumor and vascular disorders [79]. Whereas the natural PL function maintains hemostasis, their recruitment for vascular injury sites contributes to the development of cell membrane coatings applications for atherosclerosis and bacterial infections [79]. MSCs membrane-coated NPs for gene delivery have been applied mostly in cancer therapy, due to the natural tropism of MSCs membranes to cancer microenvironments and large scale culture capacity [111, 112].

Each referred cell membrane-coated NPs types provide unique properties that may be desirable to incorporate onto a single NP, therefore, a new hybrid cells membrane-coated NP concept with mixed multiple cells membranes functionalities could enhance cells membrane-coated NPs *in vivo* performance, which will require further studies for clinical translations [79]. In the hypothetic future, cells membrane-coated NPs could revolutionize human autoimmunity, diseases diagnostics and treatments [79].

In summary, this next-generation biomimetic approach of cell membrane-coated NPs provides a novel therapeutic and diagnostic modality to raise nanomedicine and subsequent nanotechnology to a different level with natural and synthetic combinations of nanosystems to a myriad of human disease treatments, presenting promising clinical future perspectives [20, 83].

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