Universidade Federal de Pernambuco – UFPE
Centro de Biociências – CB
Departamento de Farmácia – DCFar
Programa de Pós-Graduação em Inovação Terapêutica – PPGIT

YURI BASILIO GOMES PATRIOTA

DESENVOLVIMENTO E CARACTERIZAÇÃO DE SISTEMAS DE LIBERAÇÃO NANOPARTICULADOS DESTINADOS À ADMINISTRAÇÃO ORAL DE ENOXAPARINA SÓDICA

Recife
2022

YURI BASILIO GOMES PATRIOTA

DESENVOLVIMENTO E CARACTERIZAÇÃO DE SISTEMAS DE LIBERAÇÃO NANOPARTICULADOS DESTINADOS À ADMINISTRAÇÃO ORAL DE ENOXAPARINA SÓDICA

Tese apresentada ao Programa de Pós-Graduação em Inovação Terapêutica da Universidade Federal de Pernambuco, como requisito para a obtenção do título de Doutor em Inovação Terapêutica.

Área de concentração: Fármacos, Medicamentos e Insumos Essenciais para a Saúde

Orientador: Prof. Dr. José Lamartine Soares Sobrinho

Recife
2022

Dados Internacionais de Catalogação na Publicação (CIP) de acordo com ISBD

Patriota, Yuri Basilio Gomes
Desenvolvimento e caracterização de sistemas de liberação nanoparticulados destinados à administração oral de enoxaparina sódica / Yuri Basilio Gomes Patriota– 2022.

92 f. : il., fig., tab.

Orientador: José Lamartine Soares Sobrinho

Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-Graduação em Inovação Terapêutica, Recife, 2022.
Inclui referências.

1. Nanotecnologia 2. Heparina 3. Biomoléculas I. Soares Sobrinho, José Lamartine (orient.) II. Título

620.5 CDD (22.ed.) UFPE/CB – 2023 -119

Elaborado por Elaine C Barroso - CRB-4/1728

YURI BASILIO GOMES PATRIOTA

DESENVOLVIMENTO E CARACTERIZAÇÃO DE SISTEMAS DE LIBERAÇÃO NANOPARTICULADOS DESTINADOS À ADMINISTRAÇÃO ORAL DE ENOXAPARINA SÓDICA

Tese apresentada ao Programa de Pós-Graduação em Inovação Terapêutica da Universidade Federal de Pernambuco, como requisito para a obtenção do título de Doutor em Inovação Terapêutica.

Aprovada em: 21/12/2022.

BANCA EXAMINADORA

_____

Prof. Dr. José Lamartine Soares Sobrinho (Orientador)
Universidade Federal de Pernambuco

_____

Prof. Dr. Bolívar Ponciano Goulart de Lima Damasceno (Examinador Externo)
Universidade Estadual da Paraíba

_____

Profª. Drª. Luíse Lopes Chaves (Examinadora Externa)
Universidade Federal de Alagoas

_____

Prof. Dr. Lívio César Cunha Nunes (Examinador Externo)
Universidade Federal do Piauí

_____

Prof. Dr. Fábio Rocha Formiga (Examinador Externo)
Universidade de Pernambuco

Aos meus pais, minha irmã, minha esposa e principalmente, à minha filha.

DEDICO.

AGRADECIMENTOS

A Deus, pela oportunidade de viver, pela saúde e pela resiliência.

À CAPES, pelo suporte financeiro indispensável.

Aos meus pais, Maria José Gomes Patriota e Lindoval Gomes Patriota, por todo o apoio, carinho, dedicação e sacrifício prestados em todos os momentos da minha trajetória acadêmica.

À minha mulher, Isabele Fabíola pelo companheirismo, paciência e carinho nos momentos mais difíceis e à minha filha, Ana Sofia, que ressignificou minha vida completamente.

Aos meus orientadores, Professor Dr. José Lamartine Soares Sobrinho e a Professora Drª. Mônica Felts de La Roca Soares por terem aberto as portas do NCQMC, por todo o conhecimento passado, pelos conselhos, observações e incentivos, pela paciência, pela amizade, fatores imprescindíveis para o desenvolvimento de minha formação pessoal e científica.

Aos professores do Programa de Pós-Graduação em Inovação Terapêutica que contribuíram para minha formação através do conhecimento científico transmitido.

Aos amigos e colegas do Núcleo de Controle de Qualidade de Medicamentos e Correlatos pelos momentos de amizade, apoio, ensinamentos e descontração.

A todos os amigos que fiz fora da universidade, pelo acolhimento e carinho.

RESUMO

A enoxaparina é uma molécula biológica efetiva para prevenção e tratamento de distúrbios da coagulação. No entanto, ela é pouco absorvida no trato gastrointestinal. Ela está disponível apenas como solução aquosa parenteral (por via intravenosa ou subcutânea), o que limita sua aplicabilidade clínica. Além disso, ela é inativada em meio ácido e apresenta baixa permeabilidade através da parede intestinal devido ao seu alto peso molecular, sua hidrofilicidade e sua alta carga negativa e, consequentemente, baixa biodisponibilidade oral. O desenvolvimento de uma formulação oral efetiva de enoxaparina seria essencial para a terapia anticoagulante prolongada em condições crônicas, pois superaria os inconvenientes das injeções diárias (por exemplo, dor associada à agulha, infecções, hospitalização, hematomas etc.), reduzindo os efeitos colaterais e, assim, melhorando a adesão do paciente ao tratamento. O objetivo deste trabalho foi desenvolver e caracterizar sistemas de liberação nanoparticulados destinados à administração oral de enoxaparina sódica. Desenvolvemos nanopartículas núcleo/coroa de quitosana revestidas com Eudragit® L100 para administração oral de enoxaparina através de um método completamente ecológico sem empregar nenhuma técnica de homogeneização de alta energia e quaisquer solventes orgânicos. Nanocarreadores esféricos foram preparados com sucesso, com tamanho de partícula menor que 300 nm, índice de polidispersão em torno de 0,12 e potencial Zeta maior que +25 mV, eficiência de encapsulação maior que 95% e comportamento de liberação *in vitro* que indica a boa estabilidade coloidal e o sucesso do revestimento com Eudragit® L100, processo demonstrado pela liberação cumulativa de enoxaparina insignificante (<10%) quando as partículas são submetidas as condições de fluido gástrico simulado. Demonstramos que a estrutura núcleo/coroa da partícula influenciou o mecanismo de liberação do fármaco das formulações, mais uma vez indicando a presença do Eudragit® L100 na superfície das partículas. E por fim, as nanoformulações se mostraram não-tóxicas (viabilidade celular > 90%) e com atividade anticoagulante consistente indicando que o fármaco permaneceu ativo após os processos de nanoencapsulação e liberação. Esses resultados sugerem que a abordagem de revestimento entérico e a nanotecnologia de liberação de fármacos podem ser exploradas com sucesso como ferramentas potenciais para a liberação oral de enoxaparina.

Palavras-chave: Biomacromoléculas; Sistemas de liberação de fármacos; Heparina de baixo peso molecular; Nanopartículas poliméricas; Atividade anticoagulante.

ABSTRACT

Enoxaparin is an effective biological molecule for prevention and treatment of coagulation disorders. However, it is poorly absorbed in the gastrointestinal tract. Enox is only available as parenteral aqueous solution (intravenously or subcutaneously) which limit its clinical applicability. Moreover, Enox is inactivated in acidic medium and shows poor permeation through the intestinal wall due to high molecular weight, hydrophilicity and high negative charge and consequently, low oral bioavailability. The development of an oral formulation of Enox would be essential for long anticoagulant therapy in chronic conditions since it would overcome the inconveniences of the daily injections (e.g. needle-associated pain, infections, hospitalization, hematomas, etc.), reducing side effects and, thus, improving the patient adhesion to treatment. The aim of this study was to develop and characterize nanoparticulate drug delivery systems for oral administration of enoxaparin. we developed an Eudragit® L100 coated chitosan core shell nanoparticles for enoxaparin oral delivery (Eud/CS/Enox NPs) through a completely eco-friendly method without employing any high-energy homogenizer technique and any organic solvents. Spherical nanocarriers were successfully prepared with particle size lower than 300 nm, polydispersity index about 0.12 and zeta potential higher than +25 mV, entrapment efficiency greater than 95% and the in vitro release behavior confirms the good colloidal stability and the successful Eudragit® L100 coating process demonstrated by negligible cumulative enoxaparin release (<10%) when the particles are submitted to simulated gastric fluid conditions. Finally, we demonstrated that the core-shell structure of the particle influenced the drug release mechanism of the formulations, indicating the presence of the Eudragit® L100 on the surface of the particles. Finally, Enox-loaded nanoformulations showed to be safe (cell viability > 90%) and with consistent anticoagulant activity indicating that the drug remained active after the nanoencapsulation and release processes. These results suggested that enteric-coating approach and drug delivery nanotechnology can be successfully explored as potential tools for oral delivery of enoxaparin.

Keywords: Biomacromolecules; Drug delivery systems; Low-molecular weight heparin; Polymeric nanoparticles; Anticoagulant activity**.**

SUMÁRIO

1 INTRODUÇÃO

O tromboembolismo venoso (TEV) é uma doença na qual o sangue coagula inadequadamente causando uma série de complicações potencialmente fatais (Beckman *et al.*, 2010). Além disso, é considerado uma das causas mais comuns de doenças cardiovasculares e a causa mais evitável de morbidade e mortalidade em pacientes hospitalizados (Parakh e Sabath, 2019).

Os anticoagulantes são o principal tratamento para o manejo do TEV (Piran e Schulman, 2016). Entre os diferentes anticoagulantes disponíveis, as heparinas de baixo peso molecular (HBPM) apresentam características de segurança e eficácia que as distinguem dos demais anticoagulantes e, portanto, são consideradas fármacos de escolha para alguns distúrbios da coagulação, como tromboprofilaxia e tromboembolismo (Akhtar *et al.*, 2018).

Embora seja um grupo de fármacos amplamente utilizado, as HBPM estão disponíveis clinicamente somente pela via parenteral, endovenosa ou subcutaneamente, o que traz uma limitação terapêutica e uma baixa adesão do paciente ao tratamento, principalmente quando o tratamento precisa ser aplicado por longos períodos, como é em casos de tratamentos profiláticos (Paliwal *et al.*, 2012; Schluter e Lamprecht, 2014).

Portanto, o desenvolvimento de uma abordagem não-invasiva para a administração de HBPM é urgente e uma necessidade clínica que ainda não foi atendida (Motlekar e Youan, 2006), visto que neste momento que escrevo esse documento, não há no mercado formulações com essa característica. Embora diversas rotas de administração como a nasal, pulmonar, transdêrmica e tópica estão sendo investigadas e foram reportadas na literatura (Akhtar *et al.*, 2018), a via oral é a rota de administração mais conveniente e aceitável para a administração de fármacos uma vez que ela é indolor, de fácil administração e custo-efetiva (Plapied *et al.*, 2011; Das Kurmi *et al.*, 2015).

Apesar de todas as características que fazem da via oral, a via mais intensamente explorada para a administração de fármacos, o trato gastrointestinal (TGI) é um ambiente hostil para as HBPM uma vez que evolutivamente ele foi desenvolvido para quebrar nutrientes e inativar patógenos. Além de todas as características físico-químicas desfavoráveis à absorção oral (Fang e Tang, 2020), as HBPM são afetadas igualmente por todas as barreiras existentes no TGI e, portanto, a fração que sobrevive após todo esse processo de degradação é insignificante (Moroz *et al.*, 2016).

Diversas estratégias podem ser adotadas para superar as barreiras físico-químicas e biológicas à absorção oral das HBPM e melhorar a biodisponibilidade dessas macromoléculas. Essas ferramentas tecnológicas atuam no aumento da lipofilicidade, modificação das zonas de oclusão, aumento da permeabilidade celular e proteção contra o ataque ácido e enzimático do TGI (Neves *et al.*, 2016; Fang e Tang, 2020).

Uma possível absorção intestinal das HBPM só é possível quando a estabilidade dessas macromoléculas é garantida, uma vez que elas são instáveis em meio ácido (Fang e Tang, 2020). Dessa forma, a incorporação dessas macromoléculas em nanossistemas de liberação de fármacos torna-se uma alternativa promissora para o aumento da biodisponibilidade oral dessas macromoléculas através de diversas vantagens conferidas por esses nanossistemas, como a proteção do fármaco contra o ataque ácido e enzimático, aumento da permeabilidade através da camada de muco e liberação sítio-específica (Liu *et al.*, 2018).

Portanto, este trabalho propôs um sistema de liberação nanoparticulado para a administração oral de HBPM que seja capaz de promover uma melhora na biodisponibilidade oral dessas macromoléculas através da superação das diversas barreiras inerentes à administração oral dessas macromoléculas.

2 REFERENCIAL TEÓRICO

2.1 Tromboembolismo venoso: algumas considerações.

O tromboembolismo venoso (TEV) é uma desordem multifatorial na qual forma-se uma massa anormal, a partir de constituintes do sangue, no interior do sistema vascular (Kesieme *et al.*, 2011). O termo TEV abrange duas condições clínicas, ambas potencialmente fatais: (1) quando a formação do coágulo ocorre no interior dos vasos profundos do corpo, ele é denominado de trombose venosa profunda (TVP), responsável por 60 a 70% dos casos. No entanto, (2) quando o coágulo se desprende do seu local de formação e alcança as artérias pulmonares, denominamos de tromboembolismo pulmonar (TEP), acometendo cerca de 30 a 40% dos pacientes (Beckman *et al.*, 2010).

A patogênese do TEV pode ser explicada por três fatores: estase venosa, lesão vascular e um estado de hipercoagulação, conhecidos como a tríade de Virchow (Kumar *et al.*, 2010). O primeiro fator tem um papel fundamental para o desenvolvimento do TEV, porém insuficiente quando considerado sozinho. No entanto, a presença concomitante destes três fatores aumenta consideravelmente o risco de formação de coágulo e consequentemente, o surgimento do TEV (Behravesh *et al.*, 2017).

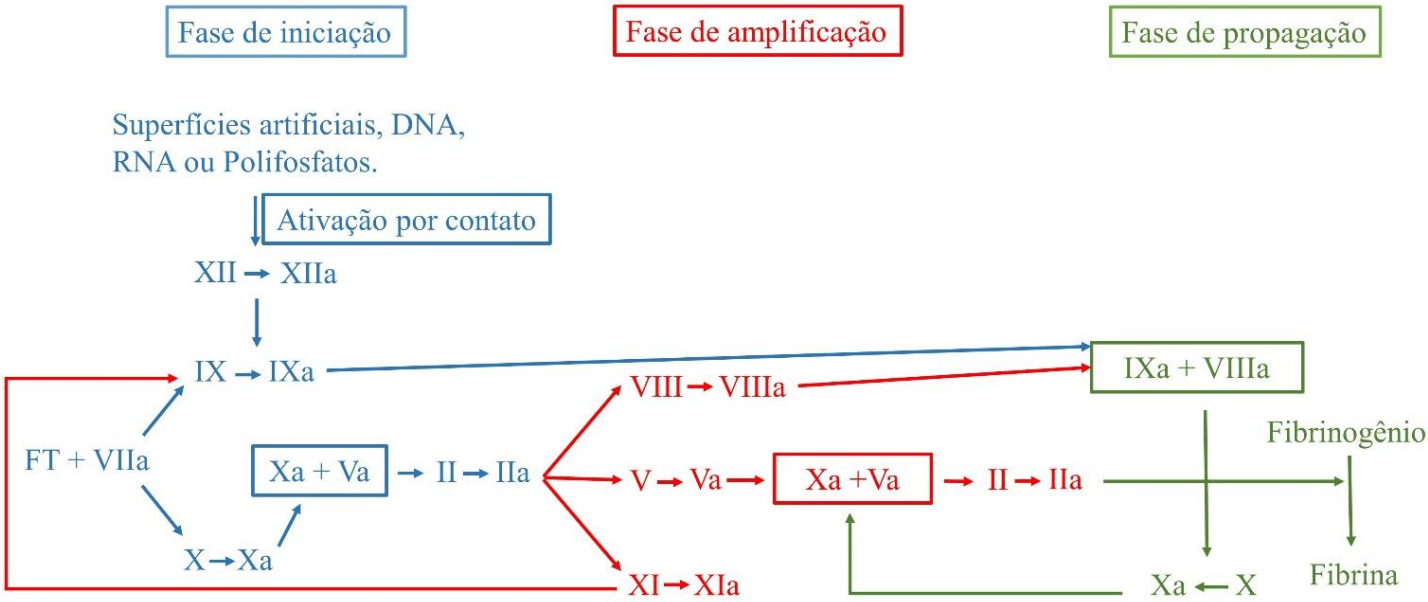
O TEV é uma doença que afeta todas as raças e etnias, assim como, ambos os gêneros e todas as idades, muito embora a taxa de incidência aumente consideravelmente nos pacientes idosos, de ambos os sexos (Beckman *et al.*, 2010). Sua incidência é estimada em 1 por 1000 pessoas, anualmente (Heit *et al.*, 2016). A taxa de mortalidade, na ausência de tratamento, supera os 3% para pacientes acometidos com TVP e 31% para pacientes com TEP (Stone *et al.*, 2017).

O TEV é uma patologia que apresenta uma alta morbidade. Neste sentido, as complicações crônicas do TEV são a síndrome pós-trombótica (SPT) e a hipertensão pulmonar tromboembólica crônica (HPTC). A SPT ocorre em 20 a 50% dos pacientes com TVP, e inclui uma série de sintomas, como: dor nos membros inferiores, edema e em casos mais graves, úlceras venosas. A HPTC ocorre em 2 a 4% dos pacientes com TEP e engloba sintomas como dispneia e intolerância ao exercício (Piran e Schulman, 2016). Portanto, o TEV é uma doença que afeta significativamente a qualidade de vida dos pacientes, mas também pode chegar a ameaça-la em casos de ausência de tratamento.

2.2 A evolução da terapia anticoagulante

A terapia anticoagulante é considerada o principal tratamento do TEV (Streiff *et al.*, 2016). Atualmente, há três tipos de anticoagulantes usados na clínica, que direta ou indiretamente afetam fatores de coagulação específicos, nas três fases da coagulação (Figura 1), inibindo o processo de formação do coágulo de fibrina (Vene e Mavri, 2018).

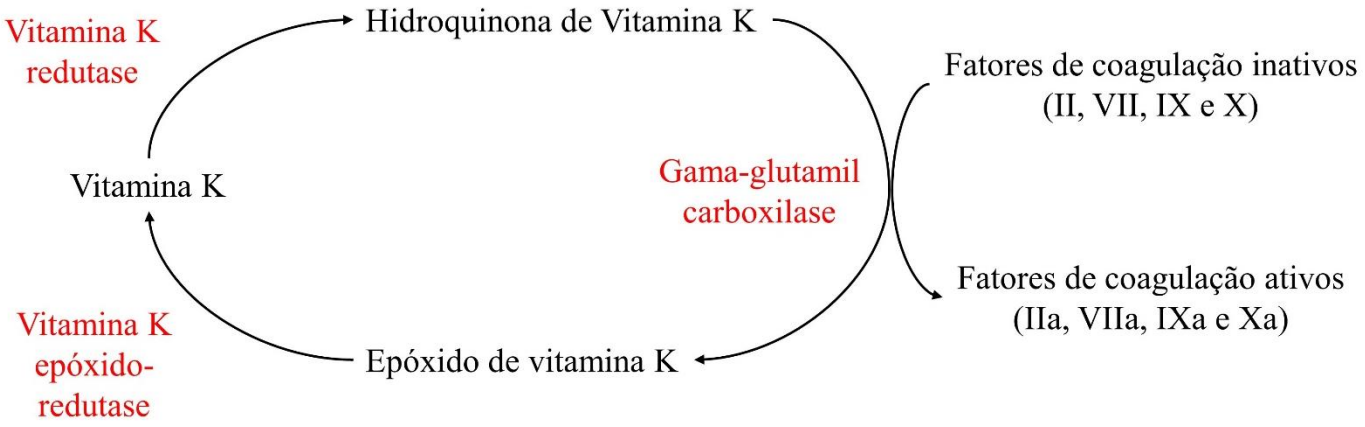
Figura 1 - Visão esquemática da coagulação sanguínea. Os retângulos, azul e vermelho, representam o complexo "protombinase"; o retângulo verde representa o complexo "tenase".



Fonte: O autor (2022).

Os antagonistas da vitamina K (AVK) são anticoagulantes orais, derivados da 4-hidroxicumarina, que agem interferindo no ciclo de interconversão da vitamina K (Figura 2), através da inativação das enzimas vitamina K epóxido-redutase e vitamina K redutase hepáticas, reduzindo a disponibilidade de vitamina K, substrato necessário para a γ-carboxilação e ativação dos fatores de coagulação II, VII, IX e X pela gama-glutamil carboxilase (Franchini *et al.*, 2016). Portanto, a produção hepática de fatores de coagulação descarboxilados ou parcialmente carboxilados resulta em proteínas com reduzida atividade coagulante (Cosmi, 2016).

Figura 2 - Mecanismo de ação dos AVK.



Fonte: O autor (2022).

A terapia com os AVK (ex: varfarina, acenocoumarol, femprocumona) é bastante problemática devido a estreita janela terapêutica, início e término da ação lentos, numerosas interações fármaco-fármaco e fármaco-alimentos, e portanto, o monitoramento laboratorial e o ajuste de doses são frequentemente necessários quando estes fármacos são utilizados (Cosmi, 2016; Franchini *et al.*, 2016).

Com o objetivo de superar as diversas limitações dos AVK, os quais até recentemente, eram os únicos anticoagulantes orais disponíveis para uso humano, os anticoagulantes orais de ação direta (ACOD) foram desenvolvidos e agem seletivamente em pontos específicos do processo de coagulação. Eles apresentam ampla janela terapêutica, rápido e previsível início e término de ação, farmacocinética previsível e por conseguinte, sem necessidade de um rígido monitoramento da coagulação e poucas interações fármaco-alimentos (Gulseth, 2016).

De acordo com seu alvo farmacológico, os ACOD podem ser divididos em inibidores orais diretos do fator Xa (FXa) e em inibidores diretos da trombina (IDT).

Os inibidores orais diretos do FXa clinicamente aprovados nas mais variadas agências regulatórias são o rivaroxabana, apixabana, edoxabana e mais recentemente, o betrixabana (Zacconi, 2018). Estes agentes agem inibindo o FXa livre ou no interior do complexo protrombinase (Figura 1). O FXa é uma serina-protease que desempenha um papel chave no processo da coagulação, uma vez que ligada ao FVa, forma o complexo

protrombinase, o qual na presença de cálcio (Ca^{2+}) catalisa a produção de trombina. Além disso, o FXa exerce um papel fundamental na fase de amplificação da coagulação, na medida que uma única molécula origina aproximadamente mil moléculas de trombina (Zacconi, 2018) .

O único IDT clinicamente aprovado é o etexilato de dabigatrana e como o próprio nome sugere, ele age inibindo a atividade intrínseca da trombina, a qual desempenha um papel central no processo da coagulação sanguínea (Figura 1) e é considerada um relevante alvo farmacológico (Franchini *et al.*, 2016). A trombina converte fibrinogênio em fibrina, tem papel fundamental na fase de amplificação da coagulação, ativando os fatores V, VIII e IX, e assim produzindo mais trombina e ativação plaquetária. Além disso, a trombina é responsável pela ativação do FXIII, o qual é responsável pela interligação das cadeias de fibrina e, consequente, estabilização do coágulo (Ibrahim *et al.*, 2016).

Embora os ACOD estejam sendo cada vez mais utilizados na prática clínica e serem relativamente seguros com relação a complicações hemorrágicas, a ausência de antídotos comercialmente disponíveis é uma imensa barreira para o uso destes fármacos. Atualmente, existem antídotos específicos sendo desenvolvidos para a terapia com ACOD e alguns encontram-se em estudos clínicos avançados (Hu *et al.*, 2016).

Outro fator que representa uma barreira importante à terapia com os ACOD é a indefinição quanto a um teste que consiga monitorar com precisão a concentração plasmática desses fármacos, condição dramaticamente necessária em certas situações, tais como, cirurgias de emergência, overdose, lesão renal aguda (Lippi e Favaloro, 2017).

As heparinas e seus derivados são definidos como anticoagulantes indiretos, uma vez que estes fármacos apresentam pouca ou nenhuma atividade anticoagulante intrínseca e exercem seus efeitos através da potencialização da atividade inibitória da antitrombina, um inibidor endógeno de vários fatores de coagulação, principalmente o FIIa, FXa e em menor grau, o FIXa (Garcia *et al.*, 2012).

De acordo com o método de obtenção aplicado, as heparinas podem ser divididas em heparinas não-fracionadas (HNF), heparinas de baixo peso molecular (HBPM) e mais recentemente, heparinas de ultrabaixo peso molecular (HUBPM), aumentando as opções para a terapia anticoagulante, com fármacos de propriedades semelhantes, no entanto, com diferenças estruturais e farmacocinéticas clinicamente relevantes (Walenga e Lyman, 2013).

As HNF podem ser definidas como uma mistura heterogênea de cadeias lineares polissacarídicas (Aláez-Versón *et al.*, 2017), e graças a esta heterogeneidade, a

farmacocinética e a farmacodinâmica das HNF são imprevisíveis. Portanto, um monitoramento laboratorial e ajustes de dose são necessários regularmente (Walenga e Lyman, 2013). Devido também as múltiplas cadeias, algumas ligam-se à outras proteínas plasmáticas e células, gerando efeitos adversos importantes, tais como, osteoporose e trombocitopenia induzida por heparina (TIH), além das complicações hemorrágicas (Oduah *et al.*, 2016).

As HBPM (ex: enoxaparina, dalterapina, tinzaparina) são obtidas através de várias reações de despolimerização enzimática ou química a partir das HNF. As cadeias de menor tamanho conferem menor ligação as células e proteínas plasmáticas quando comparadas com as HNF, resultando em maior previsibilidade farmacocinética, dose-resposta mais confiável, meia-vida plasmática mais longa e menor risco de efeitos colaterais. Por consequência, o monitoramento de rotina para as HBPM se torna desnecessário ou menos frequente na maioria das situações (Garcia *et al.*, 2012).

Recentemente, as HUBPM são desenvolvidas sob a perspectiva de desenvolver fármacos com um melhor perfil de efeitos adversos e com propriedades anticoagulantes semelhantes ou melhores do que os já existentes no mercado. Assim, o fondaparinux, um análogo sintético do fragmento pentassacarídico encontrado nas HNF e HBPM e necessário para ligação à AT, foi desenvolvido (Walenga e Lyman, 2013). Ele age inibindo especificamente o FXa através do aumento da atividade inibitória da AT. O fondaparinux apresenta um rápido início de ação, longa meia-vida plasmática e uma ação farmacológica altamente previsível, e não necessita de um monitoramento de rotina frequente (Bauer, 2001). Embora estejam clinicamente disponível, as HUBPM não estão amplamente difundidas, uma vez que seus altos custos, tornam sua utilização inviável para muitas situações clínicas (Oduah *et al.*, 2016).

As HBPM são um grupo de fármacos quimicamente e farmacologicamente distintos e cada uma tem seu próprio perfil de segurança e eficácia (Merli e Groce, 2010). Quando comparada com as diferentes classes de anticoagulantes, as HBPM são consideradas únicas devido a sua eficácia e segurança, as quais, as distinguem dos demais anticoagulantes (Park e Byun, 2016). Dentre as HBPM, a enoxaparina é o fármaco que apresenta a mais extensa evidência clínica de eficácia e segurança e, portanto, o mais amplo espectro de indicações terapêuticas e, por conseguinte, é o fármaco mais comumente usado na terapêutica (Merli e Groce, 2010).

No entanto, clinicamente, o uso das HBPM é limitado, principalmente quando o uso profilático por longos períodos é necessário, uma vez que as mesmas estão disponíveis

somente por via parenteral (intravenoso ou subcutâneo) (Paliwal *et al.*, 2012). De fato, a administração parenteral é um método invasivo que tem como consequência, a baixa adesão do paciente ao tratamento devido a diversos fatores, como o medo da dor associada a agulha, risco de infecções e hematomas (Schluter e Lamprecht, 2014). Além disso, a administração parenteral exige algumas características farmacotécnicas restritivas como ausência de pirogênios e partículas, isotonicidade e esterilidade que tornam o preparo da formulação mais complexo e, consequentemente, mais dispendioso (Motlekar e Youan, 2006).

Portanto, o desenvolvimento de uma formulação para administração não-invasiva de HBPM, particularmente de enoxaparina (Enox), é sem dúvidas, uma necessidade clínica não atendida (Neves *et al.*, 2016). Embora diversas vias de administração estão sendo exploradas, como a nasal (Yang *et al.*, 2004), pulmonar (Patel *et al.*, 2012) e transdêrmica (Jain e Jain, 2014), a via oral é, de longe, a mais conveniente e aceitável, e também a maneira mais prática de garantir uma dose diária contínua aos pacientes (Park e Byun, 2016).

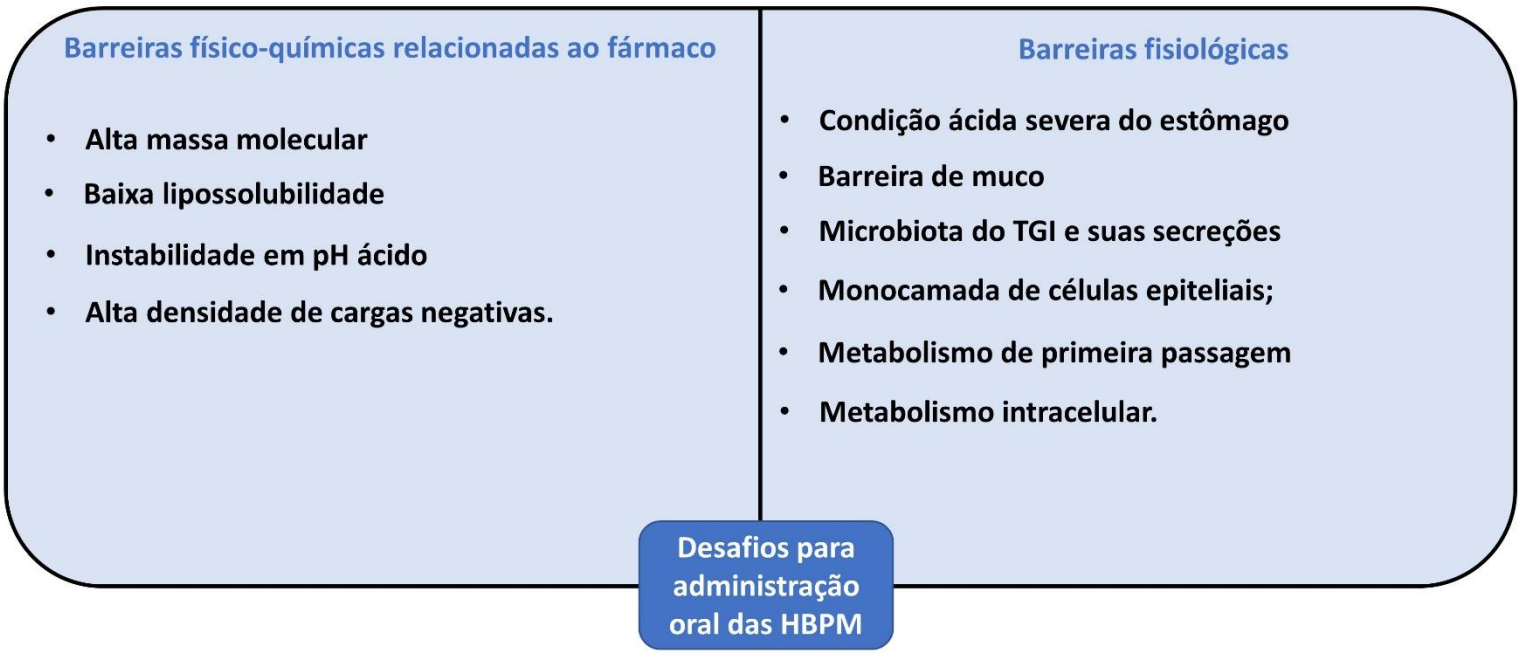
2.3 Desafios para a administração oral das HBPM

As duas principais funções fisiológicas do trato gastrointestinal (TGI) são a digestão dos alimentos em nutrientes e servir como uma barreira para impedir que materiais estranhos, como vírus, bactérias e fármacos, cheguem a corrente sanguínea. Seja administrada na forma solúvel ou formulada em formas farmacêuticas, ambas as funções do TGI representam um grande desafio para a administração oral de fármacos, particularmente, para macromoléculas como as HBPM (Lundquist e Artursson, 2016). De fato, a biodisponibilidade oral das HBPM é extremamente baixa e pode ser explicada por razões que dizem respeito as características físico-químicas do próprio fármaco, bem como as barreiras biológicas inerentes à via de administração.

Geralmente, quando administrados pela via oral, os fármacos devem seguir alguns passos até conseguirem entrar na circulação sistêmica. Primeiro, eles chegam ao estômago via esôfago e em seguida, alcançam o intestino delgado, onde são absorvidos para a veia porta ou para capilares linfáticos e transportados para o fígado ou sistema linfático, respectivamente, e finalmente para a circulação sistêmica (Fang e Tang, 2020). Durante

todo esse processo de transporte, diversas barreiras biológicas e bioquímicas devem ser superadas para a administração oral das HBPM (Figura 3).

Figura 3 - Resumo das barreiras à administração oral das HBPM.



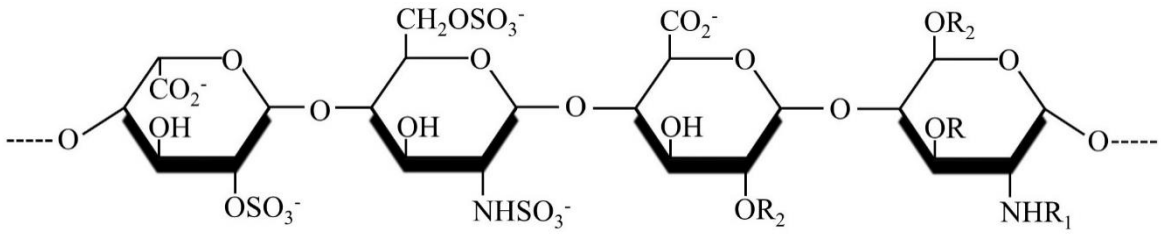
Fonte: O autor (2022).

A primeira barreira é a condição ácida severa encontrada no estômago. As heparinas, de modo geral, apresentam uma grande instabilidade química quando submetidas a pHs ácidos, sendo facilmente degradadas, levando a perda da atividade farmacológica. De fato, sob condições ácidas, ocorre a hidrólise das ligações glicosídicas, bem como uma perda dos resíduos sulfatos das heparinas (Jandik *et al.*, 1996; Motlekar e Youan, 2006).
No intestino delgado, estão situadas diversas barreiras importantes à administração oral das HBPM. A primeira delas é a degradação enzimática devido a heparinases produzidas por *Bacteroides spp.*, micro-organismos da microbiota intestinal (Ahn *et al.*, 1998; Kim *et al.*, 2000).
A camada de muco que recobre toda a superfície epitelial do TGI é outra importante barreira para a administração das HBPM. O muco é um hidrogel complexo e viscoso, composto por água, proteínas (a principal delas, mucina), carboidratos, lipídeos, sais,

anticorpos e bactérias, que apresenta pH e espessura variáveis, dependendo da região do TGI considerado (Kesinee e Bernkop-Schnürch, 2016). O muco é responsável pela proteção e lubrificação das superfícies epiteliais do TGI minimizando possíveis traumas físicos causados pelo peristaltismo de materiais ingeridos (Ensign *et al.*, 2012). Além disso, o muco age como uma barreira à patógenos e partículas estranhas, e é capaz de removê-los através de uma constante renovação de suas camadas, e assim limitar o fluxo desses materiais em direção à camada de células epiteliais subjacentes (Huckaby e Lai, 2017).

A mesma barreira imposta pela camada de muco a compostos potencialmente prejudiciais, impede que as HBPM sejam adequadamente absorvidas no TGI. As HBPM são macromoléculas lineares que apresentam uma grande quantidade de grupos sulfato e carboxilato na sua estrutura (Figura 4), os quais conferem uma alta densidade de carga negativa (Yang *et al.*, 2015; Aláez-Versón *et al.*, 2017).

Figura 4 - Estrutura química geral das HBPM. $R_1 = SO_3^-$ ou diferentes ácidos substituintes e $R_2 = SO_3^-$ ou H.



Fonte: O autor (2022).

Essas duas características, grande tamanho molecular e alta densidade de cargas negativas, tornam as HBPM mais vulneráveis a ficarem imobilizadas na malha de fibras de mucina da camada de muco por obstrução estérica ou por impedimento eletrostático, uma vez que as fibras de mucina são carregadas negativamente devido à grande densidade de cadeias de glicanos, as quais possuem uma grande quantidade de ácido siálico e grupos sulfato, que por sua vez, contribuem para essa grande densidade de cargas negativas (Huckaby e Lai, 2017).

Após conseguir difundir pela camada de muco intestinal, as HBPM devem ser absorvidas pela monocamada de células epiteliais que é considerada a maior barreira ao transporte de macromoléculas (Pridgen *et al.*, 2015). Embora existam diversos mecanismos de transporte intestinais através da monocamada epitelial, a grande massa molecular e hidrofilicidade das HBPM restringem a absorção dessas macromoléculas à via transcelular e interferem diretamente na sua biodisponibilidade

A massa molecular é um fator determinante para a permeabilidade e, consequentemente, para a absorção intestinal. A biodisponibilidade de fármacos hidrofílicos com tamanho molecular abaixo de 500-700 Da é independente do tamanho molecular. No entanto, para macromoléculas com tamanho molecular acima de 700 Da, como são as HBPM, a biodisponibilidade reduz-se acentuadamente (Goldberg e Gomez-Orellana, 2003; Hwang e Byun, 2014).

A lipofilicidade ou coeficiente de partição é outro fator que afeta diretamente a permeabilidade celular. Fármacos devem apresentar um grau mínimo de lipofilicidade para que consigam ser absorvidos através da bicamada lipídica das células intestinais por difusão passiva. Sem essa característica, a difusão passiva não ocorre, a menos que seja pela via paracelular que não é uma opção quando estamos falando sobre macromoléculas hidrofílicas como as HBPM (Goldberg e Gomez-Orellana, 2003; Hwang e Byun, 2014).

A via paracelular é o maior caminho de permeação passiva encontrado no TGI para moléculas hidrofílicas e está restrito aos espaços intercelulares encontrados entre os enterócitos (Pridgen *et al.*, 2015). As zonas de oclusão são “selos” entre as células epiteliais adjacentes formadas por diversas proteínas transmembranas e estão implicadas não só no mecanismo de aderência entre essas células epiteliais, mas também na regulação do fluxo paracelular de água, íons e solutos (Lundquist e Artursson, 2016; Liu *et al.*, 2018). Essa via utiliza canais aquosos cujos diâmetros são de 3-10 Å e, portanto, mesmo no seu estado completamente aberto, dificilmente uma macromolécula, como as HBPM, conseguiria permear a membrana celular (Liu *et al.*, 2018).

Por outro lado, a via transcelular ocorre através da internalização da molécula para o citoplasma celular pelos enterócitos ou células M intestinais, seguido pela sua liberação para o exterior da membrana basolateral em direção aos capilares subjacentes e pode ocorrer através de difusão passiva, transporte ativo ou endocitose. No entanto, quando falamos de macromoléculas, a rota endocítica é a usualmente utilizada. Endocitose é um termo mais amplo usado para descrever diferentes mecanismos de transporte celular, no qual estão incluídos, a fagocitose, a pinocitose e a endocitose mediada por receptores (Liu

et al., 2018). Conhecer como a molécula consegue acessar a camada celular intestinal é de extrema importância, uma vez que isso determinará o caminho intracelular usado pelo fármaco, a resposta biológica e até as características físico-químicas da forma farmacêutica (Donahue *et al.*, 2019; Patel *et al.*, 2019).

Após serem absorvidas, as HBPM devem difundir pelo citoplasma celular e permear a membrana basolateral e em seguida, os capilares adjacentes, até chegarem ao fígado através da veia porta hepática (Pridgen *et al.*, 2015). A primeira barreira durante todo esse processo seria a entrada no sistema endo/lisossomal celular, o qual destina o conteúdo engolfado pela célula para o lisossomo, uma organela que mantém um ambiente ácido e contém diversas enzimas hidrolíticas que poderiam causar a perda da atividade farmacológica das HBPM (Patel *et al.*, 2019). Por fim, no fígado, entre as diversas enzimas de metabolização de fármacos existentes, a heparinase é uma enzima que pode promover a degradação das HBPM e, consequentemente, a perda da atividade enzimática (Das Kurmi *et al.*, 2015; Neves *et al.*, 2016).

Com base no melhor entendimento da fisiologia gastrointestinal e consequentemente das barreiras à administração oral das HBPM, diversas estratégias vêm sendo exploradas para melhorar a biodisponibilidade oral dessas macromoléculas e elas serão discutidas na próxima seção.

2.4 Estratégias para melhorar a biodisponibilidade oral das HBPM

De acordo com todas as barreiras físico-químicas e biológicas à absorção das HBPM descritas na seção anterior, diversas estratégias podem ser adotadas, isoladas ou em combinação, para superar essas barreiras e melhorar a biodisponibilidade oral destas macromoléculas.

Os promotores de permeação (PP), são excipientes usados para melhorar a absorção intestinal de fármacos hidrofílicos através do aumento da lipofilicidade da molécula ou devido ao aumento da permeabilidade intestinal (Aungst, 2012). Eles promovem a passagem dos agentes terapêuticos através da barreira epitelial intestinal por diversos mecanismos: (1) aumento da fluidez da membrana intestinal, (2) disrupção e abertura das zonas de oclusão e (3) diminuição da viscosidade da camada de muco intestinal (Liu *et al.*, 2018).

A coadministração das HBPM com diversos promotores de permeação tais como, l-arginina (Motlekar *et al.*, 2006), tetradecil-maltosídeo (Yang *et al.*, 2005), caprato de sódio (Motlekar *et al.*, 2005), quitosana e seus derivados (Thanou *et al.*, 2007; Zhang *et al.*, 2014; Bueno *et al.*, 2015), labrasol (Rama Prasad *et al.*, 2004) foram reportados e demonstraram serem capazes de melhorar a biodisponibilidade oral destas macromoléculas.

Embora os PP sejam uma alternativa promissora para a melhora na biodisponibilidade oral das HBPM, alguns problemas tóxicos devem ser considerados e moléculas mais seguros devem ser buscadas, especialmente para o tratamento de doenças crônicas (Choonara *et al.*, 2014).

Os PP podem causar danos epiteliais irreversíveis e desorganizar as zonas de oclusão suficientemente para permitir a permeação de substâncias indesejadas e moléculas potencialmente tóxicas (Mccartney *et al.*, 2016). Os maiores problemas incluem ulceração do epitélio intestinal com erosões pontuais, problemas imunológicos e a criação de um caminho direto para bactérias e vírus ganharem acesso à circulação sistêmica e consequentemente, o desenvolvimento de uma sepse (Anilkumar *et al.*, 2011).

A conjugação química é outra estratégia que objetiva a melhora da biodisponibilidade oral das HBPM através da adição de grupos químicos capazes de aumentar a lipofilicidade e permeabilidade das macromoléculas ou direcionar a sua absorção para transportadores intestinais ou para vias de transportes mediadas por receptores (Gomez-Orellana, 2005).

A hidrofobização e a peguilação são estratégias chaves para a modificação das características das HBPM e mostraram-se estratégias promissoras para o aumento da biodisponibilidade oral dessas macromoléculas. De fato, os derivados do ácido biliar (Kim *et al.*, 2007; Lee *et al.*, 2007; Eom *et al.*, 2010; Al-Hilal *et al.*, 2014), bem como, a complexação com polietilenoglicol (PEG) (Choubey *et al.*, 2014) demonstraram ser estratégias viáveis para melhorar a absorção intestinal das HBPM.

No entanto, essa estratégia não tem a capacidade de proteger as HBPM da degradação durante a passagem pelo TGI. Além disso, alguns estudos relacionam o aumento da exposição do cólon aos ácidos biliares com o desenvolvimento de câncer colorretal (Neves *et al.*, 2016). Portanto, as concentrações dessas moléculas na formulação devem ser cuidadosamente consideradas durante o desenvolvimento das formulações.

Enquanto ambas as estratégias já discutidas não contornam o problema da instabilidade das HBPM, a incorporação dessas macromoléculas em nanossistemas de liberação de

fármacos, por sua vez, traz inúmeras vantagens para melhorar a sua baixa biodisponibilidade oral.

Os sistemas de liberação de fármacos a base de lipídeos (SLFL), como os lipossomas, as nanopartículas lipídicas sólidas (NLS), as micro/nano-emulsões (ME/NE) incluindo os sistemas de liberação de fármacos autoemulsionáveis (SLFAE) apresentam algumas características interessantes as quais tornam esses sistemas alternativas promissoras à liberação oral.

A grande versatilidade e biocompatibilidade dos excipientes lipídicos, a maioria derivados de óleos e gorduras da dieta, dão aos nanossistemas lipídicos o potencial de aumentar a internalização celular de fármacos devido a grande afinidade com as membranas biológicas (Matougui *et al.*, 2016). Além disso, esses excipientes são, em sua grande maioria, aprovados para uso farmacêutico pelo FDA e tem seu perfil de segurança bem estabelecido, garantindo uma formulação com baixo risco de toxicidade. Outro fator que favorece uma maior internalização celular é o tamanho nanométrico desses sistemas (Matougui *et al.*, 2016).

Além disso, os SLFL têm a capacidade de carregar fármacos hidrofílicos e hidrofóbicos, estabilidade, capacidade de liberar o fármaco de maneira controlada e de influenciar a rota de absorção de fármacos (Shrestha *et al.*, 2014; Niu *et al.*, 2016). Diversos SLFL incorporados com HBPM foram reportados na literatura, incluindo os lipossomas (Lavanya *et al.*, 2016), NLS (Zupančič *et al.*, 2016), ME (Kim *et al.*, 2005) e os SLFAE (Zupančič *et al.*, 2016), com resultados promissores para a administração oral das HBPM.

Outro nanocarreador bastante explorado para administração oral das HBPM são as nanopartículas poliméricas (NP). Elas apresentam uma série de características interessantes à administração oral, tais como: (1) proteção do fármaco do ambiente severo do TGI, (2) aumento do tempo de residência no intestino através da mucoadesão, (3) melhora da permeabilidade através da camada de muco, (4) endocitose das partículas e (5) liberação controlada e sítio-específica (Des Rieux *et al.*, 2006; Plapied *et al.*, 2011). Uma série de estudos foram reportados mostrando que esses nanocarreadores são capazes de melhorar a biodisponibilidade oral dessas macromoléculas (Bagre *et al.*, 2013; Fan *et al.*, 2015; Ibrahim *et al.*, 2017).

Por fim, a combinação de estratégias é outra abordagem que vem obtendo resultados promissores para a administração oral das HBPM. Eleraky e colaboradores (Eleraky *et al.*, 2020) utilizaram a combinação de um promotor de permeação com nanossistemas para melhorar a permeabilidade intestinal da enoxaparina.

O grupo de Mao publicou diversos estudos em que avaliam a combinação de um polímero promotor de permeação (quitosana) com a hidrofobização associado a um nanossistema. Eles analisaram a influência do grau de hidrofobização da quitosana, bem como, a influência do tamanho da cadeia de glicerídeo na absorção de enoxaparina (Wang *et al.*, 2013; Wang *et al.*, 2014a; Wang *et al.*, 2014b). E recentemente, esse grupo desenvolveu uma nanopartícula híbrida polímero-lipídeo e avaliou o aumento da absorção oral de enoxaparina (Dong *et al.*, 2018).

Todas as estratégias abordadas apresentam vantagens específicas que são capazes de superar alguma das barreiras impostas pela via oral. Portanto, a integração de estratégias, somando as vantagens particulares de cada abordagem em uma única formulação, precisa ser considerada para a administração oral de macromoléculas hidrofílicas, como as HBPM.

3 OBJETIVOS

3.1 Objetivo geral

Desenvolver e caracterizar sistemas de liberação nanoparticulados destinados à administração oral de enoxaparina sódica.

3.2 Objetivos específicos

- Desenvolver os nanocomplexos de quitosana/enoxaparina sódica;
- Desenvolver as nanopartículas do tipo núcleo-coroa de eudragit-L100/quitosana/enoxaparina sódica;
- Caracterizar os sistemas nanoparticulados desenvolvidos quanto ao tamanho de partícula, potencial zeta, estabilidade, eficiência de encapsulação e morfologia;
- Avaliar o perfil de liberação *in vitro* e o mecanismo de liberação em diferentes condições de pH;
- Avaliar a biocompatibilidade das nanoformulações frente a diversas linhagens celulares;
- Avaliar a eficiência anticoagulante das nanoformulações.

4 ARTIGO 1 - Applied Nanotechnologies in Anticoagulant Therapy: From Anticoagulants to Coagulation Test Performance of Drug Delivery Systems

Yuri B. G. Patriota [1], Luíse L. Chaves [1], Evren H. Gocke [2], Patricia Severino [3,4,5] , Mônica F. R. Soares [1],José L. Soares-Sobrinho [1],* and Eliana B. Souto [6,7],*

[1] Quality Control Core of Medicines and Correlates, Department of Pharmaceutical Sciences, Federal University of Pernambuco, Recife 50740-521, PE, Brazil; yuribgp@hotmail.com (Y.B.G.P.); luise@fps.edu.br (L.L.C.); monica.soares@ufpe.br (M.F.R.S.);
[2] Department of Pharmaceutical Technology, Faculty of Pharmacy, Ege University, Bornova-Izmir 35100, Turkey; evrenhomangokce@gmail.com;
[3] Laboratory of Nanotechnology and Nanomedicine (LNMed), Institute of Technology and Research (ITP), Av. Murilo Dantas, 300, Aracaju 49010-390, SE, Brazil; pattypharma@gmail.com;
[4] Institute of Technology and Research (ITP), University of Tiradentes (Unit), Av. Murilo Dantas, 300, Aracaju 49010-390, SE, Brazil;
[5] Tiradentes Institute, 150 Mt Vernon St, Dorchester, MA 02125, USA
[6] Faculty of Pharmacy, University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba,3000-548 Coimbra, Portugal;
[7] CEB-Centre of Biological Engineering, Campus de Gualtar, University of Minho, 4710-057 Braga, Portugal

***** Correspondence: jose.ssobrinho@ufpe.br (J.L.S.-S.); ebsouto@ff.uc.pt (E.B.S.)

applied nano

MDPI

Review

Applied Nanotechnologies in Anticoagulant Therapy: From Anticoagulants to Coagulation Test Performance of Drug Delivery Systems

Yuri B. G. Patriota [1], Luíse L. Chaves [1], Evren H. Gocke [2], Patricia Severino [3,4,5], Mônica F. R. Soares [1], José L. Soares-Sobrinho [1,*] and Eliana B. Souto [6,7,*]

[1] Quality Control Core of Medicines and Correlates, Department of Pharmaceutical Sciences, Federal University of Pernambuco, Recife 50740-521, PE, Brazil; yuribgp@hotmail.com (Y.B.G.P.); luise@fps.edu.br (L.L.C.); monica.soares@ufpe.br (M.F.R.S.)
[2] Department of Pharmaceutical Technology, Faculty of Pharmacy, Ege University, Bornova-Izmir 35100, Turkey; evrenhomangokce@gmail.com
[3] Laboratory of Nanotechnology and Nanomedicine (LNMed), Institute of Technology and Research (ITP), Av. Murilo Dantas, 300, Aracaju 49010-390, SE, Brazil; pattypharma@gmail.com
[4] Institute of Technology and Research (ITP), University of Tiradentes (Unit), Av. Murilo Dantas, 300, Aracaju 49010-390, SE, Brazil
[5] Tiradentes Institute, 150 Mt Vernon St, Dorchester, MA 02125, USA
[6] Faculty of Pharmacy, University of Coimbra, Pólo das Ciências da Saúde, Azinhaga de Santa Comba, 3000-548 Coimbra, Portugal
[7] CEB-Centre of Biological Engineering, Campus de Gualtar, University of Minho, 4710-057 Braga, Portugal

ABSTRACT

Heparin-based delivery systems have been explored to improve their therapeutic efficacy and to reduce toxicity for different administration routes. Regardless of the applied drug delivery system (DDS), the evaluation of anticoagulant performance is instrumental for the development of a suitable DDS. The understanding of the range of anticoagulant assays, together with their key applications and limitations, is essential both within the context of scientific research and for clinical usage. This review provides an overview of the current anticoagulant therapy and discusses the advantages and limitations of currently available anticoagulant assays. We also discuss studies involving low-molecular-weight heparin (LMWH)-based nanocarriers with emphasis on their anticoagulation performance. Conventional anticoagulants have been used for decades for the treatment of many diseases. Direct oral anticoagulants have overcome some limitations of heparins and vitamin K antagonists. However, the lack of an accurate laboratory assessment, as well as the lack of a factor "xaban" (Xa) inhibitor reversal agent, remains a major problem associated with these anticoagulants. LMWHs represent anticoagulant agents with noteworthy efficacy and safety, and they have been explored to improve their outcomes with various nanocarriers through several administration routes. The main problems related to LMWHs have been surmounted, and improved efficiency may be achieved through the use of DDSs.

Keywords: heparin; anticoagulant drugs; drug delivery systems; anticoagulant assays

1. Introduction

Investments in terms of money and time in the development of new chemical compounds have led pharmaceutical companies to explore several delivery approaches in order to improve the efficacy and minimize the side effects of drugs that are already on the market [1]. One example is heparin. This natural compound has been clinically used for the treatment and prophylaxis of coagulation disorders (e.g., venous thromboembolism (VTE) [2]) for more than 60 years [3]. Though heparins, including unfractionated heparin (UFH) and low-molecular-weight heparins (LMWHs), have also been clinically used for a long time, they show limitations because they are available only for parenteral administration, a costly and invasive method that leads to low patient compliance [4]. Non-invasive drug delivery systems are being explored to overcome these limitations. Nanotechnology-based drug carriers (nanocarriers), such as liposomes, nanogels, nanospheres, and nanocapsules, have gained increasing attention in the last few decades as a promising strategy for improving drug delivery [5] (Figure 1). Compared to conventional systems, nanocarriers have a number of advantages: (i) They are able to pass through the capillary vessels due to their submicron size and avoid clearance by phagocytes, prolonging the bloodstream circulation time; (ii) they can permeate cells and tissues to reach target organs; (iii) they can deliver the drugs in a controlled manner, reducing the frequency of administration and side effects, and they improve patient compliance [6]. Several heparin-based drug delivery systems have been developed to enhance the therapeutic efficacy of these drugs [7,8]. Although the oral route is the most desirable, it is noteworthy that there have been efforts to investigated heparin delivery through other pathways, such as nasal [9], pulmonary [10], and transdermal [11] routes.

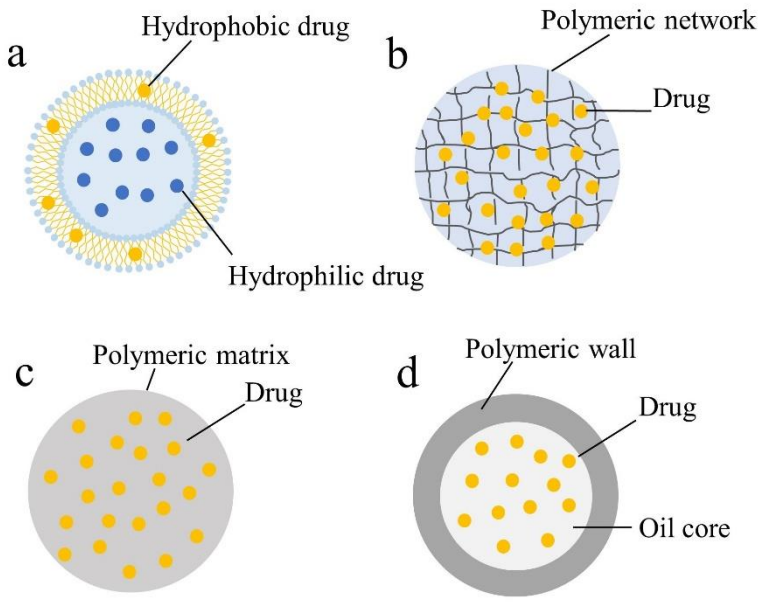


Figure 1: Schematic representation of different types of nanocarriers. (a) liposomes, (b) nanogels, (c) nanospheres and (d) nanocapsules.

The evaluation of nanocarriers efficacy comprises a critical step in the development of DDS. In particular, heparin-based nanocarriers' performance is evaluated through several anticoagulant assays based on the type of drug used. The suitability and relevance of a specific anticoagulant assay for a certain objective are also important questions [12], thus stressing the need to know their advantages and limitations [13]. Anticoagulant assays are however not comprehensively described in the perspective of DDS performance. The aim of our work has been to provide a comprehensive overview of anticoagulant drugs, together with an outline of the techniques used for monitoring the anticoagulant plasma level. The most important heparin-based DDS are also addressed, highlighting the most relevant techniques used to evaluate their anticoagulant performance.

2. Anticoagulant therapy

The main anticoagulant drugs are categorized into four classes: heparins, vitamin K antagonists (VKAs), direct thrombin inhibitors (DTIs) and direct factor "xaban" (Xa) inhibitors. For clarity, we subdivided this section into conventional anticoagulant agents (heparins and VKAs) and novel anticoagulant drugs (DTIs and direct factor Xa inhibitors).

2.1 *Conventional anticoagulant agents*

2.1.1 Unfractionated heparin (UFH), low molecular weight heparin (LMWH) and ultra-low molecular weight heparin (ULMWH)

Unfractionated heparin (UFH) is one of the oldest biopolymeric drugs that is still used in therapeutics. It is a highly sulfated glycosaminoglycan (GAG), presenting the highest negative charge density of any known biological macromolecule [14]. The anticoagulant activity of UFH is related to its affinity with a naturally occurring serine protease inhibitor, antithrombin III (ATIII), resulting in an increase in the ATIII thrombin inhibition rate, as well as an inhibition of other serine proteases involved in the coagulation process [15]. UFH is indicated for treatment and prophylaxis of several conditions, including VTE, a dangerous disorder that includes deep vein thrombosis (DVT) and pulmonary embolism (PE) [2]. UFH is administered parenterally through either intravenous or subcutaneous injection; the latter is associated with a poor bioavailability. The non-specific binding of UFH to plasma proteins explains the unpredictable anticoagulant activity among individuals, which requires continuous monitorization. UFH is rapidly cleared from the body through endothelial and macrophage cells' depolymerization mechanisms, while the kidneys are responsible for a slower UFH clearance mechanism [16]. UFH is an effective, inexpensive, and relatively safe anticoagulant agent; however, it has numerous limitations. Hemorrhage is its main life-threatening adverse effect [17]. Although bleeding can be fatal, protamine sulfate administration could reverse this adverse event. Moreover, the binding of UFH to osteoblasts and platelet factor 4 leads to the tendency to induce osteoporosis and thrombocytopenia, respectively [18,19]. The discovery of the pentasaccharide sequence [20,21], the most active core, which is involved in ATIII binding and activation and associated with the need to overcome some heparin limitations, resulted in the development of LMWH in the late 1970s to early 1980s [22,23]. LMWHs are oligosaccharide fragments that are highly sulfated, water-soluble, and negatively charged compounds, and like UFH, they are indicated for the treatment and prophylaxis of VTE [24]. LMWHs show a mean molecular weight (Mw) between 3500 and 6000 Da, with at least 60% of the material with Mw below 8000 Da. Hence, as the minimum Mw for anti-IIa activity is approximately 5000 Da, the LMWH anti-IIa activity is dramatically reduced

[17]. Currently available LMWHs are produced by several controlled chemical or enzymatic depolymerization reactions of UFH, as seen in Table 1.

These controlled reactions afford drugs with more predictable pharmacokinetic and pharmacodynamic profiles, making them more dose-dependent than UFH. As a consequence, LMWHs require less monitorization [25]. Nevertheless, LMWHs are heterogeneous compounds and have different pharmacological and biochemical features; thus, they are not clinically interchangeable [26]. Additionally, LMWHs present less adverse effects and higher bioavailability when administered subcutaneously in comparison with UFH. However, protamine sulfate administration partially reverts the anticoagulant effect, thus increasing the risk of bleeding due to overdose. LMWHs are eliminated by the kidneys; consequently, the administration in patients with renal failure prolongs its half-life [22,27].

The theory that originated the therapeutic interest in ULMWH was based on the idea that compounds with a high anti-activated factor X (FXa) to anti-activated factor II (FIIa) activity ratio would achieve similar or better efficacy than LMWH products, but with a lower risk of bleeding and thrombocytopenia [28]. ULMWHs are obtained through a more extensive controlled depolymerization reaction, which preserves the pentasaccharide active site. They contain higher percentages of short chains (Mw < 3000 Da), showing better efficacy and safety profiles. Fondaparinux was the first synthetic ULMWH, which is an analogue of the pentasaccharide sequence marketed by Sanofi in 2002 and now sold by GlaxoSmithKline under the trade name Arixtra© [29]. It has an Mw of 1728 Da, with a higher specific anti-FXa activity and longer half-life after subcutaneous injection than LMWHs. It is rapidly absorbed and 100% bioavailable after subcutaneous administration and has a half-life of approximately 14 to 18 h. It has a minimal non-specific binding and does not require monitorization. It is cleared only through the renal route; therefore, clearance may be diminished in elderly patients [30]. An advantage of fondaparinux is that it presents a low risk of virus or prion contamination because it is synthetic. On the other hand, it requires an expensive synthetic process (approximately 50 steps) with a low yield [15]. This fact has stimulated research on less expensive synthetic routes.

Idraparinux is a long-acting hypermethylated fondaparinux analogue developed by Sanofi-Aventis that binds to AT with high affinity. After subcutaneous injection, idraparinux is almost completely absorbed and shows a plasma half-life of 80 h, allowing once-weekly injection without monitoring. Idraparinux has no antidote and is excreted

unaltered through the renal route. The development of idraparinux was discontinued in phase III trials due to excessive intracranial bleeding [22,31]. The absence of an antidote for the clinical use of a long-acting anticoagulant is too dangerous. To solve this problem, a biotinylated idraparinux version was synthesized and clinically investigated. Idrabiotaparinux is a long-acting synthetic pentasaccharide analogue developed by Sanofi-Aventis. It displays the same anticoagulant properties as idraparinux. The biotin moiety allows rapid anticoagulant neutralization following the avidin infusion [31–33]. There is only one commercially available ULMWH (bemiparin). It is obtained through alkaline depolymerization from UFH, and it has been available since 1998. Bemiparin is approved for once-daily subcutaneous use in the prophylaxis of VTE. Pharmacokinetics studies reported that bemiparin showed a dose-dependent effect in healthy volunteers [34,35]. Semuloparin is a hemisynthetic ULMWH obtained through selective and controlled depolymerization. It is designed for once-daily subcutaneous administration in VTE prophylaxis in patients with cancer and to produce a higher anti-factor Xa and minimal anti-factor IIa activity compared with LWMHs. However, in 2012, the manufacturer suspended the development of semuloparin [28].

Table 1: Comparative features of LMWH, ULMWH and synthetic analogues.

Agents	M_w[a] (Da[b])	Production process	Trade name (Pharma company)	Anti-factor Xa:IIa ratio	References
LMWH					
Ardeparin	6,000	Peroxidative depolymerization	Normiflo© (Pfizer)	1.9	[15,22,46]
Certoparin	6,000	Deamine cleavage with isoamyl nitrate degradation	Sandoparin©, Mono-Embolex©, Sandoz© (Novartis)	2.4	[15,22,46]
Dalteparin	6,000	Nitrous acid depolymerization	Fragmin© (Pfizer)	2.7	[15,22,46]
Enoxaparin	4,500	Benzylation and alkaline depolymerization	Clexane©/Lovenox© (Sanofi-Aventis)	3.8	[15,22,47]
Nadroparin	5,000	Nitrous acid depolymerization	Fraxiparin© (Choay/Aspen)	3.6	[15,22]
Parnaparin	4,500	Hydrogen peroxide and cupric salt depolymerization	Fluxum© (Wasserman)	3.0	[22]
Reviparin	4,000	Nitrous acid depolymerization followed by chromatographic purification	Clivarine© (Abbott)	3.5	[15,22]

Tinzaparin	6,000	Heparinase digestion	Innohep© (Novo/Leo)	1.5	[15,22,24]
ULMWH					
Bemiparin	3,600	β-Eliminative cleavage by alkaline depolymerization	Hibor©, Ivor©, Zivor© (Sigma Tau)	8.1	[15,22,24]
Semuloparin	2,400	β-Eliminative cleavage by selective and controlled depolymerization using phosphazene base	(Sanofi-Aventis)	80	[22,28]
Synthetic analogues					
Fondaparinux	1,728	Chemical synthesis	Arixtra© (GlaxoSmithKline)	~ 850 UI anti-Xa/mg	[22,28]
Idraparinux	1,728	Chemical synthesis	(Sanofi-Aventis)	~ 1600 UI anti-Xa/mg	[22]
Idrabiotaparinux	2,052	Chemical synthesis	(Sanofi-Aventis)	~ 1600 UI anti-Xa/mg	[22]

[a] Mean molecular weight (Mw); [b] Daltons (Da).

2.1.2 Vitamin K Antagonists (VKAs)

VKAs, such as warfarin, have been the standard anticoagulant therapy for more than 60 years, and they remain the most commonly prescribed oral anticoagulants worldwide [38]. VKAs are indicated for the long-term prevention of arterial and venous thromboembolic disorders [39]. Antagonizing the vitamin K, VKAs inhibit the formation of clotting factors dependent on vitamin K, such as factor II (FII), factor VII (FVII), factor IX (FIX), factor X (FX), and proteins C and S [40]. After oral administration, VKAs are rapidly absorbed from the gastrointestinal tract; they show high bioavailability and reach the plasma concentration peak within a few hours. Furthermore, VKAs have a mean plasma half-life of 40 h, and are highly bound to plasma proteins during circulation. Biotransformation and inactivation occur through different enzymes of the cytochrome P450 system, including CYP2C9, CYP1A2, and CYP34A [41–43]. Excretion of unchanged VKAs is negligible; thus, its elimination relies on hepatic metabolism [44]. Moreover, VKA antidotes are available, and a reversal effect could be achieved through vitamin K administration or clotting factor infusion [45]. Although the VKAs' advantages are well established in several thromboembolic disorders, they have several remarkable

drawbacks. Bleeding is the most common and severe complication of VKAs [46]. In addition, the delay of onset and offset of action narrows the therapeutic index, and high inter- and intra-variability in the dose–response relationship are other issues presented by VKAs, leading to the need for frequent monitoring and dose adjustment [27,47].

2.2 *Novel Anticoagulant Drugs*

Over the last two decades, several direct oral anticoagulants (DOACs) have been developed. The research has moved directly toward the search for small molecules that bind reversibly with thrombin or FXa and are suitable for oral administration [48]. Megalatran (the prodrug was ximelagatran), developed by Hassle, followed by Astra and, subsequently, Astra-Zeneca, became the first available oral thrombin inhibitor. It shows an efficacy and bleeding rate comparable to those of LMWH and had the advantage of not needing parenteral administration or laboratory monitoring. However, ximelagatran was removed from the market due to liver toxicity [49]. Currently, there are two types of DOACs licensed for use in thromboembolic disorders: FXa inhibitors (apixaban, betrixaban, edoxaban, and rivaroxaban) and a thrombin inhibitor: dabigatran. The comparable characteristics of these agents are shown in Table 2. Dabigatran etexilate (Pradaxa®), the only direct thrombin inhibitor (DTI) licensed [47], is a selective and reversible oral nonpeptide prodrug developed by Boehringer Ingelheim Pharmaceuticals [42]. After being taken orally, dabigatran etexilate is converted into its active form, dabigatran, through esterase-mediated hydrolytic cleavage [50]. It has a rapid-action onset and low bioavailability, and it is predominantly excreted through the renal route [51]. Dabigatran etexilate has been approved for the prevention of venous thromboembolism (VTE), stroke, or systemic embolism [19]. Rivaroxaban (Xarelto®), the first direct oral FXa inhibitor clinically approved by the Food and Drug Administration (FDA) in 2008 [52], is a selective, dose-dependent, and competitive inhibitor of free and clot-based factor Xa, which was developed by Janssen Pharmaceuticals and Bayer HealthCare [53]. It is rapidly absorbed, and the blood concentration peak is achieved after 2–4 h. It has a half-life of 5–13 h, and it is excreted through the renal route (66%) and through the fecal/biliary route (28%) [54]. Rivaroxaban has been approved for the prevention of VTE [19]. Apixaban (Eliquis®) was the second FXa inhibitor to be clinically approved by the European Medicines Agency (EMA) and by the FDA for thromboembolic prophylaxis, as well as for preventing blood clots in

DVT and PE [52]. Apixaban is a direct, reversible, competitive, and selective FXa inhibitor designed by Bristol Myers Squibb and Pfizer (US). It shows good oral absorption, reaching its peak plasma concentration in 1–4 h. A total of 25% of the apixaban is excreted through the kidneys, and the remnant by the liver [55]. Edoxaban (Savaysa® in USA and Lixiana® in Canada) is another direct FXa inhibitor developed by Daiichi-Sankyo, approved in Japan and in the US for stroke and VTE prophylaxis [56,57]. It is well absorbed, reaching its plasma concentration peak after 1–2 h. Elimination of edoxaban takes place via the renal and hepatobiliary routes [58]. Betrixaban (Bevixxa®) is a more recent direct FXa inhibitor developed by Portola Pharmaceuticals Inc. (San Francisco, CA, USA). It was approved by the FDA in 2017 for the prevention of thromboembolism in high-risk hospitalized individuals [59]. Betrixaban inhibits FXa in a competitive, reversible, selective, and dose-dependent way. It is rapidly absorbed and shows its peak plasma concentration within 3–4 h. Its bioavailability is 34%, and it shows the longest half-life among the FXa inhibitors. Betrixaban is mainly excreted through the hepatobiliary system and via a P-glycoprotein efflux pump [60,61].

Table 2. Pharmacokinetic and pharmacodynamic properties of DOACs.

Features	**Factor IIa inhibitor**	**Factor Xa inhibitor**				**References**
	Dabigatran	**Apixaban**	**Rivaroxaban**	**Edoxaban**	**Betrixaban**	
M_w[a] (Da[b])	628	460	436	548	452	[62]
Bioavailability (%)	6	50	80-100	62	34	[62,63]
Protein binding (%)	35	87	92-95	55	60	[61,63]
T_{max}[c] (h)	1 - 3	1 - 4	2 - 4	1 - 2	3 - 4	[61]
Half-life (h)	12 - 17	12	5 - 13	9 - 11	19 - 27	[53,60]
Reversal agents	Yes	No	No	No	No	[62]
Renal clearance (%)	> 80	25	66	35-50	< 7	[61]

[a] Mean molecular weight (Mw); b Daltons (Da); c Time to reach the maximum plasma concentration (Tmax).

Unlike VKAs, the DOACs have a rapid onset and offset of action and a low potential for food interaction. In addition, DOACs have a wide therapeutic window and predictable anticoagulant response, and there is no need for laboratory monitoring or dose adjustments according to body weight [48]. Despite the enthusiasm for DOACs, a number of situations are noteworthy and should be considered. Pregnant patients were excluded

from the randomized trials and, consequently, the efficacy and safety concerns (e.g., teratogenic risk) in this group are unknown [39]. Cost is another issue. Drugs such as UH and VKAs are cheaper compared to DOACs [64]. Moreover, the lack of available reversal agents is another important problem. Currently, dabigatran is the only DOAC with a licensed antidote [48]. Although the DOACs have been licensed without the need for laboratory monitoring, some circumstances may require that the drugs be monitored: (i) unconscious patients, where the presence of anticoagulants may influence the management decision; (ii) in the case of suicide attempts, it may be important to establish whether there was an anticoagulant drug overdose; (iii) bridging from one anticoagulant to another; (iv) establishing whether co-medication effects are altering the anticoagulant efficacy [64]. Therefore, the lack of a reliable method for monitoring the drug level is potentially problematic [65].

3. Laboratory Assessment of Anticoagulant Therapy

There are several anticoagulant drugs available, as discussed in the previous section. Each anticoagulant agent differs in its effects on coagulation tests, and each drug may require different laboratory assays to measure/monitor drug concentration.

3.1 *Monitoring VKAs and UFH*

Routine coagulation tests are represented according to their prothrombin time/international normalized ratio (PT/INR) and their activated partial thromboplastin time (aPTT). The PT/INR and aPTT are sometimes obtained to investigate dysfunctions in secondary hemostasis; however, they are more frequently used for monitoring anticoagulant therapy [64]. The PT assay is a straightforward test that evaluates the extrinsic and common pathway factors of coagulation. It is defined as the time in seconds for plasma to clot after the addition of calcium and the thromboplastin reagent, which uses an extrinsic activator pathway (Figure 2) [66]. Commercial thromboplastin reagents have different sources, methods of preparation by their different manufacturers, and, consequently, varying sensitivities to coagulation factors, resulting in uncertainty in PT results [13]. In addition, there are various coagulation analyzers available from several manufacturers. The influence of these two factors is responsible for the variability in PT results among laboratories [66]. To bypass the variability and standardize the PT results,

in 1983, the World Health Organization (WHO) introduced the INR concept. The INR is calculated as follows: INR = (patient PT/MNPT)ISI [64], where MNPT is the mean normal PT, which is obtained from at least 20 healthy volunteers, and ISI is the international sensitivity index of the thromboplastin. The ISI reflects the ability of thromboplastin to reduce the vitamin-K-dependent factor in comparison with a standard thromboplastin defined by the WHO [13]. While the INR approach has reduced the variability of patients' interlaboratory results, on the other hand, the determination of ISI has generated a source of variability depending on the reagent/instrument utilized in the laboratory and the source of plasma used to generate MNPT [66].

Despite the fact that INR has brought an important means of standardization for interlaboratory results, the need for improvements in the technique to ensure more reliable results still exists. The PT/INR is sensitive to the presence or activity of FII, factor V (FV), FVII, FX, and fibrinogen. In that way, anticoagulant agents that alter the levels of these factors can be monitored with the PT/INR if a dose–response relationship could be defined [64]. The PT/INR responds to a lowering of the level of FII, VII, and FX caused by VKAs, leading to a prolongation of PT and, thus, a higher INR [13]. The target INR therapeutic limits are 2.0 to 3.0 for most indications, and it is well defined that subtherapeutic INR levels are related with an increased thrombotic risk. On the other hand, supratherapeutic levels correlate with an increased bleeding risk [48]. Monitoring VKA therapy is crucial due to the highly variable anticoagulant response and the narrow therapeutic window. VKAs interfere with three of the five factors of the extrinsic pathway. Thus, the PT/INR is the most sensitive technique for monitoring the VKA levels, as well as, possible fluctuations in VKA levels due to several interactions presented by these drugs [66].

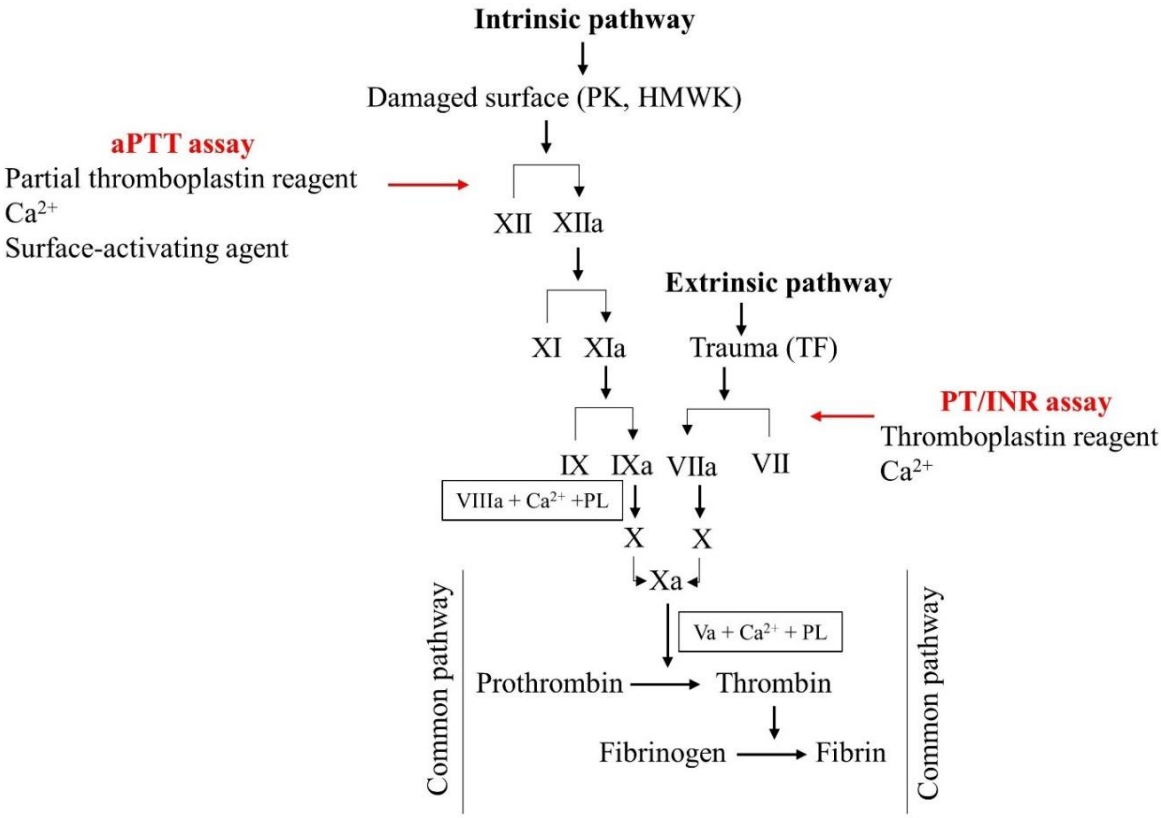


Figure 2. Intrinsic and extrinsic activator pathways.

The aPTT is a clotting assay that evaluates the intrinsic and common pathway factors of coagulation. This test was first developed by Langdell, Wagner, and Brinkhous in 1953 [67] through a modification of the whole blood clotting time test (WBCT) developed by Lee and White to test patients for hemophilia [68]. The aPTT is defined as the time in seconds for the recalcified plasma to form a clot after adding a platelet substitute (partial thromboplastin, named this way because the absence of TF) and a surface-activating agent (such as celite, ellagic acid, kaolin, or micronized silica) (Figure 2) [69,70]. The aPTT is sensitive to the presence or activity of FII, FV, factor VIII (FVIII), FIX, FX, factor XI (FXI), factor XII (FXII), and fibrinogen. Therefore, anticoagulant agents that affect the levels of these factors can be monitored with the aPTT if a dose–response relationship can be determined [64]. Most aPTT reagents shows linear sensitivity to UFH over the therapeutic range, so the aPTT is considered the main assay for monitoring UFH therapy [71]. Monitoring UFH therapy is important for avoiding plasma variation, preventing both toxic levels, which are related with an increased risk of bleeding, thrombocytopenia, and osteoporosis, or subtherapeutic levels, leading to the inefficacy of thrombosis

treatment [47]. The aPTT test offers advantages, such as its low cost, ease of performing, wide availability, and automation. These explain why it is widely accepted by clinicians [72]. However, there are some limitations that might influence the technique and are noteworthy. Regarding preanalytical conditions, several variables might affect the test results, namely, the duration of sample collection, citrate concentration, sample transport temperature, and the time between collection and plasma separation [73]. Concerning analytical conditions, the reagents, instruments, and methods of end-point detection are a source of test variability. The same reagents produced in different lots can yield variability in the test's sensibility. The partial thromboplastin reagent is available through many suppliers with either vegetable or animal phospholipid sources [74]. Thus, the high phospholipid heterogeneity, either in composition or in concentration, is another source of variation in the sensitivity of aPTT assays. Moreover, the choice of the surface-activating agent modifies the aPTT time in normal plasma [75]. The biological conditions are another factor influencing the aPTT results. Intravascular volume, protein level, coagulation factor concentration, and AT deficiency contribute to the variability in the aPTT [72]. From all of the limitations discussed above, it is remarkable that the major problem of aPTT assays is the absence of standard methods. Consequently, the aPTT shows a high interlaboratory variation, and even in-house analysis should be carefully interpreted [76]. UFH and VKAs require rigorous laboratory control because they have a very narrow therapeutic window; therefore, the correct dose is strictly related to an accurate aPTT or PT/INR assay control, respectively.

3.2 *Monitoring LMWH Therapy*

Despite the advantageous pharmacokinetic and pharmacodynamic properties of LMWHs, which grant a lesser need for laboratory monitoring in some circumstances, drug monitoring can bring some benefits for specific populations. The most commonly applied test for LMWH monitoring is the chromogenic anti-factor Xa or anti-Xa assay, mainly due to its low cost and broad availability [77]. The chromogenic anti-factor Xa is considered the gold standard for assessing the plasma LMWH level. In general, the anti-Xa assay measures the enzymatic activity of a patient's plasma in cleaving an exogenous FXa [77]. A synthetic FXa substrate linked with a chromophore is added to the plasma sample. Endogenous FXa cleaves the chromogenic substrate to release the chromophore

unit, leading to a color change that can be detected spectrophotometrically (Figure 3) [78]. In the case of a plasma sample containing LMWH, it will inhibit FXa, reducing the FXa level in order to cleave the synthetic chromophore. By comparing the results with the corresponding standard curve (samples containing known amounts of LMWH), the anticoagulant concentration can be calculated. The extent of colorimetric change is directly proportional to the enzymatic activity and inversely proportional to the anticoagulant concentration [79].

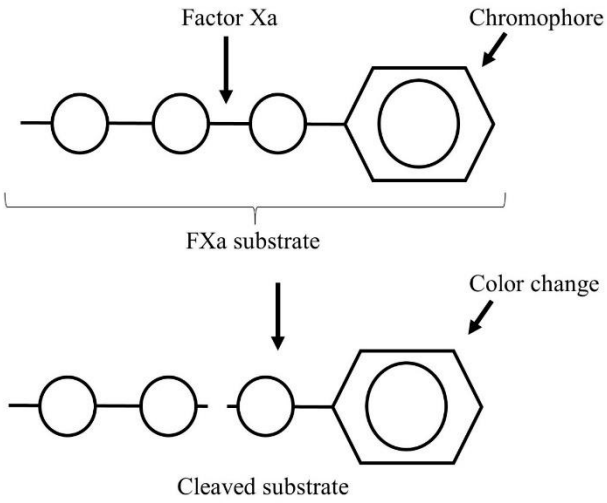


Figure 3. Schematic representation of the Endogenous FXa cleavage of chromogenic substrate releasing the chromophore unit and leading to a color change.

Drawbacks in LMWH monitoring with the anti-factor Xa assay include the lack of standardization in the (1) anti-Xa reagent, (2) standard curve construction, and (3) instrument for analysis [80]. There are numerous commercially available anti-Xa assay kits that use diverse synthetic substrates to measure FXa activity. Thus, analyses of the same sample yield discrepant results [77]. Two different anti-Xa test designs with distinct aims are available. One applies exogenous AT to correct a possibly low in vivo AT concentration. However, this approach may lead to overestimation of the in vivo anticoagulation activity because unbound LMWH, which does not produce anticoagulant activity, could bind to exogenous AT, causing a false positive result [12]. On the other hand, excess dextran sulfate may be added to mitigate in vivo protein binding to the drugs. This strategy may result in overestimated anticoagulant activity, although this is a minor

concern for LMWH due to their reduced binding to plasma proteins [77]. The construction of standard curves to calculate the drug's concentration in the plasma is essential. The same brand and lot number of LMWH administered to patients should be utilized in order to prepare the standard curve. Moreover, different reagents and instrument systems produce variable results [12,80]. The anti-FXa/anti-FIIa ratios among LMWH formulations and the time needed to obtain a blood sample are another pitfall related to the anti-factor Xa assay. In fact, because the peak plasma concentration of LMWH is at about 4 h, blood samples should be collected as close as possible to 4 h after LMWH administration [78,79]. The target therapeutic range for anti-Xa activity depends on the dosage regime and the LMWH agent utilized. With once-daily dosing, the therapeutic range of LMWH is 1.0 to 2.0 U/mL, while for a twice-daily regime, the therapeutic range of LMWH is between 0.5 and 1.0 U/mL [81]. Although anti-Xa is considered the main choice for plasma LMWH level measurement, the anti-Xa level may not correlate with the clinical results, so the results should be evaluated with caution.

3.3. *Monitoring DOAC Therapy*

Although routine laboratory monitoring of DOACs is not necessary, in some special situations, laboratory measurements may be helpful [82], such as for emergency surgeries, overdoses, extreme body weights, pregnancies, drug interactions, renal impairments, and treatment failures [83]. An ideal assay that measures DOACs accurately should show a great level of linearity within a wide range of drug concentrations. Moreover, the assay should be sensitive to low therapeutic concentrations and should be specific for the drug of interest so that no interferences affect the drug's determination. Finally, the assay should be widely available and able to give results in a short time. Currently, there is no test with these ideal characteristics [82].

Liquid chromatography/mass spectrometry (LC/MS), calibrated for each drug to be measured, is considered the standard method for DOAC measurement [84]. This technique has a superior accuracy and precision over a broad concentration range compared to coagulation-activity-based assays [85,86]. However, this technique has limited availability, is time consuming and expensive, and requires high technical expertise. Therefore, although LC/MS is a gold-standard method, it is unsuitable for routine DOAC measurement.

Conventional anticoagulant assays, such as PT and aPTT, are inappropriate for accurate DOAC measurement. However, recent guidelines and recommendations for DOAC assessment state that aPTT and PT may be utilized as a screening test for DTI and anti-Xa inhibitor (only rivaroxaban), respectively, particularly when rapid and reliable information about a possible overcoagulation is necessary [87].

Conventional monitoring tests are not suitable for apixaban, edoxaban, and betrixaban. Thrombin time (TT) is a rapid, simple, and inexpensive test that assesses the thrombin activity through conversion of fibrinogen into fibrin [88,89]. TT is highly sensitive to dabigatran, and it is not used for accurate measurement of it. Depending on the reagent and the dabigatran concentration, TT may be unmeasurable. Nevertheless, TT has an important use: Normal values are suggestive of little or no clinically relevant dabigatran levels [82]. The plasma dabigatran level can be measured with different assays, such as diluted thrombin time (dTT) and ecarin-based assays: ecarin clotting time (ECT) and ecarin chromogenic assay (ECA) [71,87]. The dTT, or its commercial version, Hemoclot® Thrombin Inhibitor (Hyphen Biomed, Neuville-sur-Oise, France), is a TT modification assay for overcoming the exquisite sensitivity to dabigatran. Diluting the test plasma with normal plasma and purified human -thrombin (to initiate clotting) ensures a rapid, simple, and commercially available quantitative method for determining plasma DTI concentration [88]. When dTT is calibrated for dabigatran measurement, the assay shows a high linearity with clinical dabigatran concentration with a good reproducibility [90]. The dTT assay has been considered the gold standard for measurement of plasma dabigatran concentration [91]. ECT (Ecarin Reagent, Diagnostica Stago, Asnieres, France) is a meizothrombin generation assay that enables accurate DTI measurement [92]. Ecarin is a metalloproteinase purified from snake venom, Echis carinatus, which, when added to citrated plasma, specifically cleaves prothrombin into an active intermediate, meizothrombin, and the time until clot formation is measured [82]. With DTI therapy, the meizothrombin generated is inactivated as long as DTI is present in the plasma. Thus, coagulation will not occur until all DTI is consumed, thus prolonging the time until clot formation [88,92]. In the ECA (ECA-T Kit, Diagnostica Stago, Asnieres, France), the meizothrombin cleaves a chromogenic substrate, which releases the chromophore unit (paranitroalanine), resulting in absorbance changes. With DTI therapy, the cleavage reaction is inhibited, and by comparing the results with the corresponding standard curve, the anticoagulant concentration can be measured [93]. Direct FXa inhibitors can be

accurately measured through a modified chromogenic anti-Xa assay. It is advocated that the anti-Xa assay calibrated with the same drug being taken by the patient is the most reliable technique for accurately measuring the anticoagulation effect of direct oral FXa inhibitors [82,87].

Although the development and introduction of DOACs in the market have revolutionized anticoagulant therapy due to their several advantages over VKAs and heparins, they have brought some challenges in laboratory monitoring [85]. Due to the differences in the sensitivity of routine coagulation tests, none of them are able to accurately measure DOACs. Nevertheless, qualitative information about the presence of DOACs or lack thereof could be obtained [88]. The absence of FDA-approved calibrators and reliable methods for quantifying DOACs has generated a lack of interest in the development of a quantitative assay. Alternative methods, such as ECT and ECA, may be useful, but have not been widely implemented. The use of commercial calibrators, as well as the use of specialty coagulation tests, to measure DOACs should be interpreted with attention [85].

4. Coagulation Test Performance of Drug Delivery Systems

4.1 *Liposomes*

Liposomes are defined as phospholipid vesicles formed by concentric lipid bilayers surrounding an aqueous inner phase [94]. Their special structure, hydrophobic and hydrophilic characteristics, and biocompatibility make liposomes a promising system for drug delivery [95].

LMWH-loaded flexible liposomes (flexosomes) (size~83 nm) and LMWH-loaded ethosomes (size~85 nm) were prepared for transdermal delivery [96]. Their physicochemical features and pharmacokinetic parameters were compared. The plasma anti-Xa activity of flexosomes, ethosomes, and LMWH solution was measured through a chromogenic anti-Xa assay (Coatest®, Chromogenix, Instrumentation Laboratory, Milano, Italy). After transdermal application, the $[\text{anti-Xa}]_{max}$ of the LMWH-loaded flexosomes was 1.11 IU/mL, while the $[\text{anti-Xa}]_{max}$ of the LMWH-loaded ethosomes was 0.32 UI/mL. The authors stated that the improved skin permeability of the flexosomes was due to their reduced size and the higher deformability than that of ethosomes (76.7% versus 46.8%). Moreover, they explained that flexosomes are able to change their shape to pass through skin barriers. In another study, Bai and Ahsan [97] prepared ardeparin-

loaded conventional liposomes and PEGylated liposomal formulations with the hydration method for the respiratory route. Pulmonary absorption of formulations was monitored through plasma anti-Xa activity and compared with LMWH saline formulations and subcutaneous administration. The antifactor Xa levels (>0.2 UI/mL) for all liposomal formulations were at therapeutic levels for more than 6 h. Moreover, the prolongation of the half-life of the drug was significantly higher when compared to that for the plain LMWH or subcutaneous route.

4.2 *Hydrogels*

Hydrogels are three-dimensional cross-linked hydrophilic polymer networks that are capable of retaining large amounts of water or biological fluids while remaining insoluble in these media [98]. Their hydrophilicity is responsible for some of the desirable features of hydrogels, such as biocompatibility and the capability of encapsulating hydrophilic molecules [99]. Because the hydrogels were mostly prepared in aqueous solutions, the drug denaturation and aggregation possibilities were mitigated. Moreover, the hydrogel network was able to protect the entrapped drug against the harsh environment [100]. Loira-Pastoriza et al. prepared nanogels to incorporate four commercial LMWHs for topical delivery [11]. For bemiparin measurement in plasma after topical application, they used a chromogenic anti-Xa assay (Stachrom® heparin, Diagnostica Stago, Asnières-sur-Seine, France). The authors could not detect bemiparin in plasma after 24 h of topical application. However, they stated that, for the route of administration and for the treatment of superficial thrombosis and hematomas, the drug should not pass into the systemic blood circulation in order to avoid a systemic effect. Matanovic et al. developed a thermoresponsive poloxamer-based platform that formed in situ combined with pH-responsive polyelectrolyte heparin/chitosan nanocomplexes for prolonged subcutaneous release of heparin. The plasma heparin concentration was measured after subcutaneous injection of selected individual formulations (thermoresponsive hydrogel and heparin/chitosan nanocomplexes) and a dual system (nanocomplexes incorporated into thermoresponsive hydrogel) through a chromogenic FXa assay and Berichrom® Heparin kit (Siemens Healthcare Diagnostics Inc., Erlanger, Germany). Plasma heparin concentration–time profiles showed a double-peak phenomenon, and this dual behavior was probably related to diffusion of heparin inside the polymer network and gel dissolution. It was demonstrated that thermoresponsive hydrogel with heparin/chitosan

nanocomplexes showed sustained release of heparin for 5 days [101]. The aPTT is the most preferred assay for monitoring the therapeutic dose of UFH in many hospitals and laboratories, mainly due to its low cost, wide availability, and ease of performing, rather than the specific advantages of this technique. However, the anti-Xa assay may be necessary either in some patients (e.g., elevated baseline aPTT or FXII deficiency) or because it offers another option with fewer interferences [71,87].

4.3 *Polymeric Nanoparticles (PNPs)*

PNPs are colloidal dispersions that have been used for drug and biomolecule delivery [102]. PNPs have several advantages: (i) They have improved stability; (ii) they can avoid the phagocyte system, thus prolonging their blood stream duration; (iii) because of their sub-cellular size, they can pass through capillary vessels; (iv) they can improve the drug properties and reduce side effects; (v) they can release the drug or biomolecule in a controlled/sustained fashion [103–105]. Dong et al. prepared chitosan-based polymer–lipid hybrid nanoparticles (PLNs) with a self-assembly method to incorporate and improve the oral absorption of enoxaparin (Enox). The PLNs were composed of chitosan as the polymer and glyceryl monooleate (GMO) as the lipid, with an optimized lipid/polymer mass ratio of 0.2 and pluronic® F127 as a stabilizer. Nanoparticles with the optimal composition were submitted to an in vivo study. The Enox anticoagulant activity was measured through the aPTT assay. The optimized Enox/PLNs showed a 4.5-fold increase in oral Enox bioavailability in comparison with an Enox solution. The authors claimed that the stability of nanoparticles is a crucial parameter for improved oral drug delivery efficiency [7]. The authors utilized the aPTT assay to measure plasma LMWH concentration; however, the aPTT was not helpful for this purpose because the anticoagulant effect of LMWH is produced mainly through FXa inhibition, and the prolongation of the aPTT is highly dependent on low thrombin activity [77]. In another work, Ramadan et al. produced and characterized cationic lipid nanocapsules (cLNCs) with a phase-inversion method intended for fondaparinux (Fp) oral absorption. In vivo studies were performed after a single oral dose in rats, and the plasma Fp measurements were carried out with the anti-Xa activity assay (HYPHEN, Biomed). The authors reported that the cLNCs significantly increased oral Fp bioavailability and improved pharmacokinetic profile in a dose-dependent manner [106].

4.4 *Other Drug Delivery Systems*

Solid lipid nanoparticles (SLNs) are lipid-based colloidal nanocarriers consisting of a hydrophobic core composed of lipids that are solid at both room and body temperatures and stabilized by a surfactant layer. SLNs are capable of affording a controlled drug release because the release of the active compound from the solid lipid matrix is much slower when compared to liquid oils [107,108]. Paliwal et al. synthetized biomimetic LMWH–lipid conjugate-loaded SLNs for oral bioavailability studies. An in vivo study was performed in albino rats, and the plasma LMWH concentration was measured with a colorimetric anti-Xa assay using a Chromogenix Coatest LMW heparin kit (Diapharma). The authors demonstrated that SLNs were within the nanometric range and were safe for oral administration. Furthermore, improved bioavailability of LMWH–lipid conjugates was observed in comparison to LMWH-loaded SLNs and LMWH solution. They attributed this result to the high loading capacity and better lymphatic drainage of the LMWH–lipid conjugate SLNs, thus achieving an improved systemic LMWH concentration [109]. Self-nano emulsifying drug delivery systems (SNEDDSs) have been explored as drug delivery systems because they are able to spontaneously form oil-in-water (o/w) nanoemulsions after administration in an aqueous environment, followed by mild agitation (e.g., the gastric motility) [110,111]. Zupancic et al. prepared and characterized various enoxaparin-loaded self-emulsifying drug delivery systems (SEDDSs) with lipids of different chain lengths for oral administration [112]. An in vivo study was carried out in rats, and the plasma enoxaparin concentration was measured with a Biophen® Heparin anti-Xa assay kit. Medium-chain lipids (MC-SEDDS) and no lipids (NL-SEDDS) showed greater mucus diffusion. MC-SEDDS and NL-SEDDS showed sustained enoxaparin release in vitro and an oral absolute enoxaparin bioavailability of 2.02%and 2.25%, respectively. A literature review focusing on anticoagulant therapies using different drug delivery systems is given in Table 3.

Table 3: Anticoagulant drug delivery systems for different routes of administration.

Drug delivery system	Active (Class)	Composition	Size range	Major outcomes	Ref
Liposomes	(LMWH)	Egg PC Tween® 20 Ethanol	80–90 nm	LMWH-loaded flexosome showed higher antifactor Xa (Anti-Xa)]max than LMWH-loaded ethosome.	[104]
	Ardeparin (LMWH)	PC Chol DSPE DSPE-PEG-2000 and DSPE–PEG-5000	100–150 nm	Liposomal formulations showed sustained release and longer half-life compared to the plain solution or subcutaneous route.	[105]
	Enoxaparin (LMWH)	Soybean PC Chol	100-200 nm	Eudragit-coated liposomes showed higher permeation and oral bioavailability when compared to uncoated liposomes.	[106]

		SA Eudragit® S 100			
Hydrogels	Bemiparin (LMWH) Nadroparin (LMWH) Tinzaparin (LMWH) Enoxaparin (LMWH)	Eudragit® RS 30D	130 nm	Gel formulations were able to delivery LMWHs across the skin barrier and after 24 h the drug was not detected in plasma.	[11]
	Heparin (UFH)	CS hydrochloride Lutrol® F127 Lutrol® F68 HPMC	150-400 nm	The dual system enabled the lowest absorption rate of heparin into systemic circulation and provided heparin concentration above the prophylaxis threshold for 5 days.	[112]
	Enoxaparin (LMWH)	CS Pluronic® F127 PEI Plys	100-1000 nm	Thermo-sensitive hydrogels were able to prolong the enoxaparin release.	[124]
	Heparin (UFH)	Gelatin MBA TEA	NA	Heparin-loaded hydrogels showed sustained release for 60 h and platelets adhesion was significantly reduced.	[125]

PNP	Enoxaparin (LMWH)	CS GMO Pluronic® F127	290-320 nm	The optimized formulation showed higher oral bioavailability compared with drug solution.	[7]
	Fondaparinux (Synthetic analogues)	Labrafac® WL 1349 Lipoïd® S75-3 Lipoïd® GMBH Solutol® HS 15 CTAB SA	40-65 nm	Cationic lipid nanocapsules showed increased oral bioavailability and longer half-life when compared to fondaparinux control solutions (oral and intravenous).	[117]
	Enoxaparin (LMWH)	Pluronic® F-68 CTAB Dextran sulfate PLGA Precirol ATO 5 E80 Tween 80 Poloxamer 407	180-195 nm	Enoxaparin/CTAB nanoparticles showed 3-fold improved gastrointestinal permeation when compared with drug solution.	[126]
	Enoxaparin (LMWH)		145-160 nm	LPHNs improved the drug intestinal permeation, enhanced the oral bioavailability and showed therapeutic efficacy.	[127]

SLN	(LMWH)	Compritol 888 ATO Stearic, palmitic and myristic acid PC	280-380 nm	The SLNs were able to improve the LMWH bioavailability in comparison to free drug solution.	[120]
SNEDDDS	Enoxaparin (LMWH)	Capmul MCM EP, Capmul PG-8 EP/NF Captex 8000, Peceol Labrafil M 1944 CS, Labrasol Maisine 35-1, Transcutol HP Myglyol 840, Cremophor EL	30-245 nm	SEDDS formulations showed sustained enoxaparin release and 2-fold bioavailability.	[128]
	Rivaroxaban (Factor Xa inhibitor)	PEG, triacetin, olive and sesame oil	50-105 nm	SNEDDS showed higher dissolution than commercial formulation. SNEDDS technology used in rivaroxaban successfully enhanced drug bioavailability in fasted condition and showed no food effect were observed in rivaroxaban-SNEDDS formulation.	[129]
	Rivaroxaban (Factor Xa inhibitor)	IPM Ethyl oleate, Tween20, and Tween80	10-115 nm	Safety SNEDDS formulations which enhanced oral and intravenous bioavailability in comparison to drug suspension.	[130]

		Cremophor, Cremophor HEL, and Transutol Transcutol HP Capryol TM 90 Maisine TM 35-1 Castor oil, oleic acid, triacetin, IPM Cremophore EL PEG 300 and PEG 400 Tween 20, Tween 80 and Span 80		Moreover, SNEDDS exhibit anticoagulant efficacy in thrombosis rat model.	

Abbreviations: CS: chitosan, Chol: cholesterol, CTAB: hexadecyltrimethyl ammonium bromide, DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, DSPE–PEG: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine polyethyleneglycol, E80: egg yolk lecithin, Egg PC: phosphatidylcholine from egg yolk, GMO: glyceryl monooleate, HPMC, hydroxypropylmethylcellulose, IPM: isopropyl myristate, MBA: N,N-methylenebis (acrylamide), NA, not available, PEI: polyethylenimine, PC: phosphatidylcholine, Plys: ε-polylysine, PNP: polymeric nanoparticle, SA: stearyl amine, SEDDS: self-emulsifying drug delivery system, SLN: solid lipid nanoparticles, SNEDDS: self-nanoemulsifying drug delivery system, TEA: triethylamine.

5. Conclusions

The conventional anticoagulant agents are well-knowledge drugs with reliable anticoagulant assays, that have been used for decades. On the other hand, after a long time without novelty in anticoagulation management, the DOACs have emerged in the last decade. De-spite the remarkable advances made in anticoagulant therapy with the introduction of DO-ACs, there are still some important issues that should be addressed, such as lack of specific antidotes and the absence of an accurate and standardized coagulation test. The anticoagulant tests were found to be suitable for measuring the anticoagulant performance of heparin-based nanocarriers through several administration routes.

Author Contributions: All authors have substantially contributed to the conceptualization, meth-odology, formal analysis, investigation, resources, and data curation. YBGP, LLC, MFRS and JLSS have contributed in the writing-original draft, data search and literature research. MFRS, JLSS, EHG, PS and EBS have contributed for the writing-review, validation and editing. E.B.S., PS, MFRS and JLSS have contributed in the project administration, supervision and funding acquisition.

Funding: This work was supported by the Banco do Nordeste (grant FUNDECI/2016.0015), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Apoio à Pesquisa e à Inovação Tecnológica do Estado de Sergipe (Fapitec) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Eliana B. Souto would like to acknowledge the Portuguese Science and Technology Foundation (FCT/MCT) and from European Funds (PRODER/COMPETE) for the project UIDB/04469/2020 (strategic fund), co-financed by FED-ER, under the Partnership Agreement PT2020.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Patel, V.F.; Liu, F.; Brown, M.B. Advances in oral transmucosal drug delivery. *J. Control. Release* **2011**, *153*, 106-116, doi:https://doi.org/10.1016/j.jconrel.2011.01.027.

2. Aláez-Versón, C.R.; Lantero, E.; Fernàndez-Busquets, X. Heparin: new life for an old drug. *Nanomedicine* **2017**, *12*, 1727-1744, doi:https://doi.org/10.2217/nnm-2017-0127.

3. Motlekar, N.A.; Youan, B.-B.C. The quest for non-invasive delivery of bioactive macromolecules: A focus on heparins. *J. Control. Release* **2006**, *113*, 91-101, doi:https://doi.org/10.1016/j.jconrel.2006.04.008.

4. Das Kurmi, B.; Tekchandani, P.; Paliwal, R.; Rai Paliwal, S. Nanocarriers in Improved Heparin Delivery: Recent Updates. *Curr. Pharm. Des* **2015**, *21*, 4509-4518, doi:https://doi.org/10.2174/1381612821666150821112808.

5. Lee, J.H.; Yeo, Y. Controlled drug release from pharmaceutical nanocarriers. *Chem. Eng. Sci* **2015**, *125*, 75-84, doi:https://doi.org/10.1016/j.ces.2014.08.046.

6. Shi, J.; Votruba, A.R.; Farokhzad, O.C.; Langer, R. Nanotechnology in drug delivery and tissue engineering: from discovery to applications. *Nano letters* **2010**, *10*, 3223-3230, doi:https://doi.org/10.1021/nl102184c.

7. Dong, W.; Wang, X.; Liu, C.; Zhang, X.; Zhang, X.; Chen, X.; Kou, Y.; Mao, S. Chitosan based polymer-lipid hybrid nanoparticles for oral delivery of enoxaparin. *Int. J. Pharm.* **2018**, *547*, 499-505, doi:https://doi.org/10.1016/j.ijpharm.2018.05.076.

8. Charoongchit, P.; Suksiriworapong, J.; Mao, S.; Sapin-Minet, A.; Maincent, P.; Junyaprasert, V.B. Investigation of cationized triblock and diblock poly(ε-caprolactone)-co-poly(ethylene glycol) copolymers for oral delivery of enoxaparin: In vitro approach. *Acta Biomater.* **2017**, *61*, 180-192, doi:https://doi.org/10.1016/j.actbio.2017.08.006.

9. Rawat, A.; Majumder, Q.H.; Ahsan, F. Inhalable large porous microspheres of low molecular weight heparin: In vitro and in vivo evaluation. *J. Control. Release* **2008**, *128*, 224-232, doi:https://doi.org/10.1016/j.jconrel.2008.03.013.

10. Patel, B.; Gupta, V.; Ahsan, F. PEG–PLGA based large porous particles for pulmonary delivery of a highly soluble drug, low molecular weight heparin. *J. Control. Release* **2012**, *162*, 310-320, doi:https://doi.org/10.1016/j.jconrel.2012.07.003.

11. Loira-Pastoriza, C.; Sapin-Minet, A.; Diab, R.; Grossiord, J.L.; Maincent, P. Low molecular weight heparin gels, based on nanoparticles, for topical delivery. *Int. J. Pharm.* **2012**, *426*, 256-262, doi:https://doi.org/10.1016/j.ijpharm.2012.01.044.

12. Walenga, J.; Hoppensteadt, D. Monitoring the new antithrombotic drugs. *Semin. Thromb. Hemost.* **2005**, *30*, 683-695, doi:https://doi.org/10.1055/s-2004-861511.

13. Samama Meyer, M.; Guinet, C. Laboratory assessment of new anticoagulants. *Clin. Chem. Lab. Med.* **2011**, *49*, 761-772, doi:https://doi.org/10.1515/CCLM.2011.134.

14. Mulloy, B.; Hogwood, J.; Gray, E.; Lever, R.; Page, C.P. Pharmacology of heparin and related drugs. *Pharmacol. Rev.* **2016**, *68*, 76-141, doi:https://doi.org/10.1124/pr.115.011247.

15. Torri, G.; Naggi, A. Heparin centenary – an ever-young life-saving drug. *Int. J. Cardiol.* **2016**, *212*, S1-S4, doi:https://doi.org/10.1016/S0167-5273(16)12001-7.

16. Onishi, A.; St Ange, K.; Dordick, J.S.; Linhardt, R.J. Heparin and anticoagulation. *Front. Biosci. (Landmark Ed).* **2016**, *21*, 1372-1392, doi:https://doi.org/10.2741/4462.

17. Gray, E.; Mulloy, B.; W Barrowcliffe, T. Heparin and low-molecular-weight heparin. *Thromb. Haemost.* **2008**, *99*, 807-818, doi:https://doi.org/10.1160/TH08-01-0032.

18. Harter, K.; Levine, M.; Henderson, S.O. Anticoagulation drug therapy: a review. *West. J. Emerg. Med.* **2015**, *16*, 11-17, doi:https://doi.org/10.5811/westjem.2014.12.22933.

19. Vene, N.; Mavri, A. An overview of the anticoagulant drugs used in routine clinical practice. In *Anticoagulant Drugs*, Božič-Mijovski, M., Ed.; IntechOpen: United Kingdom, 2018; p. 10.

20. Casu, B.; Oreste, P.; Torri, G.; Zoppetti, G.; Choay, J.; Lormeau, J.C.; Petitou, M.; Sinaɛ, P. The structure of heparin oligosaccharide fragments with high anti-(factor Xa) activity containing the minimal antithrombin III-binding sequence. *Biochem. J.* **1981**, *197*, 599-609, doi:https://doi.org/10.1042/bj1970599.

21. Lindahl, U.; Bäckström, G.; Höök, M.; Thunberg, L.; Fransson, L.-A.; Linker, A. Structure of the antithrombin-binding site in heparin. *Proc. Natl. Acad. Sci.* **1979**, *76*, 3198-3202, doi:https://doi.org/10.1073/pnas.76.7.3198.

22. Cosmi, B.; Palareti, G. Old and new heparins. *Thromb. Res.* **2012**, *129*, 388-391, doi:https://doi.org/10.1016/j.thromres.2011.11.008.

23. Kakkar, A.K. Low- and ultra-low-molecular-weight heparins. *Best Pract. Res. Clin. Haematol.* **2004**, *17*, 77-87, doi:https://doi.org/10.1016/j.beha.2004.03.001.

24. Ibrahim, S.S.; Osman, R.; Awad, G.A.S.; Mortada, N.D.; Geneidy, A.-S. Low molecular weight heparins for current and future uses: approaches for micro- and nano-particulate delivery. *Drug. Deliv.* **2016**, *23*, 2661-2667, doi:https://doi.org/10.3109/10717544.2015.1046570.

25. Park, J.; Byun, Y. Recent advances in anticoagulant drug delivery. *Expert. Opin. Drug. Deliv.* **2016**, *13*, 421-434, doi:https://doi.org/10.1517/17425247.2016.1125880.

26. Merli, G.J.; Vanscoy, G.J.; Rihn, T.L.; Groce Iii, J.B.; McCormick, W. Applying scientific citeria to therapeutic interchange: a balanced analysis of low-molecular-weight heparins. *J. Thromb Thrombolysis* **2001**, *11*, 247-259, doi:https://doi.org/10.1023/A:1011969005756.

27. Hirsh, J.; O'Donnell, M.; Eikelboom, J.W. Beyond unfractionated heparin and warfarin. *Circulation* **2007**, *116*, 552-560, doi:https://doi.org/10.1161/CIRCULATIONAHA.106.685974.

28. Walenga, J.M.; Lyman, G.H. Evolution of heparin anticoagulants to ultra-low-molecular-weight heparins: a review of pharmacologic and clinical differences and applications in patients with cancer. *Crit. Rev. Oncol. Hematol.* **2013**, *88*, 1-18, doi:https://doi.org/10.1016/j.critrevonc.2013.06.007.

29. Masuko, S.; Linhardt, R.J. Chemoenzymatic synthesis of the next generation of ultralow MW heparin therapeutics. *Future. Med. Chem.* **2012**, *4*, 289-296, doi:https://doi.org/10.4155/fmc.11.185.

30. Bauer, K.A. Fondaparinux sodium: a selective inhibitor of factor Xa. *Am. J. Health. Syst. Pharm.* **2001**, *58* S14-17, doi:https://doi.org/10.1093/ajhp/58.suppl_2.S14.

31. Gómez-Outes, A.; Suárez-Gea, M.L.; Lecumberri, R.; Rocha, E.; Pozo-Hernández, C.; Vargas-Castrillón, E. New parenteral anticoagulants in development. *Ther. Adv. Cardiovasc. Dis.* **2011**, *5*, 33-59, doi:https://doi.org/10.1177/1753944710387808.

32. Savi, P.; Herault, J.P.; Duchaussoy, P.; Millet, L.; Schaeffer, P.; Petitou, M.; Bono, F.; Herbert, J.M. Reversible biotinylated oligosaccharides: a new approach for a better management of anticoagulant therapy. *J. Thromb. Haemost.* **2008**, *6*, 1697-1706, doi:https://doi.org/10.1111/j.1538-7836.2008.03089.x.

33. Paty, I.; Trellu, M.; Destors, J.-M.; Cortez, P.; Boëlle, E.; Sanderink, G. Reversibility of the anti-FXa activity of idrabiotaparinux (biotinylated idraparinux) by intravenous avidin infusion. *J. Thromb. Haemost.* **2010**, *8*, 722-729, doi:https://doi.org/10.1111/j.1538-7836.2010.03746.x.

34. Falkon, L.; Garí, M.; Barbanoj, M.; Amiral, J.; Fontcuberta, J. Tissue factor pathway inhibitor and anti-FXa kinetic profiles of a new low-molecular-mass heparin, bemiparin, at therapeutic subcutaneous doses. *Blood Coagul. Fibrinolysis* **1998**, *9*, 137-141, doi:https://doi.org/10.1097/00001721-199803000-00004.

35. Ciccone, M.M.; Cortese, F.; Corbo, F.; Corrales, N.E.; Al-Momen, A.K.; Silva, A.; Zito, A.; Pinto, M.; Gesualdo, M.; Scicchitano, P. Bemiparin, an effective and safe low molecular weight heparin: A review. *Vascul. Pharmacol.* **2014**, *62*, 32-37, doi:https://doi.org/10.1016/j.vph.2014.03.001.

36. Pirmohamed, M.; Kamali, F.; Daly, A.K.; Wadelius, M. Oral anticoagulation: a critique of recent advances and controversies. *Trends in Pharmacol Sci* **2015**, *36*, 153-163, doi:https://doi.org/10.1016/j.tips.2015.01.003.

37. Ferreira, J.L.; Wipf, J.E. Pharmacologic therapies in anticoagulation. *Med. Clin. North. Am.* **2016**, *100*, 695-718, doi:https://doi.org/10.1016/j.mcna.2016.03.007.

38. Ibrahim, T.F.; Maxwell, S.; Iqbal, O. Current anticoagulation drugs and mechanisms of action. In *Anticoagulation and Hemostasis in Neurosurgery*, Loftus, C.M., Ed.; Springer Switzerland, 2016; pp. 33-46.

39. Hirsh, J. Oral anticoagulant drugs. *N. Engl. J. Med.* **1991**, *324*, 1865-1875, doi:https://doi.org/10.1056/nejm199106273242606.

40. Ageno, W.; Gallus, A.S.; Wittkowsky, A.; Crowther, M.; Hylek, E.M.; Palareti, G. Oral anticoagulant therapy: antithrombotic therapy and prevention of thrombosis, 9th ed:

American college of chest physicians evidence-based clinical practice guidelines. *Chest* **2012**, *141*, e44S-e88S, doi:https://doi.org/10.1378/chest.11-2292.

41. Wadelius, M.; Pirmohamed, M. Pharmacogenetics of warfarin: current status and future challenges. *Pharmacogenomics J.* **2006**, *7*, 99-111, doi:https://doi.org/10.1038/sj.tpj.6500417.

42. Ufer, M. Comparative pharmacokinetics of vitamin K antagonists. *Clin. Pharmacokinet.* **2005**, *44*, 1227-1246, doi:https://doi.org/10.2165/00003088-200544120-00003.

43. Mega, J.L.; Simon, T. Pharmacology of antithrombotic drugs: an assessment of oral antiplatelet and anticoagulant treatments. *Lancet* **2015**, *386*, 281-291, doi:https://doi.org/10.1016/S0140-6736(15)60243-4.

44. Wysowski, D.K.; Nourjah, P.; Swartz, L. Bleeding complications with warfarin use: a prevalent adverse effect resulting in regulatory action. *Arch. Intern. Med.* **2007**, *167*, 1414-1419, doi:https://doi.org/10.1001/archinte.167.13.1414.

45. Franchini, M.; Liumbruno, G.M.; Bonfanti, C.; Lippi, G. The evolution of anticoagulant therapy. *Blood. Transfus.* **2016**, *14*, 175-184, doi:https://doi.org/10.2450/2015.0096-15.

46. Akhtar, F.; Wan, X.; Wu, G.; Kesse, S.; Wang, S.; He, S. Low-Molecular-Weight Heparins: Reduced Size Particulate Systems for Improved Therapeutic Outcomes. *Molecules (Basel, Switzerland)* **2018**, *23*, 1757-1769, doi:https://doi.org/.

47. Schluter, A.; Lamprecht, A. Current Developments for the Oral Delivery of Heparin. *Current Pharmaceutical Biotechnology* **2014**, *15*, 640-649.

48. Riva, N.; Ageno, W. Pros and cons of vitamin K antagonists and non–vitamin K antagonist oral anticoagulants. *Semin. Thromb. Hemost.* **2015**, *41*, 178-187, doi:https://doi.org/10.1055/s-0035-1544231.

49. Schulman, S. Advantages and limitations of the new anticoagulants. *J. Intern. Med.* **2014**, *275*, 1-11, doi:https://doi.org/10.1111/joim.12138.

50. Bauer, K.A. Pros and cons of new oral anticoagulants. *Hematology Am. Soc. Hematol. Educ. Program.* **2013**, *2013*, 464-470, doi:https://doi.org/10.1182/asheducation-2013.1.464.

51. Stangier, J. Clinical pharmacokinetics and pharmacodynamics of the oral direct thrombin inhibitor dabigatran etexilate. *Clin. Pharmacokinet.* **2008**, *47*, 285-295, doi:https://doi.org/10.2165/00003088-200847050-00001.

52. Zacconi, F.C. FXa direct synthetic inhibitors. In *Anticoagulant Drugs*, Božič-Mijovski, M., Ed.; IntechOpen: United Kingdom, 2018; pp. 11-37.

53. Hinojar, R.; Jiménez-Natcher, J.J.; Fernández-Golfín, C.; Zamorano, J.L. New oral anticoagulants: a practical guide for physicians. *Eur. Heart. J. Cardiovasc. Pharmacother.* **2015**, *1*, 134-145, doi:https://doi.org/10.1093/ehjcvp/pvv002.

54. Laux, V.; Perzborn, E.; Kubitza, D.; Misselwitz, F. Preclinical and clinical characteristics of rivaroxaban: a novel, oral, direct factor Xa inhibitor. *Semin. Thromb. Hemost.* **2007**, *33*, 515-523, doi:https://doi.org/10.1055/s-2007-982083.

55. He, K.; He, B.; Grace, J.E.; Xin, B.; Zhang, D.; Pinto, D.J.; Luettgen, J.M.; Knabb, R.M.; Lam, P.Y.S.; Wexler, R.R.; et al. Preclinical pharmacokinetic and metabolism of apixaban, a potent and selective factor Xa inhibitor. *Blood* **2006**, *108*, 910.

56. Minguet, J.; Sims, H.M.; Smith, K.H.; Bramlage, P. The factor Xa inhibitor edoxaban for the prevention of stroke and systemic embolism in patients with atrial fibrillation. *Expert. Rev. Clin. Pharmacol.* **2017**, *10*, 5-15, doi:https://doi.org/10.1080/17512433.2016.1258301.

57. Poulakos, M.; N. Walker, J.; Baig, U.; David, T. Edoxaban: A direct oral anticoagulant. *Am. J. Health-Syst. Pharm.* **2017**, *74*, 117-129, doi:https://doi.org/10.2146/ajhp150821.

58. Parasrampuria, D.A.; Truitt, K.E. Pharmacokinetics and pharmacodynamics of edoxaban, a non-vitamin K antagonist oral anticoagulant that inhibits clotting factor Xa. *Clin. Pharmacokinet.* **2016**, *55*, 641-655, doi:https://doi.org/10.1007/s40262-015-0342-7.

59. Garland, S.G.; DeRemer, C.E.; Smith, S.M.; Gums, J.G. Betrixaban: a new oral factor Xa inhibitor for extended venous thromboembolism prophylaxis in high-risk hospitalized patients. *Ann. Pharmacother.* **2018**, *52*, 554-561, doi:https://doi.org/10.1177/1060028018754383.

60. Huisman, M.V.; Klok, F.A. Pharmacological properties of betrixaban. *Eur. Heart. J. Suppl.* **2018**, *20*, E12-E15, doi:https://doi.org/10.1093/eurheartj/suy016.

61. Thoenes, M.; Minguet, J.; Bramlage, K.; Bramlage, P.; Ferrero, C. Betrixaban – the next direct factor Xa inhibitor? *Expert. Rev. Hematol.* **2016**, *9*, 1111-1117, doi:https://doi.org/10.1080/17474086.2016.1256194.

62. Cosmi, B. An update on the pharmaceutical management of thrombosis. *Expert Opin. Pharmacother.* **2016**, *17*, 2149-2164, doi:10.1080/14656566.2016.1241233.

63. Gómez-Outes, A.; Suárez-Gea, M.L.; Lecumberri, R.; Terleira-Fernández, A.I.; Vargas-Castrillón, E. Direct-acting oral anticoagulants: pharmacology, indications, management, and future perspectives. *Eur. J. Haematol.* **2015**, *95*, 389-404, doi:doi:10.1111/ejh.12610.

64. Favaloro, E.J.; Lippi, G.; Koutts, J. Laboratory testing of anticoagulants: the present and the future. *Pathology* **2011**, *43*, 682-692, doi:https://doi.org/10.1097/PAT.0b013e32834bf5f4.

65. Czuprynska, J.; Patel, J.P.; Arya, R. Current challenges and future prospects in oral anticoagulant therapy. *Br. J. Haematol.* **2017**, *178*, 838-851, doi:https://doi.org/10.1111/bjh.14714.

66. Moffat, K.; Lewis, C. Laboratory monitoring of oral vitamin K anticoagulation. *Semin. Thromb. Hemost.* **2017**, *43*, doi:https://doi.org/10.1055/s-0036-1587690.

67. D Langdell, R.; H Wagner, R.; M Brinkhous, K. Effect of antihemophilic factor on one-stage clotting tests. A presumptive test for hemophilia and a simple one-stage antihemophilic factor assay procedure. *J. Lab. Clin. Med.* **1953**, *41*, 637-647.

68. Kitchens, C.S. To bleed or not to bleed? Is that the question for the PTT? *J. Thromb. Haemost.* **2005**, *3*, 2607-2611, doi:https://doi.org/10.1111/j.1538-7836.2005.01552.x.

69. Chee, Y.L.; Greaves, M. Role of coagulation testing in predicting bleeding risk. *Hematol. J.* **2003**, *4*, 373-378, doi:https://doi.org/10.1038/sj.thj.6200306.

70. Bonhomme, F.; Fontana, P. Laboratory testing of hemostasis. In *Perioperative hemostasis*, Marcucci, C.E., Schoettker, P., Eds.; Springer: Berlin, Heidelberg, 2015; pp. 13-24.

71. Avecilla, S.T.; Roshal, M.; Reyes Gil, M.; Erdman, P.A. Laboratory monitoring for heparins, fondaparinux, direct thrombin inhibitors, and oral anti-Xa medications. In *Transfusion medicine and hemostasis* Third ed.; Shaz, B.H., Hillyer, C.D., Reyes Gil, M., Eds.; Elsevier: Amsterdam, Netherlands, 2019; pp. 933-938.

72. Cuker, A. Unfractionated heparin for the treatment of venous thromboembolism: best practices and areas of uncertainty. *Semin. Thromb. Hemost.* **2012**, *38*, 593-599, doi:https://doi.org/10.1055/s-0032-1319770.

73. A Marlar, R.; Clement, B.; Gausman, J. Activated partial thromboplastin time monitoring of unfractionated heparin therapy: issues and recommendations. *Semin. Thromb. Hemost.* **2017**, *43*, 253-260, doi:https://doi.org/10.1055/s-0036-1581128.

74. A. Shapiro, G.; W. Huntzinger, S.; E. Wilson, J. Variation among commercial activated partial thromboplastin time reagents in response to heparin. *Am. J. Clin. Pathol.* **1977**, *67*, 477-480, doi:https://doi.org/10.1093/ajcp/67.5.477.

75. Watson, H.G.; Greaves, M. Can we predict bleeding? *Semin. Thromb. Hemost.* **2008**, *34*, 97-103, doi:https://doi.org/10.1055/s-2008-1066028.

76. Cuker, A.; Raby, A.; Moffat, K.; Flynn, G.; Crowther, M. Interlaboratory variation in heparin monitoring: lessons from the quality management program of Ontario coagulation surveys. *Thromb. Haemost.* **2010**, *104*, 837-844, doi:https://doi.org/10.1160/TH10-02-0099.

77. L Babin, J.; Traylor, K.; Witt, D. Laboratory monitoring of low-molecular-weight heparin and fondaparinux. *Semin. Thromb. Hemost.* **2017**, *43*, 261-269, doi:https://doi.org/10.1055/s-0036-1581129.

78. Gehrie, E.; Laposata, M. Test of the month: the chromogenic antifactor Xa assay. *Am. J. Hematol.* **2012**, *87*, 194-196, doi:https://doi.org/10.1002/ajh.22222.

79. Bates, S.M.; Weitz, J.I. Coagulation assays. *Circulation* **2005**, *112*, 53-60, doi:https://doi.org/10.1161/CIRCULATIONAHA.104.478222.

80. Newall, F. Anti-factor Xa (anti-Xa) assay. In *Haemostasis. Methods in Molecular Biology (Methods and Protocols)*, Monagle, P., Ed.; Humana Press: Totowa, NJ, 2013; Volume 992, pp. 265-272.

81. Funk, D.M. Coagulation assays and anticoagulant monitoring. *Hematology Am. Soc. Hematol. Educ. Program.* **2012**, *2012*, 460-465, doi:https://doi.org/10.1182/asheducation-2012.1.460.

82. Samuelson, B.T.; Cuker, A. Measurement and reversal of the direct oral anticoagulants. *Blood Rev.* **2017**, *31*, 77-84, doi:https://doi.org/10.1016/j.blre.2016.08.006.

83. Samuelson, B.T.; Cuker, A.; Siegal, D.M.; Crowther, M.; Garcia, D.A. Laboratory assessment of the anticoagulant activity of direct oral anticoagulants: a systematic review. *Chest* **2017**, *151*, 127-138, doi:https://doi.org/10.1016/j.chest.2016.08.1462.

84. Eby, C. Novel anticoagulants and laboratory testing. *Int. J. Lab. Hematol.* **2013**, *35*, 262-268, doi:https://doi.org/10.1111/ijlh.12065.

85. Adcock, D.M.; Gosselin, R. Direct oral anticoagulants (DOACs) in the laboratory: 2015 review. *Thromb. Res.* **2015**, *136*, 7-12, doi:https://doi.org/10.1016/j.thromres.2015.05.001.

86. Schmitz, E.M.H.; Boonen, K.; van den Heuvel, D.J.A.; van Dongen, J.L.J.; Schellings, M.W.M.; Emmen, J.M.A.; van der Graaf, F.; Brunsveld, L.; van de Kerkhof, D. Determination of dabigatran, rivaroxaban and apixaban by ultra-performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS) and coagulation assays for therapy monitoring of novel direct oral anticoagulants. *J. Thromb. Haemost.* **2014**, *12*, 1636-1646, doi:https://doi.org/10.1111/jth.12702.

87. Lippi, G.; Favaloro, E. Recent guidelines and recommendations for laboratory assessment of the direct oral anticoagulants (DOACs): Is there consensus? *Clin. Chem. Lab. Med.* **2015**, *53*, 185-197, doi:https://doi.org/10.1515/cclm-2014-0767.

88. Dale, B.J.; Chan, N.C.; Eikelboom, J.W. Laboratory measurement of the direct oral anticoagulants. *Br. J. Haematol.* **2016**, *172*, 315-336, doi:https://doi.org/10.1111/bjh.13810.

89. van Ryn, J.; Stangier, J.; Härtter, S.; Liesenfeld, K.-H.; Wienen, W.; Feuring, M.; Clemens, A. Dabigatran etexilate - a novel, reversible, oral direct thrombin inhibitor: interpretation of coagulation assays and reversal of anticoagulant activity. *Thromb. Haemost.* **2010**, *103*, 1116-1127, doi:https://doi.org/10.1160/TH09-11-0758.

90. Pollack, C.V. Coagulation assessment with the new generation of oral anticoagulants. *Emerg. Med. J.* **2016**, *33*, 423-430, doi:https://doi.org/10.1136/emermed-2015-204891.

91. Douxfils, J.; Mullier, F.; Robert, S.; Chatelain, C.; Chatelain, B.; Dogne, J.M. Impact of dabigatran on a large panel of routine or specific coagulation assays. Laboratory recommendations for monitoring of dabigatran etexilate. *Thromb. Haemost.* **2012**, *107*, 985-997, doi:https://doi.org/10.1160/th11-11-0804.

92. Nowak, G. The ecarin clotting time, a universal method to quantify direct thrombin inhibitors. *Pathophysiol. Haemost. Thromb.* **2003**, *33*, 173-183, doi:https://doi.org/10.1159/000081505.

93. Gosselin, R.C.; Dwyre, D.M.; Dager, W.E. Measuring dabigatran concentrations using a chromogenic ecarin clotting time assay. *Ann. Pharmacother.* **2013**, *47*, 1635-1640, doi:https://doi.org/10.1177/1060028013509074.

94. Bangham, A.D.; Standish, M.M.; Watkins, J.C. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* **1965**, *13*, 238-252, doi:https://doi.org/10.1016/S0022-2836(65)80093-6.

95. Bangham, A.D.; Standish, M.M.; Watkins, J.C.; Weissmann, G. The diffusion of ions from a phospholipid model membrane system. In Proceedings of the Symposium on Biophysics and Physiology of Biological Transport, Vienna, 1967; pp. 183-187.

96. Gregoriadis, G.; Ryman, B.E. Liposomes as carriers of enzymes or drugs: a new approach to the treatment of storage diseases. *Biochem. J.* **1971**, *124*, 58, doi:https://doi.org/10.1042/bj1240058p.

97. Gregoriadis, G. Drug entrapment in liposomes. *FEBS Lett.* **1973**, *36*, 292-296, doi:https://doi.org/10.1016/0014-5793(73)80394-1.

98. Gregoriadis, G. The carrier potential of liposomes in biology and medicine (first of two parts). *N. Engl. J. Med.* **1976**, *295*, 704-710, doi:https://doi.org/10.1056/nejm197609232951305.

99. Gregoriadis, G. The carrier potential of liposomes in biology and medicine (second of two parts). *N. Engl. J. Med.* **1976**, *295*, 765-770, doi:https://doi.org/10.1056/nejm197609302951406.

100. Sercombe, L.; Veerati, T.; Moheimani, F.; Wu, S.Y.; Sood, A.K.; Hua, S. Advances and challenges of liposome assisted drug delivery. *Front. Pharmacol.* **2015**, *6*, 13, doi:https://doi.org/10.3389/fphar.2015.00286.

101. Ahmed, K.S.; Hussein, S.A.; Ali, A.H.; Korma, S.A.; Lipeng, Q.; Jinghua, C. Liposome: composition, characterisation, preparation, and recent innovation in clinical applications. *J. Drug Target.* **2019**, *27*, 742-761, doi:10.1080/1061186X.2018.1527337.

102. Akbarzadeh, A.; Rezaei-Sadabady, R.; Davaran, S.; Joo, S.W.; Zarghami, N.; Hanifehpour, Y.; Samiei, M.; Kouhi, M.; Nejati-Koshki, K. Liposome: classification, preparation, and applications. *Nanoscale Res. Lett.* **2013**, *8*, 1 - 9, doi:https://doi.org/10.1186/1556-276X-8-102.

103. Liu, G.; Hou, S.; Tong, P.; Li, J. Liposomes: Preparation, Characteristics, and Application Strategies in Analytical Chemistry. *Critical Reviews in Analytical Chemistry* **2020**, 1-21, doi:10.1080/10408347.2020.1805293.

104. Song, Y.-K.; Hyun, S.Y.; Kim, H.-T.; Kim, C.-K.; Oh, J.-M. Transdermal delivery of low molecular weight heparin loaded in flexible liposomes with bioavailability enhancement: comparison with ethosomes. *J. Microencapsul.* **2011**, *28*, 151-158, doi:https://doi.org/10.3109/02652048.2010.507880.

105. Bai, S.; Ahsan, F. Inhalable liposomes of low molecular weight heparin for the treatment of venous thromboembolism. *J. Pharm. Sci.* **2010**, *99*, 4554-4564, doi:https://doi.org/10.1002/jps.22160.

106. Lavanya, N.; Muzib, Y.I.; Aukunuru, J.; Balekari, U. Preparation and evaluation of a novel oral delivery system for low molecular weight heparin. *International Journal of Pharmaceutical Investigation* **2016**, *6*, 148-157, doi:10.4103/2230-973X.187351.

107. Croisfelt, F.M.; Tundisi, L.L.; Ataide, J.A.; Silveira, E.; Tambourgi, E.B.; Jozala, A.F.; Souto, E.M.B.; Mazzola, P.G. Modified-release topical hydrogels: a ten-year review. *Journal of Materials Science* **2019**, *54*, 10963-10983, doi:10.1007/s10853-019-03557-x.

108. Li, D.; van Nostrum, C.F.; Mastrobattista, E.; Vermonden, T.; Hennink, W.E. Nanogels for intracellular delivery of biotherapeutics. *J. Control. Release* **2017**, *259*, 16-28, doi:https://doi.org/10.1016/j.jconrel.2016.12.020.

109. Narayanaswamy, R.; Torchilin, V.P. Hydrogels and Their Applications in Targeted Drug Delivery. *Molecules (Basel, Switzerland)* **2019**, *24*, 603.

110. Li, J.; Mooney, D.J. Designing hydrogels for controlled drug delivery. *Nat. Rev. Mater.* **2016**, *1*, 17, doi:https://doi.org/10.1038/natrevmats.2016.71.

111. Jacob, S.; Nair, A.B.; Shah, J.; Sreeharsha, N.; Gupta, S.; Shinu, P. Emerging Role of Hydrogels in Drug Delivery Systems, Tissue Engineering and Wound Management. *Pharmaceutics* **2021**, *13*, 357.

112. Matanović, M.R.; Grabnar, P.A.; Voinovich, D.; Golob, S.; Mijovski, M.B.; Grabnar, I. Development and preclinical pharmacokinetics of a novel subcutaneous thermoresponsive system for prolonged delivery of heparin. *Int. J. Pharm.* **2015**, *496*, 583-592, doi:https://doi.org/10.1016/j.ijpharm.2015.10.062.

113. Liu, Z.; Jiao, Y.; Wang, Y.; Zhou, C.; Zhang, Z. Polysaccharides-based nanoparticles as drug delivery systems. *Adv. Drug Deliv. Rev.* **2008**, *60*, 1650-1662, doi:https://doi.org/10.1016/j.addr.2008.09.001.

114. Sur, S.; Rathore, A.; Dave, V.; Reddy, K.R.; Chouhan, R.S.; Sadhu, V. Recent developments in functionalized polymer nanoparticles for efficient drug delivery system. *Nano-Structures & Nano-Objects* **2019**, *20*, 100397, doi:https://doi.org/10.1016/j.nanoso.2019.100397.

115. Singh, R.; Lillard, J.W. Nanoparticle-based targeted drug delivery. *Exp. Mol. Pathol.* **2009**, *86*, 215-223, doi:https://doi.org/10.1016/j.yexmp.2008.12.004.

116. Rizvi, S.A.A.; Saleh, A.M. Applications of nanoparticle systems in drug delivery technology. *Saudi Pharmaceutical Journal* **2018**, *26*, 64-70, doi:https://doi.org/10.1016/j.jsps.2017.10.012.

117. Ramadan, A.; Lagarce, F.; Tessier-Marteau, A.; Thomas, O.; Legras, P.; Macchi, L.; Saulnier, P.; Benoit, J.-P. Oral fondaparinux: use of lipid nanocapsules as nanocarriers and in vivo pharmacokinetic study. *Int. J. Nanomedicine* **2011**, *6*, 2941-2951, doi:https://doi.org/10.2147/IJN.S25791.

118. Souto, E.B.; Baldim, I.; Oliveira, W.P.; Rao, R.; Yadav, N.; Gama, F.M.; Mahant, S. SLN and NLC for topical, dermal and transdermal drug delivery. *Expert Opinion on Drug Delivery* **2020**, *17*, 357-377, doi: https://doi.org/10.1080/17425247.2020.1727883.

119. Mahant, S.; Rao, R.; Souto, E.B.; Nanda, S. Analytical tools and evaluation strategies for nanostructured lipid carrier based topical delivery systems. *Expert Opinion on Drug Delivery* **2020**, null-null, doi: https://doi.org/10.1080/17425247.2020.1772750.

120. Paliwal, R.; Paliwal, S.R.; Agrawal, G.P.; Vyas, S.P. Biomimetic solid lipid nanoparticles for oral bioavailability enhancement of low molecular weight heparin and its lipid conjugates: in vitro and in vivo evaluation. *Mol. Pharm.* **2011**, *8*, 1314-1321, doi:https://doi.org/10.1021/mp200109m.

121. Rehman, F.U.; Shah, K.U.; Shah, S.U.; Khan, I.U.; Khan, G.M.; Khan, A. From nanoemulsions to self-nanoemulsions, with recent advances in self-nanoemulsifying drug delivery systems (SNEDDS). *Expert Opin. Drug Deliv.* **2017**, *14*, 1325-1340, doi:https://doi.org/10.1080/17425247.2016.1218462.

122. Cherniakov, I.; Domb, A.J.; Hoffman, A. Self-nano-emulsifying drug delivery systems: an update of the biopharmaceutical aspects. *Expert Opin. Drug Deliv.* **2015**, *12*, 1121-1133, doi:https://doi.org/10.1517/17425247.2015.999038.

123. Zupančič, O.; Grieβinger, J.A.; Rohrer, J.; Pereira de Sousa, I.; Danninger, L.; Partenhauser, A.; Sündermann, N.E.; Laffleur, F.; Bernkop-Schnürch, A. Development, in vitro and in vivo evaluation of a self-emulsifying drug delivery system (SEDDS) for oral enoxaparin administration. *Eur. J. Pharm. Biopharm.* **2016**, *109*, 113-121, doi:https://doi.org/10.1016/j.ejpb.2016.09.013.

124. Fang, G.; Zhou, J.; Qian, Y.; Gou, J.; Yang, X.; Tang, B. Development and evaluation of thermo-sensitive hydrogel system with nanocomplexes for prolonged subcutaneous delivery of enoxaparin. *Journal of Drug Delivery Science and Technology* **2018**, *48*, 118-124, doi:https://doi.org/10.1016/j.jddst.2018.09.004.

125. Gritsch, L.; Motta, F.L.; Contessi Negrini, N.; Yahia, L.H.; Farè, S. Crosslinked gelatin hydrogels as carriers for controlled heparin release. *Materials Letters* **2018**, *228*, 375-378, doi:https://doi.org/10.1016/j.matlet.2018.06.047.

126. Eleraky, N.E.; Swarnakar, N.K.; Mohamed, D.F.; Attia, M.A.; Pauletti, G.M. Permeation-Enhancing Nanoparticle Formulation to Enable Oral Absorption of Enoxaparin. *AAPS PharmSciTech* **2020**, *21*, 88, doi: https://doi.org/10.1208/s12249- 020-1618-2.

127. Tang, B.; Qian, Y.; Fang, G. Development of Lipid–Polymer Hybrid Nanoparticles for Improving Oral Absorption of Enoxaparin. *Pharmaceutics* **2020**, *12*, 607.

128. Soltani, Y.; Goodarzi, N.; Mahjub, R. Preparation and characterization of self nano-emulsifying drug delivery system (SNEDDS) for oral delivery of heparin using hydrophobic complexation by cationic polymer of β-cyclodextrin. *Drug Dev. Ind. Pharm.* **2017**, *43*, 1899-1907, doi: https://doi.org/10.1080/03639045.2017.1353522.

129. Xue, X.; Cao, M.; Ren, L.; Qian, Y.; Chen, G. Preparation and Optimization of Rivaroxaban by Self-Nanoemulsifying Drug Delivery System (SNEDDS) for Enhanced Oral Bioavailability and No Food Effect. *AAPS PharmSciTech* **2018**, *19*, 1847-1859, doi: https://doi.org/10.1208/s12249-018-0991-6.

130. Abouhussein, D.M.N.; Bahaa El Din Mahmoud, D.; Mohammad F, E. Design of a liquid nano-sized drug delivery system with enhanced solubility of rivaroxaban for venous thromboembolism management in paediatric patients and emergency cases. *Journal of Liposome Research* **2019**, *29*, 399-412, doi: https://doi.org/10.1080/08982104.2019.1576732.

5 ARTIGO 2 - Synthesis of Eudragit® L100-coated chitosan-based nanoparticles for oral enoxaparin delivery

Yuri Basilio Gomes Patriota [a], Igor Eduardo Silva Arruda [a], Antônia Carla de Jesus Oliveira [a], Thaisa Cardoso de Oliveira [a], Eliadna de Lemos Vasconcelos Silva [a], Luíse Lopes Chaves [a,c], Fábio de Oliveira Silva Ribeiro [b], Durcilene Alves da Silva [b], Mônica Felts de La Roca Soares[a], José Lamartine Soares-Sobrinho [a,*]

[a] Quality Control Core of Medicines and Correlates, Federal University of Pernambuco, Recife, PE, Brazil

[b] Research Center on Biodiversity and Biotechnology – BIOTEC, Federal University of Delta of Parnaiba, Parnaiba, PI, Brazil

[c] Department of Immunology, Aggeu Magalh˜aes Institute (IAM), Oswaldo Cruz Foundation (FIOCRUZ), Brazil

* Corresponding author at: Department of Pharmaceutical Sciences, Federal University of Pernambuco, Avenue Professor Arthur de Sá, s/n, ZIP CODE: 50740-521, Cidade Universitária, Recife, Pernambuco, Brazil. E-mail address: jose.ssobrinho@ufpe.br

International Journal of Biological Macromolecules 193 (2021) 450–456

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Synthesis of Eudragit® L100-coated chitosan-based nanoparticles for oral enoxaparin delivery

Yuri Basilio Gomes Patriota [a], Igor Eduardo Silva Arruda [a], Antônia Carla de Jesus Oliveira [a], Thaisa Cardoso de Oliveira [a], Eliadna de Lemos Vasconcelos Silva [a], Luíse Lopes Chaves [a,c], Fábio de Oliveira Silva Ribeiro [b], Durcilene Alves da Silva [b], Mônica Felts de La Roca Soares [a], José Lamartine Soares-Sobrinho [a,*]

[a] Quality Control Core of Medicines and Correlates, Federal University of Pernambuco, Recife, PE, Brazil
[b] Research Center on Biodiversity and Biotechnology – BIOTEC, Federal University of Delta of Parnaiba, Parnaiba, PI, Brazil
[c] Department of Immunology, Aggeu Magalhães Institute (IAM), Oswaldo Cruz Foundation (FIOCRUZ), Brazil

ABSTRACT

Enoxaparin is an effective biological molecule for prevention and treatment of coagulation disorders. However, it is poorly absorbed in the gastrointestinal tract. In this study, we developed an Eudragit® L100 coated chitosan core shell nanoparticles for enoxaparin oral delivery (Eud/CS/Enox NPs) through a completely eco-friendly method without employing any high-energy homogenizer technique and any organic solvents. Spherical nanocarriers were successfully prepared with particle size lower than 300 nm, polydispersity index about 0.12 and zeta potential higher than +25 mV, entrapment efficiency greater than 95% and the in vitro release behavior confirms the good colloidal stability and the successful Eudragit® L100 coating process demonstrated by negligible cumulative enoxaparin release (<10%) when the particles are submitted to simulated gastric fluid conditions. Finally, we demonstrated that the core-shell structure of the particle influenced the drug release mechanism of the formulations, indicating the presence of the Eudragit® L100 on the surface of the particles. These results suggested that enteric-coating approach and drug delivery nanotechnology can be successfully explored as potential tools for oral delivery of enoxaparin.

Keywords: Chitosan, drug delivery system, eudragit® L100, low-molecular weight heparin, biomacromolecule, polymer nanoparticles.

1. Introduction

Enoxaparin (Enox) is the low molecular weight heparin (LMWH) of choice for the prevention and treatment of deep vein thrombosis and coronary syndromes [1]. It exerts your anticoagulant activity by binding to antithrombin (AT), thereby catalyzing the indirect inhibition of factor Xa (FXa) and in lesser extent, factor IIa (FIIa) (Fig. 1) [2].

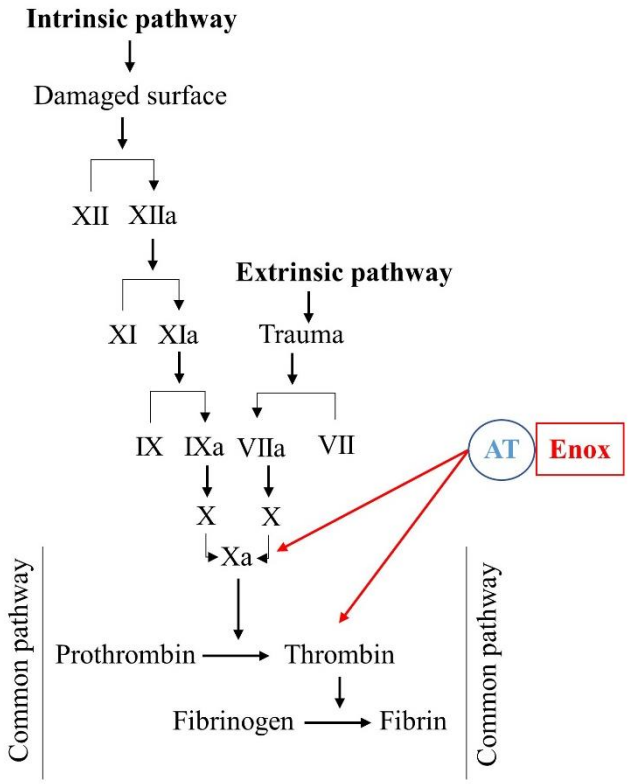


Figure 1: Coagulation cascade and Enox therapeutic targets.

Enox is only available as parenteral aqueous solution (intravenously or subcutaneously) which limit its clinical applicability. The Enox halflife (4.5 h) requires daily administration, impairing the patient compliance [3]. Moreover, Enox is inactivated in acidic medium and shows poor permeation through the intestinal wall due to high molecular weight, hydrophilicity and high negative charge and consequently, low oral bioavailability [4].

Several micro/nanoformulations for improve oral bioavailability of Enox including liposomes [5], microparticles [6], self-emulsifying drug delivery systems [7] and polymeric nanoparticles (PNPs) [8] have been explored. The development of an oral formulation of Enox would be essential for long anticoagulant therapy in chronic conditions since it would overcome the

inconveniences of the daily injections (e.g. needleassociated pain, infections, hospitalization, hematomas, etc.), reducing side effects and, thus, improving the patient adhesion to treatment [9].

Polymeric nanoparticles (PNPs) have been drawn attention as nanocarriers for oral delivery of hydrophilic macromolecules since they have some remarkable advantages: (1) protection against acidic denaturation and enzymatic degradation; (2) increase the contact and absorption area; (3) increase the intestinal membrane permeability and (4) might show controlled release properties [10].

Chitosan (CS), a natural cationic heteropolysaccharide consisting of N-acetyl-D-glucosamine and D-glucosamine linked through (1–4)- glycosidic bonds [11], has been extensively explored as a promising biomaterial for delivery of large variety of macromecules [12–14]. CS has excellent biocompatibility, biodegradability, and nonimmunogenicity properties [15]. Moreover, CS-based biomaterials exhibit mucoadhesive and absorption-enhancing properties [16]. In fact, CS is capable to interact with the mucus and epithelial cells to prolong the residence time in the small intestine [17] and promote the opening of tight junctions (TJs) reversibly, facilitating the paracellular transport [18,19], respectively. However, oral delivery of CS-based materials is limited due to CS is easily solubilized at gastric pH medium, leading to loss of its mucoadhesive and absorption-enhancing properties [20] and in addition, allowing the leakage the drug to the harsh gastric environment [21].

Eudragit® L100 (Eud) is a synthetic anionic copolymer based on methacrylic acid and methyl methacrylic acid (ratio 1:1) [22]. Eud is an enteric pH-dependent product, soluble above pH 6, that has been widely used as controlled drug release excipient [23]. In fact, the use of Eud in various formulations such as liposomes [24], nanoparticles [21,25] for oral delivery of hydrophilic macromolecules has been reported.

The aim of this study was to develop Enox-loaded Eud-coated CS core/shell nanoparticles through an eco-friendly method for oral delivery.

2. Materials and methods

2.1. Materials

Enoxaparin sodium (Endocrisis® 40 mg/0.4 mL, 4000 IU) was a kind gift from Cristalia (Brazil). Eudragit® L100 (M_w of 135 kDa, apparent viscosity of 50-200 mPas) was purchased from Evonik (Essen, Germany). Chitosan (M_w of 105 g/mol, degree of deacetylation ~ 81%,) was purchased from Polymar (Fortaleza, Brazil). All other chemicals and reagents were of analytical grade and used without any further purification.

2.2. Preparation of CS/Enox nanocomplexes

CS/Enox nanocomplexes (F1, CS/Enox NCs) were prepared through polyelectrolyte complexation (PEC) technique . Briefly, Enox aqueous solution (0.55 mg/mL, pH 6.5) was dripped into 2 mL of CS aqueous acetic acid solution (0.5 mg/mL, pH 4.5) under magnetic stirring for 30 min at room temperature [10]. The system pH was adjusted with 1 N HCl or 1 N NaOH as required. Formulation variables such as initial CS pH and Enox to CS weight ratio (w/w) were studied on the basis of their effect on particle size and drug entrapment.

2.3 Preparation of Eud coated core shell CS nanoparticles

Eud coating on CS/Enox NCs was carried out in accordance with the method described by Xu and coworkers [21]. To prepare Eud-coated CS/Enox NPs, Eud phosphate buffer solution (PBS) (0.05 mg/mL) were added dropwise to CS/Enox NCs under mild agitation at room temperature and left under agitation for 20 min (F2, NC-loading) or Eud PBS solution were premixed with Enox aqueous solution before complexation with CS solution (F3, Anion-loading) [3].

2.4 Colloidal dispersions characterization

2.4.1 *Particle size (PS), polydispersity index (PDI) and zeta potential (ZP)*

The average particle size and zeta potential of undiluted colloidal dispersions were determining by dynamic light scattering (DLS) using ZetaSizer Nano-ZS90 (Malvern Instruments Ltd., UK). Measurements were carried out after an equilibration time of 120 s at a cell temperature of 25ºC with a detection angle of 90º. Particle size distribution was reported as a polidispersity index (PDI) [8].

2.4.2 Morphological characterization

The morphology of the colloidal dispersions were observed by atomic force microscopy (AFM) using a TT-AFM instrument (AFM workshop, USA) in intermittent contact mode using TED PELLA tips (TAP300-G10) at an amplitude frequency of aproximately 239 kHz. The samples were diluted with ultra-pure water and left in ultrasound bath for 30 min. Further, 10 µL of diluted samples was applied to a freshly cleaved mica surface and oven dried at 36ºC for 15 min. Images were analyzed using Gwyddion software 2.45 [26].

2.4.3 Stability study

To evaluate the stability of CS/Enox NCs and Eud/CS/Enox NPs in gastrointestinal tract (GIT), the colloidal dispersions were submitted to simulated GIT pH conditions (0.1 M HCl, pH 1.2, PBS pH 6.8 and 7.4). Briefly, 0.5 mL of colloidal dispersions were dispersed into 2.5 mL of simulated GIT fluids under continuous stirring at room temperature. The integrity of nanoformulations was monitored at predetermined time intervals through any change of PS, PDI and ZP using ZetaSizer Nano-ZS90 (Malvern Instruments Ltd., UK) [21].

2.4.4 Estimation of Enox entrapment efficiency (EE)

The EE of Enox was calculated based in the difference between the total amount of Enox added to the colloidal dispersions and the untrapped drug amount remaining in the aqueous supernatant after the centrifugation step. The EE was determined by an indirect turbidimetric/ nephelometric method based on the quantitative precipitation reaction occurring between Enox's sulfate and carboxyl groups and the amine groups of cetylpyridinium chloride. Briefly, 250 µL of each sample was reacted for 1 h at room temperature with 250 µL of sodium acetate buffer (1 M, pH 4.8) and 1 mL of cetylpyridinium chloride (0.1% w/v) in NaCl aqueous solution (0.94% w/v). The precipitates were assayed spectrophotometrically (Varian, 50 UV-VIS, Australia) at 290 nm [6]. This method was validated according to the ICH guide [27] through parameters such as linearity, specificity, precision and accuracy, and robustness (data not shown).

2.5 Enox *in vitro* release study

The Enox in vitro release studies were performed through dialysis bag method [28]. The Enox in vitro release from selected colloidal dispersions was determining in enzyme-free and gradient pH medium in air shake incubator at 150 rpm. Briefly, Eud/CS/Enox NPs and CS/Enox NCs

were placed inside a dialysis membrane (MWCO 12–14 kDa). The dialysis membrane was suspended in 5 mL of simulated gastric fluid (SGF, pH 1.2) at 37 ± 0.5 ◦C for 2 h. Then, the SGF was replaced by 5 mL of PBS pH 6.8 for 4 h and finally, for PBS pH 7.4 until the end of 12 h both at 37 ± 0.5 ◦C. Aliquots (500 μL) were collected at predetermined time intervals and replaced with equal volumes of respective medium. The concentration of Enox released was determined by turbidimetric/nephelometric method previously described.

2.6 Evaluation of the mechanism of drug release

To examine the drug release behavior from nanoformulations, it was evaluated changes in the PS upon incubation in simulated GIT conditions. For this study, the nanoformulations were submitted to the same protocol described in Section 2.5. The volume-based mean diameters (Dv_t) of the nanoformulations were measured at predetermined time intervals using ZetaSizer Nano-ZS90 (Malvern Instruments Ltd., UK). The Dvt was subtracted from that volume-based mean diameters observed at time zero (Dv_0) and the differences between the diameters were then plotted against time [29].

2.7 Statistical analysis

All results were expressed as the mean value ± standard deviation (SD) from at least three measurements. Significance of difference was evaluated using one-way ANOVA at the probability level of 0.05.

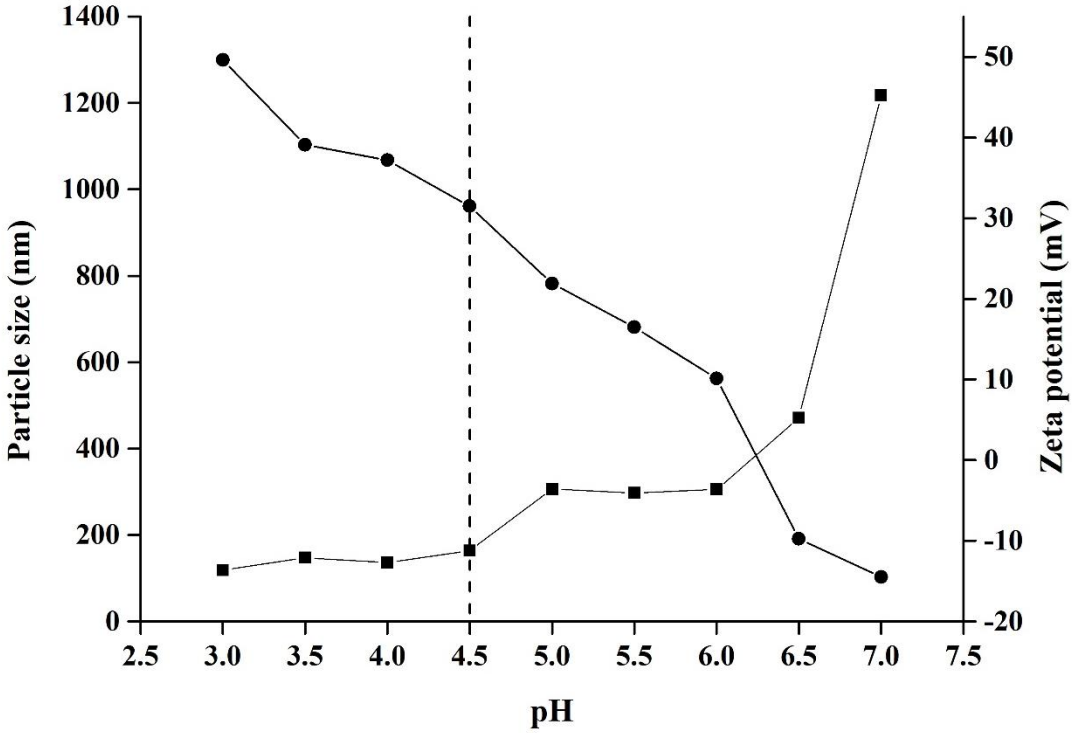
3. Results and discussion

3.1 Preparation of CS/Enox NCs

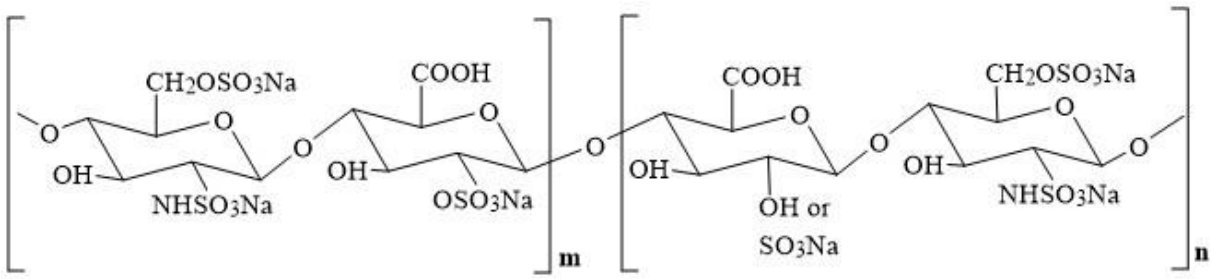
3.1.1 Effect of system pH

Since the NCs formation is mainly driven by electrostatic interactions, both polymers have to be ionized and own opposite charges. The self-assembly reaction should be performed at pH values close to the pKa interval of the two polymers (pKa of Enox is approximately 3.1 and the pKa of CS is 6.5) [30]. Thus, this investigation was performed in pH range of 3–7 for CS since the pH of CS solution will influence its charge density and therefore, the properties of resulting NCs. To study the influence of changing CS solution pH values on PS and ZP of CS/Enox NCs (Fig. 2), both Enox to CS weight ratio and concentration were kept constant.

(A)



(B)



(C)

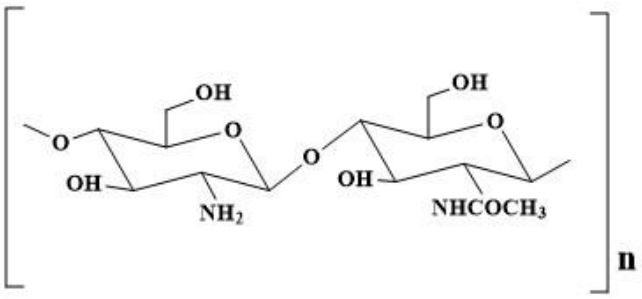


Figure 2: Influence of CS pH values on PS (■) and zeta potential (●) for CS/Enox NCs (a) and chemical structure of Enox (b) and CS (c) showing their ionization sites.

As depicted in Fig. 2, the PS and ZP of CS/Enox NCs is dependent on the initial CS solution pH. It was observed that rising the CS solution pH values, an increase in the PS of colloidal dispersions accompanied by a corresponding decrease in their positive surface charge was obtained.

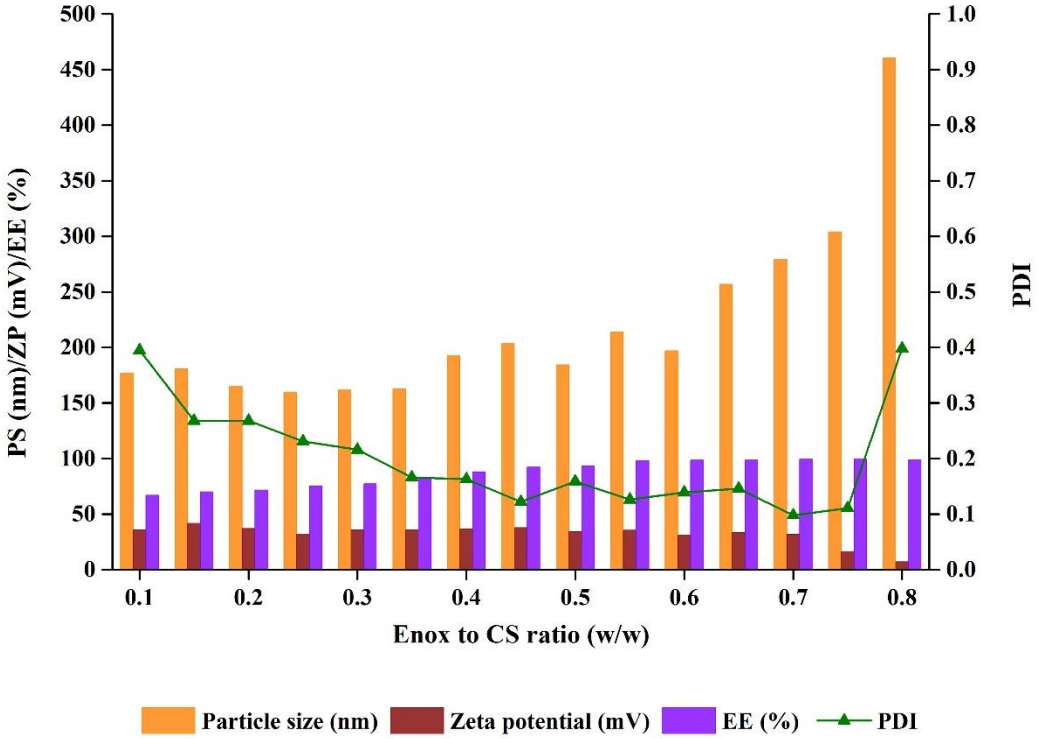
Addition of Enox solution led to a turbid aggregating dispersion when the initial CS solution pH range was 6 to 7. Even adjusting the initial CS solution pH to 5.5, the precipitation was observed. This can probably be attributed to the fact that in the formulation prepared at pH values near to CS isoelectric point, the poor ionization of CS amino groups, reduced its charge density, consequently, decreasing the electrostatic interaction with Enox molecules, resulting to unstable dispersions [3].

On the other hand, when the CS solution pH was adjusted to 5 and 4.5, spontaneously formed an opalescent and stable colloidal dispersions. At these pH values, the CS amino groups were more protonated, highly positively charged, favoring the inter-cross-linkages with negatively charged Enox molecules [14].

The PS is a crucial parameter regarding efficient uptake by intestinal cells [31]. In fact, smaller particles can penetrate the gastrointestinal (GI) mucus gel layer and reach the underlying epithelium to a higher extent than larger particles [32]. In addition, surface charge is another parameter that affects the intestinal absorption [31]. Positive charge nanoparticles interact with negatively GI mucus layer, hence, enhancing the intestinal residence time, increasing particle uptake, as well as improving drug absorption [8]. Thus, the formulation prepared with the initial CS solution pH of 4.5 was selected to further studies.

3.1.2 Effect of Enox to CS weight ratio

The drug/polymer ratio is another parameter that has significant influence on the properties of NCs [30]. Therefore, the influence of the stoichiometry on PS, ZP, PDI and EE was investigated at pH 4.5 (Fig. 3).



.

Figure 3: Influence of Enox to CS mass ratio on the PS, ZP, PDI and EE for CS/Enox NCs.

As shown in Fig. 3, an increase in the PS and EE of CS/Enox NCs were observed with successive increase in the Enox concentration. As the Enox concentration is increased, CS molecules could be linked with more Enox molecules, thereby increasing the PS as well as the EE of the CS/Enox NCs [33], until that a critical point was achieved (Enox to CS mass ratio of 0.6 and 0.5, respectively), from which the PS increased suddenly and the EE reach a plateau phase, in which, it is worth nothing that all CS/Enox NCs showed high EE values (>95%). The positive ZP values remained unchanged almost all investigation ranging from +21.3 to +16.6 mV (Fig. 3), except in the final mass ratio, where a lower ZP value was observed (+7.4 mV), probably due to the large proportion of free negatively charged Enox groups [34]. On the other hand, the PDI showed a decreasing behavior until an Enox to CS mass ratio of 0.7, from which the PDI increased abruptly, possibly related to a decrease in the ZP values due to excess of Enox molecules, favoring agglomeration of the particles [33].

According to these results, it was defined that Enox to CS mass ratio of 0.55 is the optimal proportion to further studies as it produced colloidal dispersions with appropriate PS (208 ± 21.7 nm), minimum PDI (0.125 ± 0.01) and a maximum EE (98.3 ± 1.1).

3.2 Preparation of Eud/CS/Enox NPs

In order to protect the integrity of Enox in the harsh gastric acid milleu, optimized CS/Enox NCs formulations were coated with Eud layers through electrostatic interaction (Table 1). Several Eud solution concentrations were previously evaluated to potentialize the benefit of Eud in the electrostatic interaction equilibrium (data not shown). It is worth to mentioning that Eud concentrations as high as utilized on this work resulted in unstable aggregated colloidal dispersions leading to precipitation. In addition, it is noteworthy that this method was completely eco-friendly without employing any high-energy homogenizer methods and any organic solvents, contributing to the physicalchemical stability of the drug.

Two Eud loading approaches were applied to investigate the influence of the order of addition of the reactants on nanoformulation characteristics. Eud (0.05 mg/mL) was dissolved in PBS pH 6.8 where the carboxyl groups become negatively charged facilitating the adsorption to the F1 positive amino groups. As the addition of Eud PBS solution, the pH value of the final dispersion raised to 4.7 and as consequence, the solubility of Eud decreased and formed the protective layer, forming the Eud/CS/Enox NPs [21].

NC-loading method showed an increase in particle size which clearly indicates the presence of Eud on the surface of nanoparticle. This effect was mediated through the reduction of F1 positive surface charge density by neutralization of CS amino groups by negatively charged Eud through electrostatic interaction [35].

On the other hand, Eud coating through anion-loading method produced NPs much smaller which could be explained by the competitive ionic interaction between negatively charged carboxyl and carboxyl/sulfate groups of Eud and Enox, respectively on positively charged amino groups of CS. In fact, as Eud was utilized on pH which it would be negatively charged, hence, Eud could compete with Enox to interact with CS electrostatically. This interaction, provided by the multi-ionic sites of the large CS molecules, may have collaborated to the more compact nanoparticles [36].

F2 and F3 showed non-aggregating nanosized colloidal dispersions with positive ZP values (Table 1). The surface charge is an important parameter since it influences not only the stability of the colloidal preparation but also can affects the in vivo fate of the nanocarriers, influencing the opsonization process, blood circulation time as well as biodistribution [37]. In addition, the surface charge can be tuned to improve the nanocarries-mucin interaction prolonging the transit and retention times, allowing more time for drug release in the target site [38].

The EE for all colloidal dispersions was comparable and nearly 100% (Table 1), which indicates a strong ionic interaction between CS and Enox molecules. Once the interaction between those entities are driven by electrostatic interaction and an exceeding amount of CS was used in the formulation (weight ratio of CS to Enox was 2:1), therefore, the chance of all Enox molecules being ionically complexed with CS counterparts is too high, leading to a high EE [28]. All formulations showed in Table 1 were chosen for the subsequent studies.

Table 1: Characterization of different formulations.

Code	Formulations	PS (nm)	PDI	ZP (mV)	EE (%)
F1	CS/Enox NCs	208.4 ± 21.7	0.125 ± 0.01	+ 28.0 ± 1.8	98.3 ± 1.1
F2	Eud/CS/Enox NPs[a]	293.6 ± 5.7	0.103 ± 0.03	+ 25.6 ± 0.9	96.2 ± 1.0
F3	Eud/CS/Enox NPs[b]	200.4 ± 4.2	0.124 ± 0.01	+ 31.0 ± 0.4	95.2 ± 1.5

[a] NC-loading: Eud PBS pH 6.8 solution were added to the freshly prepared CS/Enox NCs.
[b] Anion-loading: Eud PBS pH 6.8 solution were premixed with Enox aqueous solution before complexation with CS solution.

3.3 Morphological characterization

The morphology of Enox-loaded nanoformulations was observed with atomic force microscopy (AFM) and is shown in Fig. 4. All the nanoformulations are spherical or sub spherical in shape and well separated from each other. In addition, the PS obtained from AFM is smaller than that determined by DLS technique (Table 1) which can be attributed to the different measurement mechanism and sample treatment. For DLS, nanoformulations were evaluated in the hydrated environment, so the polymer chains were well swelled. On the other hand, for AFM visualization, nanoformulations were previously dried and possibly the nanocarriers shrunk [39].

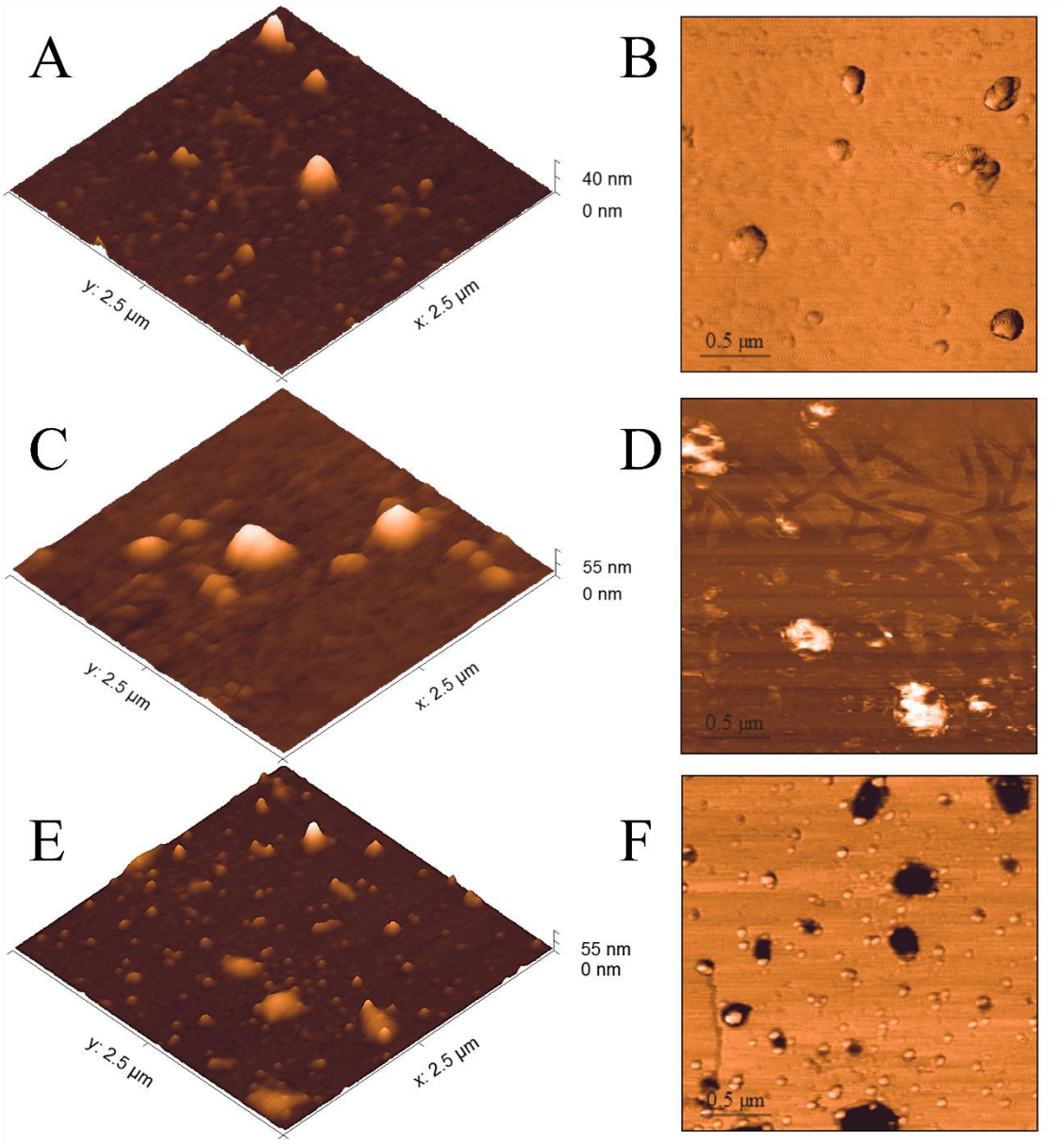


Figure 4: AFM images of Enox-loaded nanoformulations: F1 (A-B), F2 (C-D) and F3 (E-F).

3.4 Stability study

The nanoformulations were evaluated for stability at different pH conditions by detecting their particle sizes as depicted in Fig. 5. As showed in Fig. 5A, the PS of F2 and F3 had a negligible increase after 2 h of incubation in SGF indicating that they were able to maintain the integrity, which could be attribute to the Eud layers upon particle surface. On the other hand, when the same formulations were dispersed in pH 6.8 and 7.4 (Fig. 5B and C, respectively), the PS decreased which could be related to the beginning of Eud layers erosion. Once the Enox is inactivated in acidic medium, it is highly desirable that nanocarriers maintain the structure and

hence could protect the stability of the drug when passing in the stomach [21,40]. For F1, a more pronounced PS change was observed in all pH conditions comparatively with F2 and F3. However, in pH 7.4 a sudden increase in PS was observed which could be related to the destabilization and consequently, the disintegration of the system. As the CS amino groups become deprotonated, the electrostatic interaction between Enox and CS is weakened, leading the systems to swelling and destabilization process.

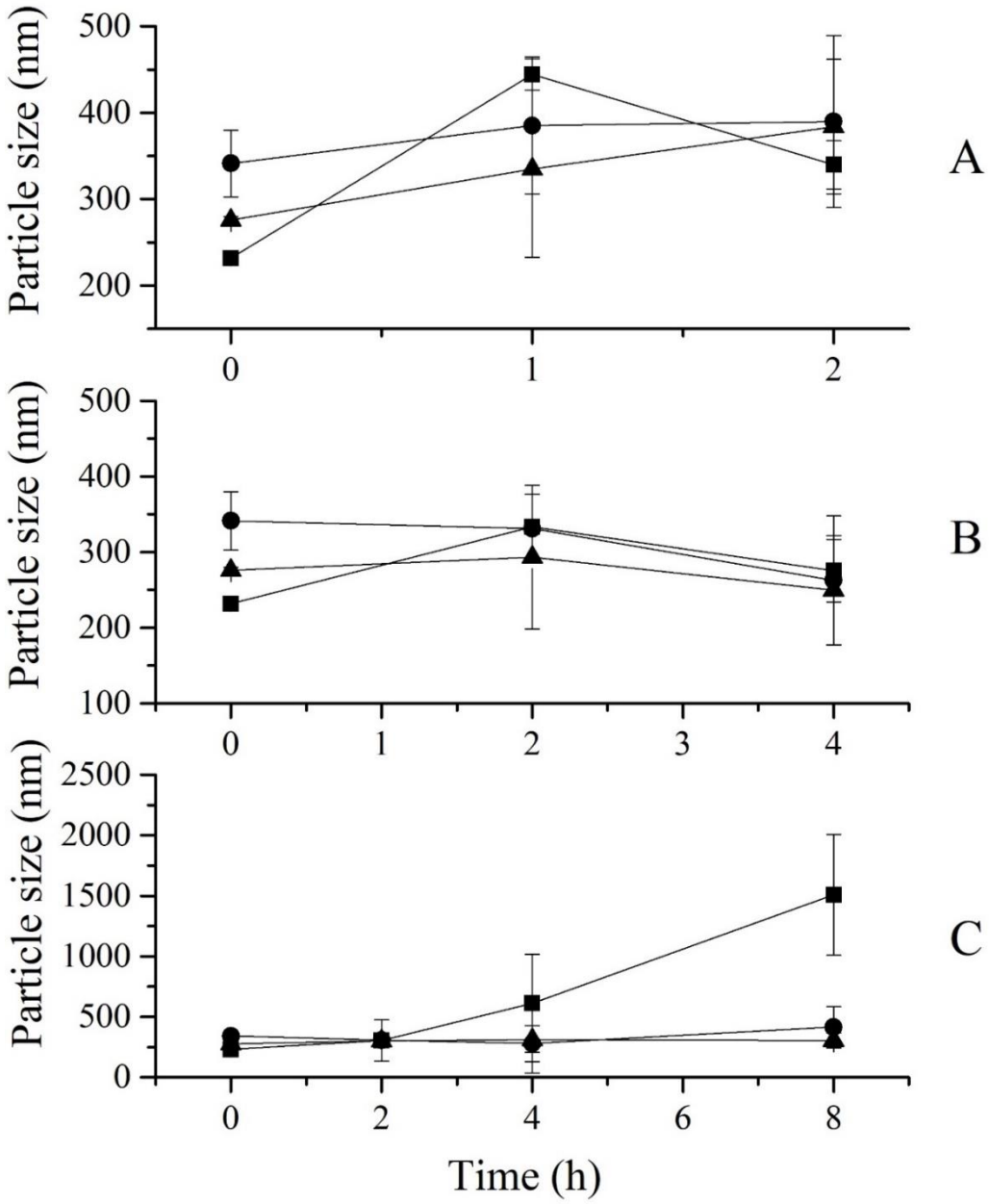


Figure 5: Particle size monitoring of Enox-loaded nanoformulations in different simulated pH conditions: (A) pH 1.2, (B) pH 6.8, and (C) pH 7.4. (■) F1, (●) F2 and (▲) F3.

3.5 Enox in vitro release study

Fig. 6 shows the Enox *in vitro* release profile from selected colloidal dispersions in simulated GIT fluid at different pH. The Enox release from all nanoformulations was similar ($p < 0.05$), low and incomplete after 12 h.

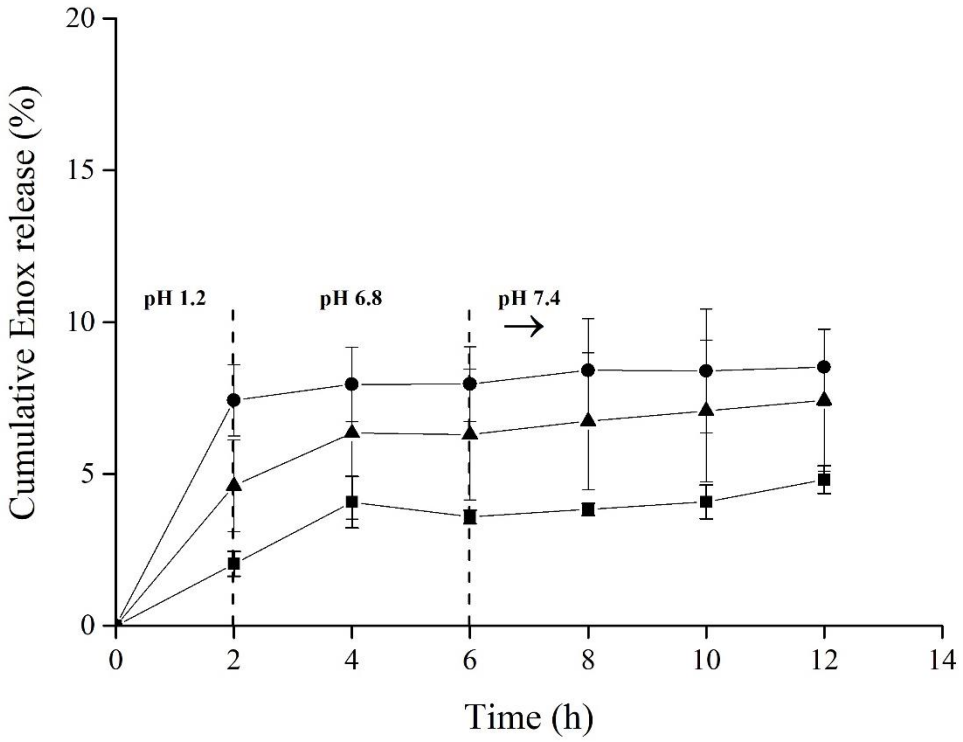


Figure 6: *In vitro* release profile of Enox-loaded nanoformulations in different simulated pH environments. (■) F1, (●) F2 and (▲) F3.

All nanocarriers exhibited a very poor cumulative Enox release at gastric medium. In addition, at pH 6.8 and 7.4 the cumulative Enox release showed a slight increase, but still it was very slow, achieving a cumulative Enox release less than 10% for all nanoformulations studied i.e. in formulations F1, 4.8% (13.2 μg); F2, 8.5% (23.4 μg) and F3, 7.4% (20.4 μg).

In comparison with F2 and F3, F1 showed lower cumulative Enox release. This might be attributed to acid mucopolysaccharide nature of Enox molecules, which are able to maintain the electrostatic attraction with positively charged CS molecules in a wider pH range, and hence maintaining the F1 stability even at pH 1.2 [10]. Furthermore, it is worth to note that the Enox was exposed directly to acidic medium on F1 formulation and it may have suffered inactivation

by low pH condition since this formulation is not able to protect the drug from acidic environment [41].

Regard to F2 and F3, slight increase in cumulative Enox release was noticed, which might be related to the Eud-coating procedure. In fact, the addition of Eud PBS pH 6.8 solution possibly weakened the interaction force between positively charged CS molecules and negativelycharged Enox molecules [35]. In addition, the slower cumulative Enox release showed by F3 in comparison with F2, could be attributed to the more compact structure formed by the polymers difficulting the penetration of the incubating medium into nanocarrier matrix [3].

A slow Enox release behavior could be of interest for oral Enox administration. Once Enox must exert your anticoagulant effect in the bloodstream, it is interesting that the release phenomena take place only when the nanocarriers achieve and interact with the proximity of absorbing intestinal epithelium [10].

As a strategy for prolonging the drug residence time of formulations at intestinal mucosal surface, CS, a well-known mucoadhesive biomaterial, was applied to allow more time for drug release at the target site, resulting in improved bioavailability [38,42]. In fact, CS presents strong bioadhesiveness properties which is related to the interaction between sialic acid residue of mucus intestinal barrier and amino groups of CS molecules [42]. It is worth mentioning that a similar Enox release behavior was reported by other authors [3,28,43].

3.6 Mechanism of drug release

The changes in mean volume diameter of nanocarriers suspended in simulated GIT fluids are depicted in Fig 7.

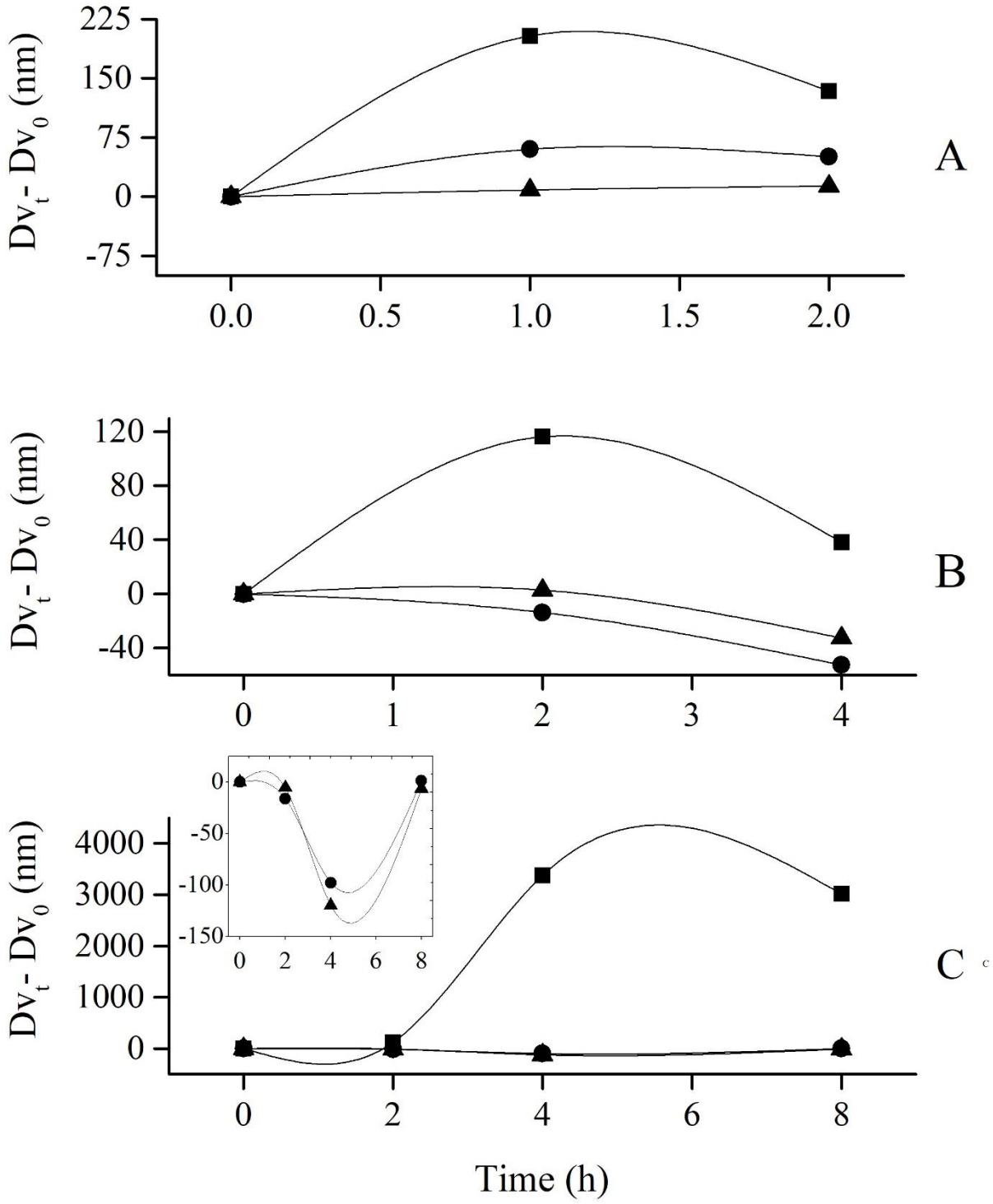


Figure 7: Mean volume diameter behavior of Enox-loaded nanoformulations in different simulated pH environments: (A) pH 1.2, (B) pH 6.8, and (C) pH 7.4. (■) F1, (●) F2 and (▲) F3. Inset graph emphasizes the F2 and F3 behavior.

Among all conditions, the F1 showed a higher increase in the mean volume diameters compared to F2 and F3. In fact, acrylic polymers such as Eud are pH-dependent polymers extensively used for enteric effect that dissolves at pH 6 above. It is expected that they withstand the lower gastric pH values and the degradation process takes place at neutral or slightly alkaline pH [23]. For F1, at pH 1.2 and 6.8, initially an increase in the mean volume diameters was observed followed by a little reduction in size. However, at pH 7.4, there was a rapid increase in mean volume diameters followed by a significant reduction. On the other hand, for F2 and F3, at pH 1.2, nanocarriers showed a smooth change in mean volume diameters demonstrating the gastric resistance which might be attributed to the Eud coating process. Further, at pH 6.8 and 7.4, the nanocarriers showed a more pronounced reduction in the mean volume diameters followed by a considerable increase in the diameter. In fact, as Eud has a pH-dependent solubility, its erosion and swelling increase as the pH increases [44].

Several studies demonstrated that the mechanism that controls/governs the drug release from CS-containing systems is the diffusion or swelling/erosion or both mechanisms [45]. In the swelling/erosioncontrolled release, the system exhibits an increase in particle size followed by a reduction in the diameters [46]. This behavior could be observed in the F1 suggesting that the drug was released by this manner. However, for F2 and F3, a different mechanism could be observed suggesting an initial erosion process and as the medium reaches the more hydrophilic core of the particle begins the swelling process [29].

4. Conclusion

In this study, we developed an spherical Eud-coated nanoformulations using an eco-friendly method which could entrapped high amount of drug and maintain the integrity against gastric acid environment. In addition, the *in vitro* release perfomance confirms the stability and the ability of Eud-coated nanocarriers to protect the drug from low pH conditions, demonstrated by negligible cumulative Enox release when the particles are submitted to SGF conditions. Finally, we demonstrated that the core-shell structure of the particle influenced the drug release mechanism of the formulations. Based on our study, it suggests that the combination of enteric-coating appoach and drug delivery systems could be successfully explored for the oral delivery of Enox.

References

[1] E. Gray, B. Mulloy, T.W. Barrowcliffe, Heparin and low-molecular-weight heparin, Thromb. Haemost. 99 (2008) 807–818, https://doi.org/10.1160/TH08-01-0032.
[2] R. Paliwal, S.R. Paliwal, G.P. Agrawal, S.P. Vyas, Recent advances in search of oral heparin therapeutics, Med. Res. Rev. 32 (2012) 388–409, https://doi.org/10.1002/med.20217.
[3] S.S. Ibrahim, R. Osman, G.A.S. Awad, N.D. Mortada, A.-S. Geneidi, Polysaccharides-based nanocomplexes for the prolonged delivery of enoxaparin: In-vitro and in-vivo evaluation, Int. J. Pharm. 526 (2017) 271–279, https://doi.org/10.1016/j.ijpharm.2017.05.007.
[4] N.A. Motlekar, B.-B.C., Youan the quest for non-invasive delivery of bioactive macromolecules: a focus on heparins, J. Control. Release 113 (2006) 91–101, https://doi.org/10.1016/j.jconrel.2006.04.008.
[5] N. Lavanya, Y.I. Muzib, J. Aukunuru, U., Balekari preparation and evaluation of a novel oral delivery system for low molecular weight heparin, Int. J. Pharm. Invest. 6 (2016) 148–157, https://doi.org/10.4103/2230-973X.187351.
[6] D. Hales, L. Vlase, S.A. Porav, A. Bodoki, L. Barbu-Tudoran, M. Achim, A quality by design (QbD) study on enoxaparin sodium loaded polymeric microspheres for colon-specific delivery, Eur. J. Pharm. Sci. 100 (2017) 249–261, https://doi.org/10.1016/j.ejps.2017.01.006.
[7] O. Zupancic, J.A. Grieβinger, J. Rohrer, I.Pereira de Sousa, L. Danninger, A. Partenhauser, Development, in vitro and in vivo evaluation of a self-emulsifying drug delivery system (SEDDS) for oral enoxaparin administration, Eur. J. Pharm. Biopharm. 109 (2016) 113–121, https://doi.org/10.1016/j.ejpb.2016.09.013.
[8] W. Dong, X. Wang, C. Liu, X. Zhang, X. Zhang, X. Chen, et al., Chitosan based polymer-lipid hybrid nanoparticles for oral delivery of enoxaparin int, J. Pharm. 547 (2018) 499–505, https://doi.org/10.1016/j.ijpharm.2018.05.076.
[9] A. Schluter, A. Lamprecht, Current developments for the oral delivery of heparin, Curr. Pharm. Biotechnol. 15 (2014) 640–649.
[10] W. Sun, S. Mao, Y. Wang, V.B. Junyaprasert, T. Zhang, L. Na, et al., Bioadhesion and oral absorption of enoxaparin nanocomplexes int, J. Pharm. 386 (2010) 275–281, https://doi.org/10.1016/j.ijpharm.2009.11.025.
[11] Y. Yang, S. Wang, Y. Wang, X. Wang, Q. Wang, M. Chen, Advances in selfassembled

chitosan nanomaterials for drug delivery, Biotechnology Advances 32 (2014) 1301–1316, https://doi.org/10.1016/j.biotechadv.2014.07.007.
[12] E.D.L.Vasconcelos Silva, A.C.D.J. Oliveira, Y.B.G. Patriota, A.J. Ribeiro, F. Veiga, F. Hallwass, Solvent-free synthesis of acetylated cashew gum for oral delivery system of insulin, Carbohydr. Polym. 207 (2019) 601–608.
[13] Y. Han, Q. Duan, Y. Li, Y. Li, J., Tian preparation and characterization of chitosanbased nanoparticles as protein delivery system, Adv. Polym. Technol. 37 (2018) 1214–1220, https://doi.org/10.1002/adv.21782.
[14] B. Fan, Y. Xing, Y. Zheng, C. Sun, G. Liang, pH-responsive thiolated chitosan nanoparticles for oral low-molecular weight heparin delivery: in vitro and in vivo evaluation, Drug delivery 28 (2015) 1–10.
[15] T. Kean, M., Thanou biodegradation, biodistribution and toxicity of chitosan, Adv. Drug Deliv. Rev. 62 (2010) 3–11, https://doi.org/10.1016/j.addr.2009.09.004.
[16] M.-C. Chen, F.-L. Mi, Z.-X. Liao, C.-W. Hsiao, K. Sonaje, M.-F. Chung, Recent advances in chitosan-based nanoparticles for oral delivery of macromolecules, Adv. Drug Deliv. Rev. 65 (2013) 865–879, https://doi.org/10.1016/j.addr.2012.10.010.
[17] S. T.A., C.P. Sharma, Chitosan and Its Derivatives for Drug Delivery Perspective, 2011.
[18] M. Amidi, E. Mastrobattista, W. Jiskoot, W.E., Hennink chitosan-based delivery systems for protein therapeutics and antigens, Adv. Drug Deliv. Rev. 62 (2010) 59–82, https://doi.org/10.1016/j.addr.2009.11.009.
[19] K. Sonaje, K.-J. Lin, M.T. Tseng, S.-P. Wey, F.-Y. Su, E.-Y. Chuang, et al., Effects of chitosan-nanoparticle-mediated tight junction opening on the oral absorption of endotoxins, Biomaterials 32 (2011) 8712–8721, https://doi.org/10.1016/j. biomaterials.2011.07.086.
[20] A.P. Bagre, K. Jain, N.K. Jain, Alginate coated chitosan core shell nanoparticles for oral delivery of enoxaparin: in vitro and in vivo assessment, Int. J. Pharm. 456 (2013) 31, https://doi.org/10.1016/j.ijpharm.2013.08.037.
[21] B. Xu, W. Zhang, Y. Chen, Y. Xu, B. Wang, L. Zong, Eudragit® L100-coated mannosylated chitosan nanoparticles for oral protein vaccine delivery, Int. J. Biol. Macromol. 113 (2018) 534–542, https://doi.org/10.1016/j.ijbiomac.2018.02.016.
[22] C.N. Patra, R. Priya, S. Swain, G. Kumar Jena, K.C. Panigrahi, D. Ghose, Pharmaceutical significance of Eudragit: A review, Future J. Pharm. Sci. 3 (2017) 33–45, https://doi.org/10.1016/j.fjps.2017.02.001.

[23] S. Thakral, N.K. Thakral, D.K. Majumdar, Eudragit®: a technology evaluation, Expert Opin. Drug Deliv. 10 (2013) 131–149, https://doi.org/10.1517/17425247.2013.736962.
[24] K.M. Hosny, O.A.A. Ahmed, R.T., Al-abdali enteric-coated alendronate sodium nanoliposomes: a novel formula to overcome barriers for the treatment of osteoporosis, Expert Opin. Drug Deliv. 10 (2013) 741–746, https://doi.org/10.1517/17425247.2013.799136.
[25] Y. Zhang, X. Wu, L. Meng, Y. Zhang, R. Ai, N. Qi, Thiolated Eudragit nanoparticles for oral insulin delivery: Preparation, characterization and in vivo evaluation, Int. J. Pharm. 436 (2012) 341–350, https://doi.org/10.1016/j.ijpharm.2012.06.054.
[26] A.C.D.J. Oliveira, L.L. Chaves, F.D.O.S. Ribeiro, L.R.M. de Lima, T.C. Oliveira, F. García-Vill´en, Microwave-initiated rapid synthesis of phthalated cashew gum for drug delivery systems, Carbohydr. Polym. (2020) 117226, https://doi.org/10.1016/j.carbpol.2020.117226.
[27] International Conference on Harmonization (ICH) of Technical Requirements for registration of Pharmaceuticals for Human Use, Topic Q2 (R1): Validation of analytical procedures: Text and methodology Q2(R1), in, Geneva, 2005.
[28] M.-C. Chen, H.-S. Wong, K.-J. Lin, H.-L. Chen, S.-P. Wey, K. Sonaje, et al., The characteristics, biodistribution and bioavailability of a chitosan-based nanoparticulate system for the oral delivery of heparin, Biomaterials 30 (2009) 6629–6637, https://doi.org/10.1016/j.biomaterials.2009.08.030.
[29] B. Patel, V. Gupta, F., Ahsan PEG–PLGA based large porous particles for pulmonary delivery of a highly soluble drug, low molecular weight heparin, J. Control. Release 162 (2012) 310–320, https://doi.org/10.1016/j.jconrel.2012.07.003.
[30] W. Sun, S. Mao, D. Mei, T., Kissel self-assembled polyelectrolyte nanocomplexes between chitosan derivatives and enoxaparin, Eur. J. Pharm. Biopharm. 69 (2008) 417–425, https://doi.org/10.1016/j.ejpb.2008.01.016.
[31] L.M. Ensign, R. Cone, J., Hanes Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers, Adv. Drug Deliv. Rev. 64 (2012) 557–570, https://doi.org/10.1016/j.addr.2011.12.009.
[32] K. Maisel, L. Ensign, M. Reddy, R. Cone, J., Hanes effect of surface chemistry on nanoparticle interaction with gastrointestinal mucus and distribution in the gastrointestinal tract following oral and rectal administration in the mouse, J. Control. Release 197 (2015) 48–57, https://doi.org/10.1016/j.

jconrel.2014.10.026.
[33] R. Mahjub, T.Heidari Shayesteh, M. Radmehr, S.Y. Vafaei, M. Amini, R. Dinarvand, Preparation and optimization of N-trimethyl-O-carboxymethyl chitosan nanoparticles for delivery of low-molecular-weight heparin, Pharm. Dev. Technol. 21 (2016) 14–25, https://doi.org/10.3109/10837450.2014.965320.
[34] M. Radivojˇsa, I. Grabnar, P., Ahlin grabnar thermoreversible in situ gelling poloxamer-based systems with chitosan nanocomplexes for prolonged subcutaneous delivery of heparin: design and in vitro evaluation eur, J. Pharm. Sci. 50 (2013) 93–101, https://doi.org/10.1016/j.ejps.2013.03.002.
[35] S. Mouftah, M.M.A. Abdel-Mottaleb, A., Lamprecht buccal delivery of low molecular weight heparin by cationic polymethacrylate nanoparticles, Int. J. Pharm. 515 (2016) 565–574, https://doi.org/10.1016/j.ijpharm.2016.10.039.
[36] Y. Chen, V.J. Mohanraj, F. Wang, H.A. Benson, Designing chitosan-dextran sulfate nanoparticles using charge ratios, AAPS PharmSciTech 8 (2007) E98, https://doi.org/10.1208/pt0804098.
[37] Z. Zhao, A. Ukidve, V. Krishnan, S., Mitragotri effect of physicochemical and surface properties on in vivo fate of drug nanocarriers, Adv. Drug Deliv. Rev. (2019) pp, https://doi.org/10.1016/j.addr.2019.01.002.
[38] J.T. Huckaby, S.K., Lai PEGylation for enhancing nanoparticle diffusion in mucus, Adv. Drug Deliv. Rev. 124 (2017) 125–139, https://doi.org/10.1016/j.addr.2017.08.010.
[39] L. Wang, L. Li, Y. Sun, Y. Tian, Y. Li, C. Li, Exploration of hydrophobic modification degree of chitosan-based nanocomplexes on the oral delivery of enoxaparin, Eur. J. Pharm. Sci. 50 (2013) 263–271, https://doi.org/10.1016/j.ejps.2013.07.009.
[40] F. Akhtar, X. Wan, G. Wu, S. Kesse, S. Wang, S. He, in: Low-Molecular-Weight Heparins: Reduced Size Particulate Systems for Improved Therapeutic Outcomes Molecules (Basel, Switzerland) 23, 2018, pp. 1757–1769, doi:.
[41] G. Fang, B. Tang, Advanced delivery strategies facilitating oral absorption of heparins, Asian Journal of Pharmaceutical Sciences 15 (2020) 449–460, https://doi.org/10.1016/j.ajps.2019.11.006.
[42] F. Kesinee, A., Bernkop-Schnürch mucoadhesive vs. mucopenetrating particulate drug delivery, Eur. J. Pharm. Biopharm. 98 (2016) 76–89, https://doi.org/10.1016/j.ejpb.2015.11.003.
[43] R. Paliwal, S.R. Paliwal, G.P. Agrawal, S.P., Vyas chitosan nanoconstructs for

improved oral delivery of low molecular weight heparin: in vitro and in vivo evaluation, Int. J. Pharm. 422 (2012) 179–184, https://doi.org/10.1016/j.ijpharm.2011.10.048.

[44] M. Cetin, A. Atila, Y., Kadioglu formulation and in vitro characterization of Eudragit® L100 and Eudragit® L100-PLGA nanoparticles containing diclofenac sodium, AAPS PharmSciTech 11 (2010) 1250–1256, https://doi.org/10.1208/s12249-010-9489-6.

[45] R. Safdar, A.A. Omar, A. Arunagiri, I. Regupathi, M. Thanabalan, Potential of chitosan and its derivatives for controlled drug release applications – a review, J. Drug Deliv. Sci. Technol. 49 (2019) 642–659, https://doi.org/10.1016/j.jddst.2018.10.020.

[46] J.H. Lee, Y. Yeo, Controlled drug release from pharmaceutical nanocarriers, Chem. Eng. Sci 125 (2015) 75–84, https://doi.org/10.1016/j.ces.2014.08.046.

6 CONSIDERAÇÕES FINAIS E PERSPECTIVAS FUTURAS

Os anticoagulantes convencionais são fármacos bem conhecidos e com testes anticoagulantes bem definidos, enquanto os ADOC são uma classe de fármacos relativamente recente que chegou trazendo notáveis mudanças na terapia anticoagulante, porém, com alguns questionamentos em relação a sua utilização principalmente devido a inexistência de antídotos específicos e ausência de um teste de monitoramento preciso e confiável. Os nanocarreadores a base de HBPM vem sendo desenvolvidos e testados por diversas vias de administração e os testes anticoagulantes se mostraram adequados para quantificar a performance desses nanossistemas.

As nanopartículas do tipo core/shell de eudragit® L100/quitosana/enoxaparina sódica foram obtidas com sucesso através de um método ecologicamente correto, sem o uso de solventes orgânicos, e foram capazes de incorporar elevada quantidade de fármaco e manter sua integridade contra o pH ácido simulado. Além disso, através dos estudos de liberação *in vitro* e mecanismo de liberação confirmamos não só a estabilidade dessas partículas e a habilidade desses sistemas em proteger o fármaco das condições severas do pH ácido, como também, que o recobrimento com eudragit® L100 foi obtido com sucesso e que a estrutura core/shell da partícula influenciou o mecanismo de liberação das nanoformulações.

Pretendemos aprofundar o estudo desses nanossistemas empregando uma abordagem *in vivo* e *ex-vivo*, investigando o comportamento dessas nanopartículas em um organismo vivo, tanto de uma perspectiva farmacológica quanto toxicológica.

Por fim, e não menos importante, queria deixar registrado para que futuros leitores dessa tese, possam ter uma noção, mesmo que mínima, dos desafios que enfrentamos, em todos os aspectos, com a pandemia da COVID-19 causada pelo novo coronavírus, SARS-CoV-2. O mundo em isolamento social, jornais noticiando mortes aos milhares em todo o planeta todo dia, por uma doença desconhecida da comunidade científica que não havia tratamento e nem cura. Nós, pesquisadores em formação, com nossos prazos esgotando, as universidades fechadas, laboratórios parados ou com acesso restrito, parcerias inviabilizadas e, aliado a tudo isso, o medo de se contaminar e adoecer nossos familiares, amigos e colegas de trabalho. Após esforços globais, vieram as vacinas e pudemos ver uma luz no fim do túnel.

É por isso que a finalização deste trabalho é tão comemorada porque não significa somente o fechamento de um ciclo, mas uma nova chance de viver que muitos não tiveram, significa a resiliência da raça humana, significa a vitória da ciência.

REFERÊNCIAS

AHN, M. Y. et al. Characterization of a Bacteroides species from human intestine that degrades glycosaminoglycans. **Canadian Journal of Microbiology,** v. 44, n. 5, p. 423-429, 1998.

AKHTAR, F. et al. Low-Molecular-Weight Heparins: Reduced Size Particulate Systems for Improved Therapeutic Outcomes. **Molecules,** v. 23, n. 7, p. 1757-1769, 2018.

AL-HILAL, T. A. et al. Oligomeric bile acid-mediated oral delivery of low molecular weight heparin. **Journal of Controlled Release,** v. 175, p. 17-24, 2014.

ALÁEZ-VERSÓN, C. R.; LANTERO, E.; FERNÀNDEZ-BUSQUETS, X. Heparin: new life for an old drug. **Nanomedicine,** v. 12, n. 14, p. 1727-1744, 2017.

ANILKUMAR, P. et al. A rationalized description on study of intestinal barrier, drug permeability and permeation enhancers. **Journal of Global Trends in Pharmaceutical Sciences,** v. 2, n. 4, p. 431-449, 2011.

AUNGST, B. J. Absorption enhancers: applications and advances. **American Association of Pharmaceutical Scientists Journal,** v. 14, n. 1, p. 10-18, 2012.

BAGRE, A. P.; JAIN, K.; JAIN, N. K. Alginate coated chitosan core shell nanoparticles for oral delivery of enoxaparin: In vitro and in vivo assessment. **International Journal of Pharmaceutics,** v. 456, n. 1, p. 31-40, 2013.

BAUER, K. A. Fondaparinux sodium: a selective inhibitor of factor Xa. **American Journal of Health-System Pharmacy,** v. 58 n. Suppl 2, p. S14-17, 2001.

BECKMAN, M. G. et al. Venous Thromboembolism: A Public Health Concern. **American Journal of Preventive Medicine,** v. 38, n. 4, p. S495-S501, 2010.

BEHRAVESH, S. et al. Pathogenesis of Thromboembolism and Endovascular Management. **Thrombosis,** v. 2017, p. 1-13, 2017.

BUENO, P. V. A. et al. N,N-Dimethyl chitosan/heparin polyelectrolyte complex vehicle for efficient heparin delivery. **International Journal of Biological Macromolecules,** v. 75, p. 186-191, 2015.

CHOONARA, B. F. et al. A review of advanced oral drug delivery technologies facilitating the protection and absorption of protein and peptide molecules. **Biotechnology Advances,** v. 32, n. 7, p. 1269-1282, 2014.

CHOUBEY, A. K. et al. Development and evaluation of PEGylated Enoxaparin: A novel approach for enhanced anti-Xa activity. **Bioorganic Chemistry,** v. 54, p. 1-6, 2014.

COSMI, B. An update on the pharmaceutical management of thrombosis. **Expert Opinion on Pharmacotherapy,** v. 17, n. 16, p. 2149-2164, 2016.

DAS KURMI, B. et al. Nanocarriers in Improved Heparin Delivery: Recent Updates. **Current Pharmaceutical Design,** v. 21, n. 30, p. 4509-4518, 2015.

DES RIEUX, A. et al. Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach. **Journal of Controlled Release,** v. 116, n. 1, p. 1-27, 2006.

DONAHUE, N. D.; ACAR, H.; WILHELM, S. Concepts of nanoparticle cellular uptake, intracellular trafficking, and kinetics in nanomedicine. **Advanced Drug Delivery Reviews,** v. 143, n. 15, p. 68-96, 2019.

DONG, W. et al. Chitosan based polymer-lipid hybrid nanoparticles for oral delivery of enoxaparin. **International Journal of Pharmaceutics,** v. 547, n. 1, p. 499-505, 2018.

ELERAKY, N. E. et al. Permeation-Enhancing Nanoparticle Formulation to Enable Oral Absorption of Enoxaparin. **AAPS PharmSciTech,** v. 21, n. 3, p. 88, 2020.

ENSIGN, L. M.; CONE, R.; HANES, J. Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers. **Advanced Drug Delivery Reviews,** v. 64, n. 6, p. 557-570, 2012.

EOM, J. S. et al. Antithrombotic efficacy of an oral low molecular weight heparin conjugated with deoxycholic asset on microsurgical anastomosis in rats. **Thrombosis Research,** v. 126, n. 3, p. e220-e224, 2010.

FAN, B. et al. pH-responsive thiolated chitosan nanoparticles for oral low-molecular weight heparin delivery: in vitro and in vivo evaluation. **Drug delivery,** v. 28, n. 1, p. 1-10, 2015.

FANG, G.; TANG, B. Advanced delivery strategies facilitating oral absorption of heparins. **Asian Journal of Pharmaceutical Sciences,** v. 15, n. 4, p. 449-460, 2020.

FRANCHINI, M. et al. The evolution of anticoagulant therapy. **Blood Transfusion,** v. 14, n. 2, p. 175-184, 2016.

GARCIA, D. A. et al. Parenteral anticoagulants: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. **Chest,** v. 141, n. 2 p. e24S-e43S, 2012.

GOLDBERG, M.; GOMEZ-ORELLANA, I. Challenges for the oral delivery of macromolecules. **Nature Reviews Drug Discovery,** v. 2, n. 4, p. 289-295, 2003.

GOMEZ-ORELLANA, I. Strategies to improve oral bioavailability. **Expert Opinion on Drug Delivery.,** v. 2, p. 419-33, 2005.

GULSETH, M. P. Overview of direct oral anticoagulant therapy reversal. **American Journal of Health-System Pharmacy,** v. 73, n. Supplement 2, p. S5-S13, 2016.

HEIT, J. A.; SPENCER, F. A.; WHITE, R. H. The epidemiology of venous thromboembolism. **Journal of Thrombosis and Thrombolysis,** v. 41, n. 1, p. 3-14, 2016.

HU, T. Y.; VAIDYA, V. R.; ASIRVATHAM, S. J. Reversing anticoagulant effects of novel oral anticoagulants: role of ciraparantag, andexanet alfa, and idarucizumab. **Vascular Health and Risk Management.,** v. 12, p. 35-44, 2016.

HUCKABY, J. T.; LAI, S. K. PEGylation for enhancing nanoparticle diffusion in mucus. **Advanced Drug Delivery Reviews,** v. 124, n. 15, p. 125-139, 2017.

HWANG, S. R.; BYUN, Y. Advances in oral macromolecular drug delivery. **Expert Opinion on Drug Delivery.,** v. 11, n. 12, p. 1955-1967, 2014.

IBRAHIM, S. S. et al. Polysaccharides-based nanocomplexes for the prolonged delivery of enoxaparin: In-vitro and in-vivo evaluation. **International Journal of Pharmaceutics,** v. 526, n. 1–2, p. 271-279, 2017.

IBRAHIM, T. F.; MAXWELL, S.; IQBAL, O. Current anticoagulation drugs and mechanisms of action. In: LOFTUS, C. M. (Ed.). **Anticoagulation and Hemostasis in Neurosurgery**. Switzerland: Springer 2016. p.33-46.

JAIN, A.; JAIN, N. Preparation and Optimization of Transdermal Delivery System for Low Molecular Weight Heparin Using Ethonolic Liposomes. **Journal of Biomaterials and Tissue Engineering,** v. 4, n. 5, p. 416-422, 2014.

JANDIK, K. A. et al. Accelerated stability studies of heparin. **Journal of Pharmaceutical Sciences,** v. 85, n. 1, p. 45-51, 1996.

KESIEME, E. et al. Deep vein thrombosis: a clinical review. **Journal of Blood Medicine,** v. 2, p. 59-69, 2011.

KESINEE, F.; BERNKOP-SCHNÜRCH, A. Mucoadhesive vs. mucopenetrating particulate drug delivery. **European Journal of Pharmaceutics and Biopharmaceutics,** v. 98, p. 76-89, 2016.

KIM, B. T. et al. Purification and characterization of a novel heparinase from Bacteroides stercoris HJ-15. **Journal of Biochemistry,** v. 128, n. 2, p. 323-328, 2000.

KIM, S. K. et al. Absorption study of deoxycholic acid-heparin conjugate as a new form of oral anti-coagulant. **Journal of Controlled Release,** v. 120, n. 1, p. 4-10, 2007.

KIM, S. K. et al. Tricaprylin microemulsion for oral delivery of low molecular weight heparin conjugates. **Journal of Controlled Release,** v. 105, n. 1, p. 32-42, 2005.

KUMAR, D. R. et al. Virchow's Contribution to the Understanding of Thrombosis and Cellular Biology. **Clinical Medicine & Research,** v. 8, n. 3-4, p. 168-172, 2010.

LAVANYA, N. et al. Preparation and evaluation of a novel oral delivery system for low molecular weight heparin. **International Journal of Pharmaceutical Investigation,** v. 6, n. 3, p. 148-157, 2016.

LEE, D. Y. et al. Liphophilic complexation of heparin based on bile acid for oral delivery. **Journal of Controlled Release,** v. 123, n. 1, p. 39-45, 2007.

LIPPI, G.; FAVALORO, E. J. Laboratory monitoring of direct oral anticoagulants (DOACs) - the perfect storm? **Annals of Translational Medicine,** v. 5, n. 1, p. 6, 2017.

LIU, C. et al. Strategies and industrial perspectives to improve oral absorption of biological macromolecules. **Expert Opinion on Drug Delivery.,** v. 15, n. 3, p. 223-233, 2018.

LUNDQUIST, P.; ARTURSSON, P. Oral absorption of peptides and nanoparticles across the human intestine: Opportunities, limitations and studies in human tissues. **Advanced Drug Delivery Reviews,** v. 106, n. Part B, p. 256-276, 2016.

MATOUGUI, N. et al. Lipid-based nanoformulations for peptide delivery. **International Journal of Pharmaceutics,** v. 502, n. 1, p. 80-97, 2016.

MCCARTNEY, F.; GLEESON, J.; BRAYDEN, D. Safety concerns over the use of intestinal permeation enhancers: A mini-review. **Tissue Barriers,** v. 4, p. 00-00, 2016.

MERLI, G. J.; GROCE, J. B. Pharmacological and clinical differences between low-molecular-weight heparins: implications for prescribing practice and therapeutic interchange. **Pharmacy & Therapeutics** v. 35, n. 2, p. 95-105, 2010.

MOROZ, E.; MATOORI, S.; LEROUX, J.-C. Oral delivery of macromolecular drugs: Where we are after almost 100years of attempts. **Advanced Drug Delivery Reviews,** v. 101, p. 108-121, 2016.

MOTLEKAR, N. A. et al. Oral delivery of low-molecular-weight heparin using sodium caprate as absorption enhancer reaches therapeutic levels. **Journal of Drug Targeting,** v. 13, n. 10, p. 573-583, 2005.

MOTLEKAR, N. A. et al. Modulation of gastrointestinal permeability of low-molecular-weight heparin by L-arginine: in-vivo and in-vitro evaluation. **Journal of Pharmacy and Pharmacology,** v. 58, n. 5, p. 591-598, 2006.

MOTLEKAR, N. A.; YOUAN, B.-B. C. The quest for non-invasive delivery of bioactive macromolecules: A focus on heparins. **Journal of Controlled Release,** v. 113, n. 2, p. 91-101, 2006.

NEVES, A. R. et al. Strategies to Overcome Heparins' Low Oral Bioavailability. **Pharmaceuticals,** v. 9, n. 3, p. 3-16, 2016.

NIU, Z. et al. Lipid-based nanocarriers for oral peptide delivery. **Advanced Drug Delivery Reviews,** v. 106, p. 337-354, 2016.

ODUAH, E. I.; LINHARDT, R. J.; SHARFSTEIN, S. T. Heparin: Past, present, and future. **Pharmaceuticals,** v. 9, n. 3, p. 38-50, 2016.

PALIWAL, R. et al. Recent advances in search of oral heparin therapeutics. **Medicinal Research Reviews,** v. 32, n. 2, p. 388-409, 2012.

PARAKH, R. S.; SABATH, D. E. Venous Thromboembolism: Role of the Clinical Laboratory in Diagnosis and Management. **The Journal of Applied Laboratory Medicine,** v. 3, n. 5, p. 870-882, 2019.

PARK, J.; BYUN, Y. Recent advances in anticoagulant drug delivery. **Expert Opinion on Drug Delivery.,** v. 13, n. 3, p. 421-434, 2016.

PATEL, B.; GUPTA, V.; AHSAN, F. PEG–PLGA based large porous particles for pulmonary delivery of a highly soluble drug, low molecular weight heparin. **Journal of Controlled Release,** v. 162, n. 2, p. 310-320, 2012.

PATEL, S. et al. Brief update on endocytosis of nanomedicines. **Advanced Drug Delivery Reviews,** v. 144, p. 90-111, 2019.

PIRAN, S.; SCHULMAN, S. Management of venous thromboembolism: an update. **Thrombosis Journal.,** v. 14, n. Suppl 1, p. 23, 2016.

PLAPIED, L. et al. Fate of polymeric nanocarriers for oral drug delivery. **Current Opinion in Colloid & Interface Science,** v. 16, n. 3, p. 228-237, 2011.

PRIDGEN, E. M.; ALEXIS, F.; FAROKHZAD, O. C. Polymeric nanoparticle drug delivery technologies for oral delivery applications. **Expert Opinion on Drug Delivery.,** v. 12, n. 9, p. 1459-1473, 2015.

RAMA PRASAD, Y. V. et al. In situ intestinal absorption studies on low molecular weight heparin in rats using labrasol as absorption enhancer. **International Journal of Pharmaceutics,** v. 271, n. 1-2, p. 225-232, 2004.

SCHLUTER, A.; LAMPRECHT, A. Current Developments for the Oral Delivery of Heparin. **Current Pharmaceutical Biotechnology,** v. 15, n. 7, p. 640-649, 2014.

SHRESTHA, H.; BALA, R.; ARORA, S. Lipid-Based Drug Delivery Systems. **Journal of Pharmaceutics,** v. 2014, p. 801-820, 2014.

STONE, J. et al. Deep vein thrombosis: pathogenesis, diagnosis, and medical management. **Cardiovascular Diagnosis and Therapy,** v. 7, n. Suppl 3, p. S276-S284, 2017.

STREIFF, M. B. et al. Guidance for the treatment of deep vein thrombosis and pulmonary embolism. **Journal of Thrombosis and Thrombolysis,** v. 41, n. 1, p. 32-67, 2016.

THANOU, M. et al. N-sulfonato-N,O-carboxymethylchitosan: A novel polymeric absorption enhancer for the oral delivery of macromolecules. **Journal of Controlled Release,** v. 117, n. 2, p. 171-178, 2007.

VENE, N.; MAVRI, A. An overview of the anticoagulant drugs used in routine clinical practice. In: BOŽIČ-MIJOVSKI, M. (Ed.). **Anticoagulant Drugs**. United Kingdom: IntechOpen, 2018. p.10.

WALENGA, J. M.; LYMAN, G. H. Evolution of heparin anticoagulants to ultra-low-molecular-weight heparins: a review of pharmacologic and clinical differences and applications in patients with cancer. **Critical Reviews in Oncology/Hematology,** v. 88, n. 1, p. 1-18, 2013.

WANG, L. et al. In vitro and in vivo evaluation of chitosan graft glyceryl monooleate as peroral delivery carrier of enoxaparin. **International Journal of Pharmaceutics,** v. 471, n. 1–2, p. 391-399, 2014a.

WANG, L. et al. Exploration of hydrophobic modification degree of chitosan-based nanocomplexes on the oral delivery of enoxaparin. **European Journal of Pharmaceutical Sciences,** v. 50, n. 3–4, p. 263-271, 2013.

WANG, L. et al. Uptake, transport and peroral absorption of fatty glyceride grafted chitosan copolymer–enoxaparin nanocomplexes: Influence of glyceride chain length. **Acta Biomaterialia,** v. 10, n. 8, p. 3675-3685, 2014b.

YANG, T.; ARNOLD, J. J.; AHSAN, F. Tetradecylmaltoside (TDM) enhances in vitro and in vivo intestinal absorption of enoxaparin, a low molecular weight heparin. **Journal of Drug Targeting,** v. 13, n. 1, p. 29-38, 2005.

YANG, T. et al. Cyclodextrins in nasal delivery of low-molecular-weight heparins: in vivo and in vitro studies. **Pharmaceutical Research,** v. 21, n. 7, p. 1127-1136, 2004.

YANG, X. et al. Advanced Nanocarriers Based on Heparin and Its Derivatives for Cancer Management. **Biomacromolecules,** v. 16, n. 2, p. 423-436, 2015.

ZACCONI, F. C. FXa direct synthetic inhibitors. In: BOŽIČ-MIJOVSKI, M. (Ed.). **Anticoagulant Drugs**. United Kingdom: IntechOpen, 2018. p.11-37.

ZHANG, H. et al. Absorption enhancing effects of chitosan oligomers on the intestinal absorption of low molecular weight heparin in rats. **International Journal of Pharmaceutics,** v. 466, n. 1, p. 156-162, 2014.

ZUPANČIČ, O. et al. Development, in vitro and in vivo evaluation of a self-emulsifying drug delivery system (SEDDS) for oral enoxaparin administration. **European Journal of Pharmaceutics and Biopharmaceutics,** v. 109, p. 113-121, 2016.