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FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

THE CONTRIBUTION OF DRUG-MEMBRANE BIOPHYSICAL STUDIES FOR THE DEVELOPMENT OF SAFER NSAID

THE CASE OF DICLOFENAC

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DISSERTAÇÃO DE MESTRADO APRESENTADA
À FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO EM
BIOENGENHARIA

Integrated Masters in Bioengineering – Molecular Biotechnology

Master's Thesis

The contribution of drug-membrane biophysical studies for the development of safer NSAID: the case of diclofenac

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July 2015

Acknowledgements

First of all I would like to thank my supervisor, Prof. Salette Reis, for giving me the opportunity to work in the Department of Chemical Sciences of the Faculty of Pharmacy, for her constant encouragement and for creating a good working environment. I am also thankful to my co-supervisor Cláudia Nunes, for helping me put all the pieces of this work together and for her availability.

The most invaluable person for the success of this project was Catarina Pereira Leite, for following every step of the project and supporting me throughout these months. I would like to thank her for teaching me so much on the scientific and personal level, for her friendship, for constantly encouraging me to go further and for her thorough review of this dissertation.

To all of the MB^2 group, I express my gratitude for their warm welcome and for making every working day a pleasure.

Special thanks to my friends Catarina, Diana, Filipe and Helena for their companionship.

And last, but not least, I thank my family for their unconditional support.

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List of Abbreviations

ANS	1-Anilinonaphthalene-8-Sulfonic Acid
ASA	Acetylsalicylic acid
ATB-337	H_2S-diclofenac
ATB-346	H_2S-naproxen
ATB-346	H_2S-indomethacin
B	Cooperativity
CD	Cyclodextrins
COX	Cyclooxygenase
Coxibs	Selective inhibitors of COX-2
CV	Cardiovascular
DLPC	Dilinoleoyl-phosphatidylcholine
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMPE	1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine
DMSO	Dimethyl sulfoxide
DPH	1,6-Diphenyl-1,3,5-Hexatriene
DPPC	Dipalmitoyl-phosphatidylcholine
DSC	Differential scanning calorimetry
EPC	Egg yolk phosphatidylcholine
FDA	Food and Drugs Administration
FMLP	N-formyl-methionyl-leucyl-phenylalanine
FRET	Fluorescence resonance energy transfer
FTIR	Fourier transform infrared spectroscopy
GI	Gastrointestinal
GS-HCl	Glucosamine hydrochloride
GUV	Giant unilamellar vesicles
H_2S-NSAID	Hydrogen sulfide-releasing NSAID
K_p	Partition coefficient
LOX	Lipoxygenase
LT	Leukotrienes
LUV	Large unilamellar vesicles
MD	Molecular dynamics

MEDAL	Multinational Etoricoxib and Diclofenac Arthritis Long-term
MLV	Multilamellar vesicles
mPGES-1	Microsomal prostaglandin E synthase 1
NCX-4016	Nitroaspirin
NMR	Nuclear magnetic resonance
NNU-hpda	(2-(4-hydroxylphenyl)-3-(3,5-dihydroxylphenyl)pro-penoic acid)
NO-NSAID	Nitric oxide-releasing NSAID
NSAID	Nonsteroidal anti-inflammatory drugs
OA	Osteoarthritis
PC	Phosphatidylcholine
PE	Phospatidylethanolamine
PG	Prostaglandins
PMN	Polymorphonuclear neutrophils
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
PS	Phosphatidylserine
RA	Rheumatoid arthritis
SEM	Scanning electron microscopy
SUV	Small unilamellar vesicles
T_m	Main phase transition temperature
TMA-DPH	1-(4-Trimethylammoniumphenyl)-6-Phenyl-1,3,5-Hexatrienep-Toluenesulfonate
TNF-α	Tumour necrosis factor-alpha
TXA	Thromboxanes

Abstract

As the persistence of inflammatory response can result in harmful effects to the organism, nonsteroidal anti-inflammatory drugs (NSAID) have emerged to treat inflammatory conditions. Indeed, these drugs represent one of the most widely consumed pharmaceuticals due to their anti-inflammatory, analgesic and antipyretic properties. Although they are used in acute situations and for minor aches, they are particularly relevant in chronic cases. Yet, their chronic consumption is associated with a wide spectrum of unwanted effects, including gastrointestinal, cardiovascular, hepatic and renal toxicity. In this context, two approaches were established in the attempt of reducing NSAID toxicity: the modification of conventional drugs and the development of novel drugs to act on new targets. Some of these strategies have already resulted in the commercialization of pharmaceuticals, while others are in clinical trials or under development.

Interestingly, the currently well-described NSAID main mechanism of action – inhibition of the prostaglandins (PG) biosynthesis – does not fully explain their adverse effects. The investigation of additional mechanisms is crucial to understand how safer NSAID can be developed. The hypothesis that the therapeutic and toxic actions of NSAID can derive from their relation to cellular membranes has triggered the *in vitro* assessment of NSAID-membrane interactions. Effectively, different studies have shown their ability to alter the membrane hydrophobicity, fluidity and structure. Therefore, the study of NSAID-membrane interactions may enlighten the action of these drugs at the membrane level and eventually predict their capability to cause adverse effects *in vivo*.

This work aims to assess if diclofenac, one of the most widely consumed NSAID worldwide, has the ability to alter biological membranes. This experimental study evaluated diclofenac interactions with a membrane model system, DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) liposomes, and under relevant pH (7.4, 5.0 and 3.0) and temperature (37 ºC) conditions that mimic microenvironments where the drug is found after oral administration. The evaluation of the drug's partition was accomplished by derivative spectrophotometry; membrane-binding was assessed by a fluorescence titration technique; the drug membrane location was determined through steady-state and time-resolved fluorescence quenching; the study of the lipid phase transition and fluidity were both evaluated using fluorescence anisotropy measurements; and finally, the influence of the drug on membrane permeability was verified by a carboxyfluorescein leakage assay which encompassed a size-exclusion chromatography followed by steady-state fluorescence experiments. It was effectively verified that diclofenac interacts with phospholipid bilayers and alters its biophysical properties. The drug's actions depend on its ionization state, as experiments have demonstrated that the protonated form (assessed at pH 3.0) exerts a

more relevant effect. Diclofenac location in the membrane was confirmed to be in the phospholipid headgroups region at all pH conditions. Overall, its effects on the membrane biophysical properties include a decrease of the lipid main phase transition temperature and cooperativity, an increase of the fluidity within the phospholipids headgroup region and an augmented membrane permeability.

Finally, gathered results were discussed, firstly, in terms of relevance for diclofenac's therapeutic and toxic effects verified *in vivo* and, secondly, considering the contribution of biophysical studies for responding to the vital need imposed by chronic inflammatory diseases: the development of safer and more tolerable NSAID.

Resumo

Uma vez que a subsistência da resposta inflamatória pode resultar em efeitos prejudiciais no organismo, os anti-inflamatórios não-esteroides (AINE) surgiram para tratar condições inflamatórias. Efetivamente, estes fármacos são amplamente consumidos graças às suas propriedades anti-inflamatórias, analgésicas e antipiréticas. Apesar de serem usados em casos agudos e para aliviar dores leves, os AINE são particularmente revelantes para o tratamento de doenças crónicas. Todavia, o seu consumo crónico estás associado a um vasto espectro de efeitos indesejados, incluindo toxicidade gastrointestinal, cardiovascular, hepática e renal. Neste contexto, duas abordagens foram estabelecidas para tentar reduzir a toxicidade dos AINE: a modificação dos fármacos convencionais e o desenvolvimento de novos fármacos para atuarem em novos alvos terapêuticos. Algumas destas estratégias já resultaram na comercialização de fármacos mais seguros, enquanto outras ainda se encontram em ensaios clínicos ou em desenvolvimento.

Curiosamente, o mecanismo de ação dos AINE – a inibição da biossíntese das prostaglandinas (PG) – não explica os seus efeitos adversos na totalidade. A investigação de mecanismos adicionais é crucial para o desenvolvimento de AINE mais seguros. A hipótese de que as ações tóxicas e terapêuticas dos AINE podem derivar das suas relações com as membranas celulares desencadeou o estudo *in vitro* de interações AINE-membranas. De facto, vários estudos mostraram a capacidade dos AINE em alterar a hidrofobicidade, fluidez e estrutura das membranas. Portanto, a avaliação das interações dos AINE com membranas pode elucidar a ação destes fármacos a nível membranar e eventualmente prever a sua capacidade de causar efeitos adversos *in vivo*.

Este trabalho visa avaliar se o diclofenac, um dos AINE mais utilizados mundialmente, é capaz de alterar as propriedades biofísicas das membranas biológicas. Este estudo experimental avaliou as interações do diclofenac com um sistema mimético membranar, lipossomas de 1,2-dimistroil-*sn*-glicero-3-fosfocolina (DMPC), e em condições de pH (7.4, 5.0 e 3.0) e temperatura (37 ºC) relevantes que mimetizam os microambientes em que o fármaco se encontra após administração oral. A avaliação da partição do fármaco foi executada por espetrofotometria derivativa; a ligação à membrana foi avaliada através duma técnica de titulação fluorescente; a determinação da localização membranar do fármaco foi feita por medições de desativação de fluorescência e tempo-de-vida resolvidos no tempo; estudos relativos à transição de fase e à fluidez do lípido foram ambos obtidos por medições anisotropia; e finalmente, a influência do fármaco na permeabilidade membranar foi verificada por um ensaio de libertação da carboxifluoresceína que envolveu a execução de uma cromatografia por exclusão de tamanho seguida por medições de fluorescência. Foi efetivamente verificado que o diclofenac interage com bicamadas

fosfolipídicas e altera as suas propriedades biofísicas. As ações do fármaco dependem do seu estado de ionização, visto que vários ensaios demonstraram que a sua forma protonada (aqui estudada a pH 3.0) exerce um efeito mais relevante. A localização do diclofenac na membrana foi confirmada ser na região das cabeças polares dos fosfolípidos. Além disso, os seus efeitos na membrana incluem um decréscimo da temperatura e cooperatividade da transição de fase principal do lípido; um aumento da fluidez na zona das cabeças polares dos fosfolípidos e ainda uma permeabilidade membranar aumentada.

Finalmente, os resultados obtidos foram discutidos, em primeiro lugar, relativamente à sua relevância para os efeitos terapêuticos e tóxicos do diclofenac verificados *in vivo* e, em segundo lugar, tendo em conta a contribuição dos estudos biofísicos para responder à necessidade vital imposta pelas doenças inflamatórias crónicas: o desenvolvimento de AINE mais seguros e toleráveis.

1. Introduction

1.1) Nonsteroidal anti-inflammatory drugs (NSAID)

1.1.1) Inflammation

Inflammation is an important biological event that occurs as a response of the immune system to infection or injury. The inflammatory response is triggered by mediators, which include cytokines, vasoactive amines, plasma protein systems and eicosanoids (prostaglandins (PG), leukotrienes (LT) and thromboxanes (TXA)). (1) Inflammatory processes can be divided into two distinct phases. The acute phase is dominated by neutrophils and the influx of monocytes that differentiate into macrophages and it is characterized by redness, heat, swelling and pain. The prolonged presence of monocytes, macrophages and lymphocytes, along with the proliferation of blood vessels and connective tissue, mark the chronic phase. (2, 3) Although these events are generally seen as beneficial, once they lead to the removal of pathogenic factors and allow the recovery of the tissue's physiological function, they can also result in a persistent and dysfunctional response, originating scarring and loss of organ function. (3) For this reason, anti-inflammatory drugs (e.g. corticosteroids and nonsteroidal anti-inflammatory drugs) are crucial for controlling inflammatory processes.

1.1.2) General characterization of NSAID

Nonsteroidal anti-inflammatory drugs (NSAID) are a group of pharmaceutical agents that provide symptomatic relief from pain and inflammation as a result of their analgesic, anti-inflammatory and antipyretic properties. (4) These type of drugs are available over-the-counter and can be used for minor aches, in cases of acute inflammatory conditions or as a therapeutic for chronic inflammatory diseases, such rheumatoid arthritis (RA) and osteoarthritis (OA). (5)

The use of the anti-inflammatory compounds to treat fever and inflammation dates from about 3500 years ago. While the identification of salicylic acid (active ingredient of aspirin) occurred in the 17th century, the mass production of salicylic acid only began in 1860. This compound was found to have some unpleasant properties, as it possessed a bitter taste and caused dyspepsia. Hence, its improvement gave birth to aspirin as we know it today, acetylsalicylic acid (ASA), which was firstly commercialized around 1897. (6) Over the time, other NSAID emerged as better alternatives for the treatment of rheumatic diseases, considering that ASA implies high dosages that lead to central nervous signs of overdose. (4) The first non-aspirin NSAID to be introduced in the market were indomethacin

and ibuprofen in the late 60's. Diclofenac and naproxen followed, with launches in 1974 and 1976, respectively. These still remain some of the most widely used anti-inflammatory drugs. (7, 8) Nowadays, more than 50 NSAID are available in the global market with formulations that can vary from tablets to gels and injections. Their individual potencies differ and the choice between the many NSAID is usually dictated by their pharmacokinetic behaviour and their adverse effects. (5, 9)

Despite the early widespread use of NSAID, its main mechanism of action was only unravelled in 1971. (10) The knowledge of the drugs' mode of action later contributed to the explanation of some of their adverse effects. Due to their inherent toxicity, the effort to create safer and better tolerated anti-inflammatory agents stands until today.

1.1.3) Mechanism of Action

The NSAID mechanism of action was reported by Vane and Piper that demonstrated their ability to hamper the prostanoids biosynthesis, specifically through the inhibition of the cyclooxygenase (COX) enzyme. (7)

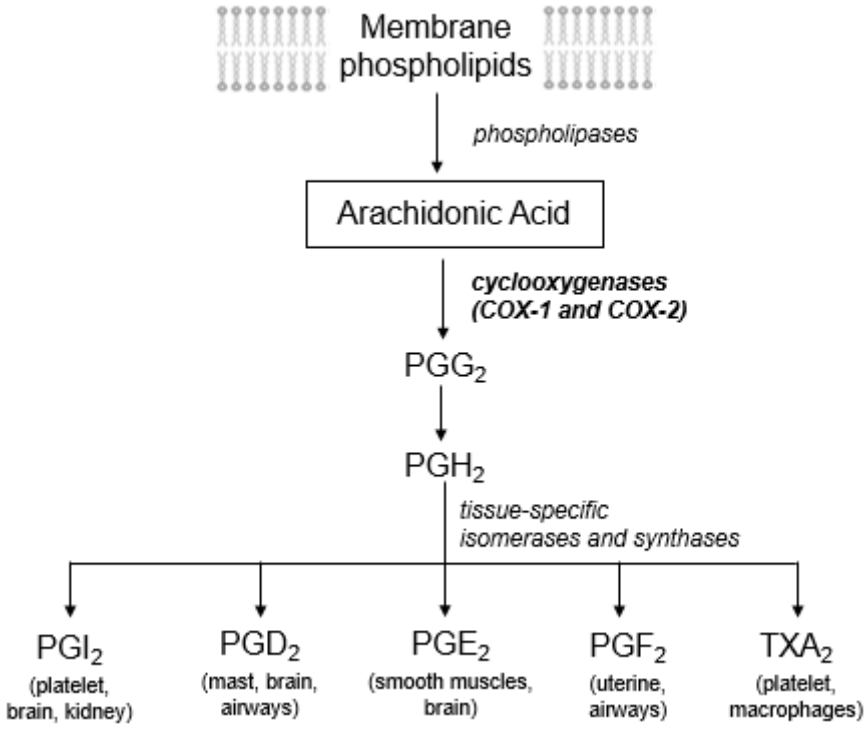


Figure 1 – Eicosanoids biosynthesis pathway: representation of the prostanoids branch (conversion of arachidonic acid by COX-1 and COX-2).

The prostanoids include PG and TXA, which are hormone-like lipids that derive from arachidonic acid. This fatty acid, released from the membrane phospholipids by

phospholipases upon stimulus, is metabolized by the consecutive action of COX and PG isomerases and synthases (Figure 1). The prostanoids biosynthesis pathway is not only responsible for the inflammatory response, but also regulates tissue homeostasis (e.g. renal blood low, gastric cytoprotection and vasodilation), since PG are involved in a wide range of processes and present in many different cell types (Figure 1). (3, 9)

After the discovery of the NSAID mechanism of action, the COX enzyme isoforms were defined. While COX-1 was described in 1976, the COX-2 gene was only identified in 1991. (7) Nowadays, both isoforms are well-characterized and are known to have more than 60% of sequence identity. Nevertheless, they possess some significant structural and functional differences. (8) These homodimers possess three domains in each subunit: an epidermal growth factor, a membrane binding domain and a catalytic domain that contains the active sites. Although three high mannose oligosaccharides can be found in both isoforms, COX-2 contains a fourth oligosaccharide that is able to regulate its degradation. (11) Another relevant structural divergence concerns the COX active site, where the NSAID binding occurs. In the COX-2 enzyme the active site channel is larger and more accessible due to differences in the aminoacid sequence. The exchange of a valine in position 523 in COX-2 for a more bulky aminoacid, isoleucine, in COX-1 at the active site creates a side pocket in COX-2 (Figure 2). This aminoacid exchange has implications for the selectivity profile of COX inhibitors. (12)

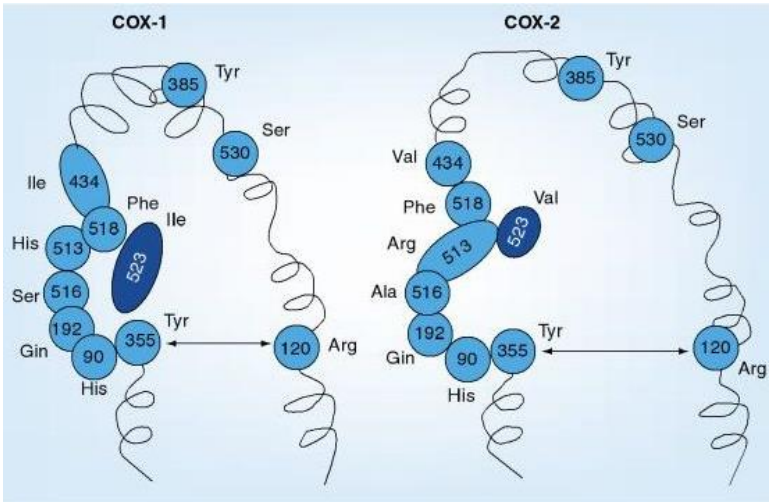


Figure 2 – COX-1 and COX-2 structural differences. Taken from (13).

Regarding their functional role, COX-1 is a constitutive enzyme that is involved in tissue homeostasis, including gastric cytoprotection, platelet aggregation, renal blood flow auto-regulation and initiation of parturition. Differently, COX-2 expression is induced when

inflammatory cells are injured or activated by cytokines, such as interleukin-1 and tumour necrosis factor-alpha (TNF-α), thereby being mainly associated with the production of prostanoids in inflammatory conditions. (5)

In general, conventional NSAID inhibit both COX-1 and COX-2 in a variable degree. While the anti-inflammatory action of the drugs are mainly related to their inhibition of COX-2, the blockage of COX-1 is more likely to be the cause of unwanted effects, particularly at the gastrointestinal (GI) level. This concept triggered the development of the selective inhibitors of COX-2 (coxibs). Even though these drugs are presently commercialized, their launch was quite controversial (5) – as it will be discussed in section 1.1.5.

In sum, the NSAID therapeutic properties result from the inhibition of prostanoid biosynthesis through the COX pathway. In particular, its anti-inflammatory action is mainly associated with the decrease of PG that amplify the inflammatory processes. Also, it can also be attributed to their oxygen radical scavenging capacity that decreases tissue damage. Their analgesic effects occur by decreasing PG that sensitise nociceptors to certain inflammatory mediators. Finally, the NSAID antipyretic effect is achieved by resetting the hypothalamic thermo-regulating function, without affecting normal body temperature. (5)

1.1.4) Toxicity

Despite NSAID huge commercial impact and therapeutic potential, this group of pharmaceuticals causes adverse effects that are relevant when extensively used in elderly populations and for long periods of time, making them particularly hazardous for chronic consumers. (5) Their toxicity relates to the role of PG in physiological processes besides their mediation of inflammation (Figure 3). (14)

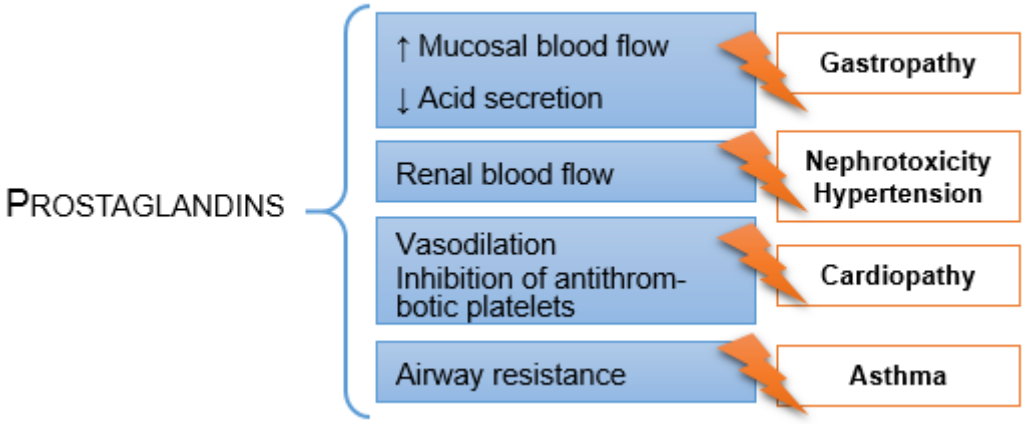


Figure 3 – Prostaglandins functions and respective adverse effects caused by NSAID-induced inhibition. Adapted from (9).

GI disturbances are the most common side effect of NSAID and include gastric discomfort, dyspepsia, diarrhoea, nausea, vomiting and, in more severe cases, gastric bleeding and ulceration. This occurs because PG are very important mediators of mucosal defence and repair of the GI tract, therefore their inhibition increases the tract susceptibility to damage, meaning that NSAID can frequently cause gastric mucosal injury with risk of peptic ulceration and enteropathy. (8, 14) In addition to the systemic effects of NSAID that comprise these PG downregulation mechanism, NSAID topical actions are also relevant for NSAID-induced GI toxicity. Local action includes the death of epithelial cells by the uncoupling of the oxidative phosphorylation, and the decrease of the protective epithelium mucosa through the reduction of mucus and bicarbonate secretion. Moreover, NSAID have been shown to disrupt the surface-active phospholipid layer on the surface of the gastric mucosa, facilitating the penetration of noxious agents on the gastric mucosa. (15)

NSAID cardiovascular (CV) side effects are also very relevant as they include myocardial infarction, hearth failure and hypertension. This can occur because the drugs may induce systemic vasoconstriction, particularly in patients with pre-existing heart failure. Importantly, the imbalance of the production of the prostanoids PGI_2 and TXA_2 may trigger the occurrence of CV toxicity. The first, produced by endothelial and smooth muscle cells, is an antithrombotic and vasodilator agent capable of modulating the interaction between activated platelets and the endovascular wall. In contrast, TXA_2 is a pro-thrombotic platelet agonist and vasoconstrictor biosynthesized in platelets through COX-1. (16)

Renal adverse effects, like renal ischemia or acute renal failure, may occur in more susceptible patients, as the release of PGI_2 and PGE_2 is increased in cases of renal dysfunction, despite the low basal values of PG synthesis rate. Furthermore, patients with hypertension often present the release of renal vasodilator PG to reduce renal ischemia; as NSAID inhibit this compensatory mechanism, a further elevation in blood pressure is likely to occur in response to the increase in both renal and systemic vascular resistance. (16)

Moreover, asthma attacks possibly caused by the lack of bronchodilating PG have also been verified in predisposed patients. (9) Less commonly, skin reactions, central nervous system effects, bone marrow disturbances and liver disorders can take place. (5)

In this context, researchers have been attempting to develop a safer and effective NSAID, meaning a drug capable of fighting inflammation and pain causing minimal toxicity. An overview of the explored strategies is presented in the next section.

1.1.5) Strategies to overcome NSAID toxicity

Being the NSAID toxicity a topic of growing concern, further mechanisms that could be responsible for causing side effects, besides the downregulation of PG, have been explored to develop safer treatments. As more insights on the biological actions of these anti-inflammatory drugs were gained, the creation of safer and more tolerable NSAID emerged through two distinct approaches: the modification of traditional NSAID and the development of novel drugs to reach new therapeutic targets.

1.1.5.1) Modification of conventional drugs

PC-NSAID: One of the first explored subjects concerning the modification of conventional NSAID was phosphatidylcholine (PC), an abundant and surface-active phospholipid in the gastric mucosa with which NSAID possess a strong ability to associate. Indeed, tradition NSAID were shown to alter the lipids hydrophobicity and fluidity (17), leading to the destabilization of the protective hydrophobic lining of the GI mucosa. (18, 19)

The pre-association of NSAID to PC was tested in order to verify if these interactions could be prevented, once the drugs binding site becomes unavailable to associate with the mucosa's phospholipids. In preliminary studies using rodent models, a protective gastric effect of diclofenac-DPPC (dipalmitoyl-phosphatidylcholine) complex was verified in the upper GI tract (20) and a superior anti-inflammatory and analgesic effect of ibuprofen-PC vs the unmodified drug was revealed – possibly due to the augmented half-life of the drug in the complexed form. (21) Later, various NSAID were evaluated and it was proven that, when compared to the equivalent unmodified NSAID: indomethacin-PC presents therapeutic effects and reduces GI injury (22); aspirin-PC (specifically PL2200, developed by PLx Pharma Inc.) can diminish gastroduodenal ulcers by 70%, while maintaining antiplatelet efficacy (23); ibuprofen-PC presents similar bioavailability but reduced gastroduodenal injury (significant for patients over 55 years). (21) Even though these drugs reveal promising results, only aspirin-PC has been FDA approved; other drug derivatives are still under clinical development.

CD-NSAID: Cyclodextrins (CD) are cyclic oligosaccharides produced from starch currently recognized as a relevant class of pharmaceutical excipients. Their solubility can be increased through chemical modification that originates, among others, hydroxy-propyl-β-CD derivatives. Due to their chemical structure (Table 1), they can include whole drug molecules within its cavity and consequently augment their solubility and availability. (24) For this reason, their application is relevant for poor soluble drugs, since NSAID tend to concentrate in its undissolved form in the gastric mucosa, eventually killing its epithelial cells

and inducing GI injury. (15, 18) So, NSAID complexation with CD allows the administration of drugs' lower dosages with greater solubility, which may prevent such toxicity.

A comparative study of ketoprofen and keptoprofen-β-CD exhibited a greater solubility, higher bioavailability and also higher anti-inflammatory action for the complexed form. (25) Similarly, evidences of increased anti-inflammatory effect were found for piroxicam and its inclusion in β-CD. This work also revealed lower gastric damage for the CD-piroxicam in animal models. (26) Nevertheless, a study in rats showed no differences in terms of renal and hepatic lesions when indomethacin was administered complexed with hydroxyl-propyl-β-CD, indicating that there is a similar GI absorption. (27)

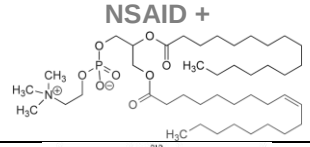
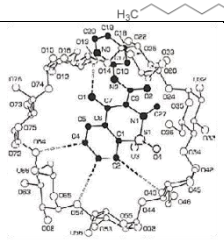
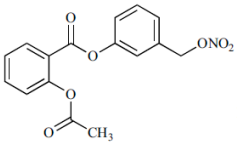
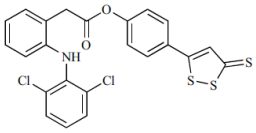
NO-NSAID: the incorporation of chemical moieties in NSAID, allowing the release of gastroprotective agents, has been considered as an approach to reduce GI damage. Nitric oxide-releasing NSAID (NO-NSAID) have been developed, since NO possesses gastric protection capacities that include the promotion of mucus secretion, the increase of mucosal blood flow and reduced adherence of neutrophils to the gastric vasculature endothelium. (28) NO-NSAID consist in a parent NSAID covalently linked to a spacer which, in turn, is associated to a NO-releasing moiety. Many different NO-NSAID have been synthetized since the concept emerged and their pharmacological and pharmacokinetic properties vary with the type of spacer present in the structure (aliphatic, aromatic or heterocyclic). As NO-NSAID are pro-drugs, they suffer a rapid cleaving after oral administration, resulting in the separation of the parent drug and the NO-releasing moiety attached to the spacer, followed by a progressive metabolism of NO and its slow liberation. (29, 30)

In the last decade, nitroaspirin (NCX-4016) was one of leading NO-NSAID (Table 1) and its investigation achieved studies in healthy humans. Although it exhibited low gastric toxicity and high potency in the inhibition of platelet activation in comparison to its parent drug, the discovery of mutagenicity of one of its main metabolites led to the arrest of its development. (31, 32) *In vivo* studies with NO-NSAID that couple a nitrooxybutyl moiety to the parental drug (diclofenac, aspirin and flurbiprofen) have been performed. Coherently, all show significantly less gastric mucosal injury following acute and chronic administration, while retaining comparable anti-inflammatory activity. (33, 34) Many diclofenac derivatives of this kind have also been studied in the past years. Two *in vivo* studies conducted in rats compared both the anti-inflammatory activity and the ulcerogenic effect of the NO-derivatives with diclofenac and a better gastric tolerability was verified for the novel compounds. (35, 36) Gastrosparing effects have been observed with NO-naproxen in both animals and humans. In fact, one drug, naproxcinod, has undergone short-term clinical studies as a potentially safe treatment option for OA patients. Still, it failed to be approved

since FDA acknowledged apprehensions about its CV safety, hence recommending additional long term studies concerning this matter. (37) Notwithstanding, since NO plays several vital roles in the CV system, including the maintenance of vascular integrity, drugs that release this compound are most likely to have cardioprotective effects. (38)

H_2S-NSAID: Hydrogen sulfide (H_2S) is a long-known compound but its biological effects have only recently been discovered. In fact, it plays an important physiological role acting as an endogenous anti-inflammatory agent, being generated in the gastric mucosa. It does not only protects the mucosa – promoting its integrity and repair and controlling its blood flow – but also regulates the adherence of leukocytes to the vascular endothelium. Besides, the reduction of H_2S generation has been shown to be a COX-independent mechanism of action of NSAID. For these reasons, this gaseous mediator presents itself as an attractive candidate to be used as a releasing group from NSAID. (39, 40)

Table 1 – Summary of the strategies employed with example of a novel compound chemical structure.

Drug complex	Chemical moiety	Chemical Structure	Features of drug complex vs parent drug
PC-NSAID	Phosphatidylcholine	NSAID + 	Therapeutic efficacy Reduced GI toxicity
CD-NSAID	β-Cyclodextrin	 CD-piroxicam	Increased drug solubility and bioavailability Augmented anti-inflammatory action
NO-releasing NSAID	Nitric oxide	 NCX-4016	Anti-inflammatory action Diminished gastric ulceration
H_2S-releasing NSAID	Hydrogen sulfide	 ATB-337	Anti-inflammatory action Higher gastrointestinal safety Lack of CV adverse effects

Pre-clinical studies carried on with H_2S-releasing NSAID (H_2S-NSAID) *per os* revealed promising results. For ATB-346 (H_2S-naproxen), experimental data showed similar anti-inflammatory activity to naproxen with reduced gastric damage, including the decrease of PGE_2 levels, but less gastric damage than the parent compound. (41, 42) Likewise, ATB-

337 (H_2S-diclofenac – Table 1), revealed similar results: PG synthesis was inhibited by both drugs, while dose-dependent haemorrhagic lesions were only found associated with the traditional NSAID. (43) Evidence for ATB-343 (H_2S-indomethacin) corroborates the GI safety of these drugs, as this novel compound significantly reduces both the gastric damage and the leukocyte adherence, in comparison to indomethacin alone. (44) Furthermore, the co-administration of the unconjugated parent drug with the specific H_2S-related group was performed to clarify the mechanism of action of these H_2S-releasing drugs. Interestingly, the gastric-sparing properties of these compounds were not found after their administration as separate entities. (42, 43) This may relate to the fact that the conjugated drug exhibits enhanced generation of H_2S. (43) Indeed, H_2S-releasing drugs present favourable features to be employed as substitutes of traditional NSAID. Nevertheless, their clinical development is still in a premature stage.

In sum, preliminary evidence supports that PC-NSAID, NO-NSAID and H_2S-NSAID are promising strategies reducing GI toxicity, while maintaining the drugs' therapeutic efficacy to inhibit pain and inflammation.

1.1.5.2) Novel drugs aiming at new targets

COX-2 selective inhibitors: the characterization of both COX isoforms led to the belief that the inhibition of COX-1 was responsible for NSAID toxicity, while the inhibition of COX-2 explained the drugs' therapeutic effects, since the latter is produced in inflammatory conditions. This line of thought was the rationale behind the development of coxibs and the idea that sparing gastric COX-1 inhibition had the potential to decrease gastric damage, while maintaining anti-inflammatory action, generated great enthusiasm. (5, 8) Hence, a fast and intense development of coxibs took place. Celecoxib and rofecoxib were the first to enter clinical trials in 1995 and, 4 years later, the first to reach the market. (10) Etoricoxib and lumiracoxib are part of the second generation of coxibs. Notably, these drugs had a great socio-economic impact as they generated high volumes of sales. (45)

Although these compounds effectively benefit over GI toxicity, they still interfere with some COX-2 dependent PG, namely PGI_2 which is cardioprotective. This explains the increased risk of CV disturbances for these drugs and why they are not indicated for patients with ischemic heart disease or cerebrovascular disease. (16) In 2004, rofecoxib was voluntarily withdrawn from the market, after the observation of significant incidence of CV hazards. Later, the sale of lumiracoxib and valdecoxib was also suspended due to their hepatic and dermatological toxicity. (12) Yet, as no increased risk of CV thrombotic events

was evident for celecoxib and etoricoxib, they remain commercialized and are valuable options for patients with pre-existing gastric damage.

Dual COX-LOX inhibitors: Leukotrienes (LT), a paracrine hormone that act as complementary mediators of inflammation, is metabolized in the arachidonic acid pathway by the lipoxygenase (LOX) enzyme (Figure 4). (46) LOX are lipid peroxidising enzymes that work as dioxygenases. Three known isozymes belong to this group: 5-, 12- and 15-LOX. The best characterized and thought to be the most biologically relevant is 5-LOX. (47) Taking in consideration the pro-inflammatory profile of LT and prostanoids, it is expected that a drug capable of inhibiting the generation of both types of molecules will not only present fewer side effects but also an improved anti-inflammatory activity. (47, 48) Numerous and diverse molecules have been synthetized to act as dual COX/LOX inhibitors over the years. Some of the studied classes of compounds include pyrazolines, reported to have moderate to good *in vitro* inhibiting activity (49); derivatives of phenylpropenoic acids, from which the synthetic analogue NNU-hpda (2-(4-hydroxylphenyl)-3-(3,5-dihydroxylphenyl)pro-penoic acid) stands out for having a potent inhibitory effects on the production of PGE_2 and LTB_4 and presenting a better toxicity profile than most NSAID (50); ASA analogues, which were found to be more potent anti-inflammatory agents than aspirin and both were devoid of any gastric ulcerogenicity (51); rofecoxib derivatives, from which two furanones with optimal COX and LOX inhibition presented higher anti-inflammatory activity than the reference LOX inhibitors and moderate analgesic activity. (52)

Licofelone, another novel dual COX/LOX inhibitor agent, presented note-worthy clinical results. Vidal *et al.* showed the compound's *in vivo* ability to inhibit both COX-2 and LOX-5 protein expression in vascular lesions, attenuate PGE_2 and inhibit LTB_4 generation in neutrophils. (53) Relatively to indomethacin, licofelone demonstrated a longer duration of action, higher effectiveness, reduced gastric ulceration and prevention of NSAID-related increase of LT levels. (54) When assessed as a treatment for OA, the drug proved similar effectiveness as naproxen. (55) Currently, phase III trials have been successfully completed for licofelone. Results revealed comparable efficacy to traditional NSAID and general tolerability (56), avoiding the downside of coxibs. (53) Hence, while many dual COX/LOX inhibitors are in early development stages, licofelone gathers all the conditions to be commercialized in a near future.

mPGES inhibitors: the microsomal prostaglandin E synthase 1 (mPGES-1) acts down the COX enzyme in the prostanoid biosynthesis pathway (Figure 4), converting PGH_2 into PGE_2, which is the most important PG involved in inflammation, fever and pain. mPGES-1 is induced by pro-inflammatory cytokines and growth factors. What makes this target better than the COX enzyme is the fact that it is downstream in the pathway,

suppressing only PGE_2 and no other physiologically important prostanoids, thus avoiding adverse effects. This synthase has been proposed as a drug target in 1999, but the process of bringing drugs to the market has been very slow. (57)

Nevertheless, potent compounds with good selectivity have been recently reported. (58-60) Some demonstrated a reduction in the biosynthesis of PGE_2 both *in vitro* (59, 60) and *in vivo* (59), due to the upregulation of PGI_2 and PGF_2. The most potent studied compound (PF-4693627) was found to be orally active and assays using naproxen as a reference revealed a good combination of *in vitro* cell potency, pharmacokinetic, *in vivo* efficacy (using the air pouch model of inflammation) in addition to an attractive synthesis. For these reasons, it was selected as a clinical candidate for the treatment of inflammation caused by OA and RA. (58) Presently, the most advanced research belongs to Glenmark Pharmaceuticals Ltd. that has completed preclinical studies and filed for Phase 1 human trials with the molecule GRC 27864. (61) This is a potent, selective, and orally bioavailable mPGES-1 inhibitor, currently developed in the pursuance of an effective and safe therapy for chronic inflammatory disorders, such as RA and OA. (62)

Anti-TNF agents: other anti-inflammatory agents have been explored as potential solutions to relief the burden of chronic inflammatory diseases. Since TNF-α is one of the most relevant pro-inflammatory agents, it has been indicated as a target for novel therapeutics. Actually, some protein bases injectables, like etanercept, infliximab and adalimumab, have already been successfully introduced in the market – all of them are anti-TNF-α monoclonal antibodies that ground their effects on the scavenging of the cytokine. (6) Recently, an oral drug that binds and neutralizes TNF (AVX-470) has been approved as safe and tolerable under clinical trials for a long-term treatment of patients with active ulcerative colitis. (63) One study indicates that anti-TNF-α therapy reduces small bowel damage and have a gastroprotective effect, although a wound-healing effect remains undefined. (64) Overall, anti-TNF agents are strong candidates to replace NSAID, particularly in more vulnerable patients.

Glucosamine hydrochloride: glucosamine is relevant for the alleviation of arthritic symptoms, since it is a glycosaminoglycan precursor – one of the major components of cartilage. Glucosamine hydrochloride (GS-HCl) has revealed the ability to inhibit COX-2, possibly by hampering N-glycosylation or enabling the protein turnover during translation. (65, 66) Still, few works prove its efficacy for the treatment of inflammation. In 2013, i*n vitro* and *in vivo* experiments assessed the effects of GS-HCl alone, indomethacin alone and the conjugation of both. The decrease of TNF-α stimulated by COX-2, the anti-arthritic efficacy and gastric sparing effects were all significantly superior when both compounds were

administrated. (66) Briefly, GS-HCl presents an interesting strategy but still has a long way to go to be viewed as a possible alternative to traditional NSAID.

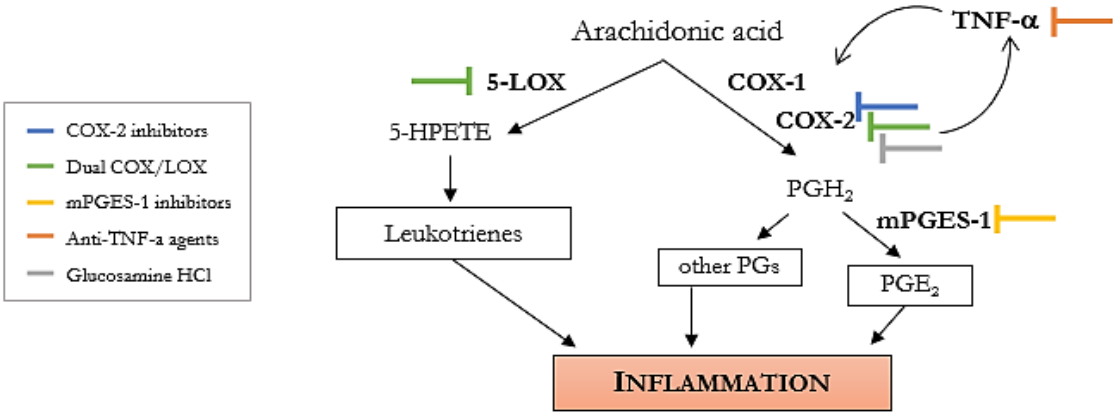


Figure 4 – Inflammation induction by the arachidonic acid pathway (ILT and PG). The targets inhibited by novel drugs are indicated in the scheme.

In conclusion, the development of coxibs did not solve the NSAID toxicity issue, but contributed for the intensification of the research for safer drugs. Among novel molecules under investigation, the dual COX/LOX inhibitor licofelone has proven to be the most likely to reach the market in first place, as well as the mPGES-1 inhibitor from Glenmark Pharmaceutical Ltd.

1.2) NSAID-membrane interactions

1.2.1) The importance of drug-membrane interactions

The analysis of drug-membrane interactions has emerged as a branch of the study of NSAID mechanisms of action. Although the hampering of the prostanoid biosynthesis is a crucial action of NSAID, it does not fully explain their pharmacologic and toxic effects. Lichtenberger *et al.* were the first to suggest that both beneficial and deleterious effects of NSAID could arise from its interaction with membranes, in particular with phospholipids. (67)

In fact, there are several reasons that rationalise the importance of these interactions. First of all, after entering the organism drugs have to be absorbed, distributed, metabolized and even eliminated, and these steps require the interaction with biological membranes. These processes are especially relevant for NSAID since they have an intracellular and membrane target – COX enzymes can be found at the endoplasmic reticulum or by the nuclear envelope (11) – and also because they are frequently administered orally. Secondly, although pharmacological actions may occur through the

association to proteins (e.g. receptors, transporters or enzymes), the relationship between drugs and lipids may induce biophysical alterations of the membranes that in turn modify protein conformation and alter their activity. In other words, by influencing the membranes' structure, drugs may indirectly modulate protein location and function and ultimately change cell signalling and gene expression. In this context, lipids have gained more attention and the recognition of their central role in cellular events and certain pathologies has led them to be considered novel drug targets. (68)

As the behaviour of drugs in membranes is highly conditioned by their physicochemical properties, for instance their lipophilicity and pK_a, NSAID will presumably have a strong association with membranes since they are mostly weak acids and amphiphilic molecules. (68, 69) On the other hand, drug-membrane interactions also influence the drug itself by altering their diffusion, binding and conformation, which can culminate in selectivity, efficacy and toxicity changes. (70) In fact, studies with analgesic drugs have demonstrated that interactions with lipids can influence a drug's pharmacokinetic properties and efficacy. (71) Additionally, NSAID-membranes studies have revealed that these interactions can be involved with both GI and CV side effects, through their topical action on the gastric mucosa and their role in the development of several CV pathologies. (15, 72)

In sum, drug-membrane interactions can influence both membrane and bioactive molecules properties. So, the study of these interactions is necessary to understand the drugs bioavailability, entrance into cellular compartments, efficacy and to contribute to the knowledge of drug-induced toxicity mechanisms.

1.2.2) Biological membranes and cell membrane models

Biological membranes have a dynamic nature and a complex composition. Many fundamental physiological events take place within cell membranes, such as signalling, transport, fusion and fission. They act as a selective barrier, protecting the cell from the external environment, and defining the cell's boundaries. (68, 73) Their composition consists in a lipid bilayer with linked proteins and carbohydrates. Since they possess a highly hydrophobic core and a hydrophilic surface due to the amphipathic nature of the lipids, membranes are considered impermeable barriers. In fact, lipids are fundamental for the dynamics of the bilayer, besides serving as scaffolds for membrane proteins and regulating signal transduction. (74) The main lipid constituents of eukaryotic cell membranes are glycerophospholipids, such as PC, phospatidylethanolamine (PE) and phosphatidylserine (PS). Among these, PC is the most abundant representing over 50% of

all phospholipids (75) and for that reason it is frequently selected to mimic cell membranes; nevertheless, their composition varies between different cell types.

The originally proposed fluid mosaic model for the plasma membrane structure, which proposed that membranes consisted of dynamic and fluid lipid bilayers with embedded proteins (76), has been revisited. The idea of homogeneous membranes has been replaced by fluid bilayers with rigid microdomains, frequently referred to as lipid rafts, explained by the great variety of lipids present in biological membranes. This heterogeneity is essential to maintain all membrane functions. (74) Overall, the cell membrane determines a balanced environment and, therefore, any modification of its structure or properties by a bioactive molecule may affect its functions and integrity. (75)

Due to their heterogeneity, biological membranes are considered very complex structures. Since this reality is very difficult to investigate at the biophysical level, simplified artificial membrane model systems that mimic the natural phospholipid bilayer are fundamental to study drug-membrane interactions. (68, 75) The use of protein-free lipid membranes, such as micelles, liposomes and lipid monolayers (Figure 5) – the most widely used models – allows an easier manipulation of the membrane lipid properties and to focus on the interaction between the drug in study and the membrane lipids. (69) Micelles consist in a hydrocarbon core and a polar surface with close hydrophilic groups. Besides having a simple preparation, their surface and hydrophobicity are easily manipulated. Liposomes are closed spherical vesicles with an internal aqueous compartment that consist in one or more lipid bilayers. On the other hand, monolayers are formed by amphipathic molecules that spontaneously orientate themselves in an interface (e.g. air/water), resulting in a planar geometry. (71)

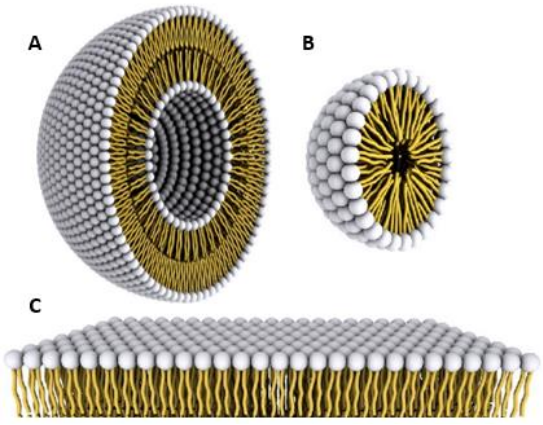


Figure 5 – Model membrane systems: liposomes (A), micelles (B) and monolayers (C).

Biophysical techniques are very useful to understand the processes occurring in the membrane, once they enable the assessment of membrane responses to drugs' presence.

There is a large amount of techniques that complement each other for the elucidation of biological questions as they provide a detailed image of molecular events. (75)

1.2.3) Studies with NSAID

Until now, several studies concerning NSAID interaction with membranes have took place, as reviewed by Pereira-Leite *et al*. (68) These works include *in vitro* studies with the above-mentioned membrane model systems, *in vitro* studies with cell cultures and molecular dynamics (MD) simulations.

1.2.3.1) Common models and techniques

The full evaluation of a drug interaction with membranes is only possible when several complementary techniques are employed. Liposomes are the most widely used membrane model systems for these *in vitro* studies and different types of liposomes are advantageous for the application of specific techniques. For assays that require a high amount of sample material, such as X-ray diffraction, differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR), multilamellar vesicles (MLV) are beneficial. However, large unilamellar vesicles (LUV) are more often chosen to assess drug-membrane interactions for having an identical curvature of cell membranes and being a homogeneous sample regarding vesicles size. The determination of NSAID partition coefficient (K_p) has been done by derivative spectrophotometry, fluorescence quenching or electrophoretic light scattering. Fluorescence experiments have also been useful for studying many other parameters: the drug membrane location was assessed by fluorescence quenching and fluorescence resonance energy transfer; NSAID effect on membrane properties was studied through fluorescence anisotropy; and the influence of NSAID on membrane permeability was investigated by performing fluorescence intensity measurements. The temperature and thermodynamic parameters of the lipid's phase transition was assessed by calorimetric techniques, namely DSC and isothermal titration calorimetry. Methods like the Fourier transform infrared spectroscopy (FITR), Raman spectroscopy, NMR and X-ray diffraction have allowed the evaluation of the NSAID disturbing effect on the phospholipidic bilayer dynamics and structure. (68)

1.2.3.2) Main conclusions and significance for therapeutic and toxic effects

To primarily understand NSAID-membranes interactions, their lipophilicity and hydrophobicity are determined by the study of the drug partition. The K_p is generally

expressed as “logD” and has been established to be between 2.0 and 4.5 at physiological pH for NSAID. This indicates a good bioavailability and places NSAID in the class II drugs (lipophilic with low solubility and high permeability). (77, 78) When considering NSAID transportation across cellular membranes, it is important to contemplate both absorption and permeation steps, seen that these drugs are mainly driven by passive diffusion. These processes are not only affected by the drugs’ lipophilicity but also by their charge. NSAID adopt different ionization states at different pH because they are mostly weak acids. Hence, their behaviour differs according to the surrounding microenvironment as demonstrated by partitioning studies. While drugs with pK_a < physiological pH (e.g. indomethacin and acemetacin) have a superior K_p for lower pH (79, 80), drugs with pK_a > physiological pH (e.g. celecoxib) show no significant differences for partitioning at different pH (81). Furthermore, NSAID that are negatively charged tend to interact with the phospholipid headgroups, as seen with clonixin, tolmetin and diclofenac (82-84), and drugs in the protonated form are able to further penetrate the lipids acyl chains, which has been verified for indomethacin, meloxicam and piroxicam at pH 5.0 (85).

Regarding the drugs’ ability to induce alterations in membrane fluidity and order, several studies confirm this hypothesis. In fact, both the lipids’ pre-transition and the main transition were found to be affected by acemetacin (86), celecoxib (81), ibuprofen (87), naproxen (88) and piroxicam (85), among others, through the decrease of the transitions’ temperature and cooperativity. The disruption of the bilayers structural organization at different physical states has also been reported. For instance, indomethacin modifies membrane heterogeneity (89), which may lead to changes in both protein location and cell function, and oxicams-induced membrane fusion. These disordering effects may some explain NSAID beneficial effects, including their antioxidant (90, 91) and antitumoral (92, 93) activity. Indeed, the permeability increase of mitochondrial membrane in presence of piroxicam can ultimately cause apoptosis, which may be on the basis of its antitumoral activity. (94) Additionally, the inhibition of phospholipase A_2 activity by NSAID has been demonstrated to occur not only by direct interaction with the enzyme but also through the alterations of the membrane structure. (95, 96)

In contrast, the induction of changes in membranes’ fluidity, permeability and biomechanical properties, as verified by both *in vitro* and *in vivo* studies, are related to the prejudicial action of indomethacin and naproxen on the GI mucosa hydrophobicity and the NSAID-induced disruption of the gastric surface-active phospholipids protective layer. (97) The anti-inflammatory agents’ cytotoxicity is also explained by their ability to increase membrane permeability. (98) Furthermore, naproxen, ibuprofen and diclofenac were shown to alter the structure of erythrocytes membranes, which possibly explains CV side effects.

(88, 99, 100) Also, mitochondrial impairment can be seen as a general toxicity mechanism of NSAID. For instance, it can relate to GI topical damage since NSAID are uncouplers of oxidative phosphorylation leading to epithelial cell death of the GI mucosa or it can justify their hepatic and renal toxicity. (101, 102) In particular, nimesulide was found to be distributed across the mitochondrial membrane, interfering with its structure and compromising fundamental processes, such as permeability, protein/lipid distribution and proteins' activity. (103)

The research applied to cell cultures, although less extensively, has corroborated the results obtained with the model systems, thus validating them. (68) Finally, it was possible to obtain a molecular description of the NSAID-membrane interactions and assess the drug location using MD simulations. This field already allowed the evaluation of some NSAID partition and provided indications of drugs' effects dependent of their ionization state. (104-106)

1.3) Motivation and aim

1.3.1) Diclofenac

Diclofenac, 2-[(2,6-dichlorophenyl)amino]phenylacetate (Figure 6), is a widely commercialized NSAID due to its anti-inflammatory, analgesic and antipyretic properties. This phenylacetic acid derivative is a nonselective COX inhibitor and similarly to other NSAID, it leads to the occurrence of GI, CV, renal and hepatic adverse effects. (8, 107, 108) This drug can be found in diverse formulations, including ophthalmic preparations, topical gels, tablets, rectal suppositories and even for intramuscular administration. (8) Its topical application has a potent analgesic action and is thought to minimize toxicity (109); on the other hand, its consumption *per os*, one of the most generally used, is the most concerning in terms of side effects.

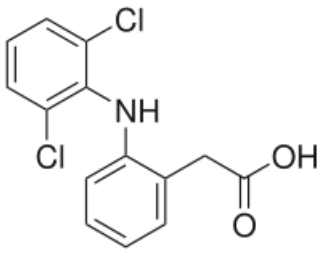


Figure 6 – Diclofenac chemical structure.

Regarding its pharmacokinetics, diclofenac is almost completely absorbed when administered orally but after suffering the first-pass effect only about 50% reaches the systemic circulation in the unchanged form. At therapeutic concentrations, this NSAID is over 99% bound to plasma proteins and its plasma half-life is between 1 to 2 hours. The drug can be metabolized into different compounds, including 4'-hydroxydiclofenac (major metabolite), 5-hydroxydiclofenac, 3'-hydroxydiclofenac and 4',5-dihydroxydiclofenac. Its excretion occurs in the form of glucuronide and sulphate conjugates through urine and bile and less than 1% is excreted in the unchanged form. (110)

Concerning the drug's therapeutic effects, diclofenac has been proven to be effective in treating OA and RA (111, 112) and a meta-analysis supports that diclofenac should remain as a reference medication for OA treatment, since therapeutic doses provide a similar efficacy to comparators, which include coxibs, meloxicam, nimesulide and etodolac. (113) Its analgesic properties have been confirmed in a trial of acute pain in children. (114) Nevertheless, diclofenac's adverse effects have also been observed in several trials. The MEDAL (Multinational Etoricoxib and Diclofenac Arthritis Long-term) program revealed that etoricoxib has a similar behaviour to the traditional NSAID concerning the incidence of gastroduodenal ulcers (115); yet diclofenac-induced GI damage is more prevalent than coxibs-induced GI toxicity. (111, 112, 115) CV risk was also demonstrated to be relevant for diclofenac. (115) Moreover, diclofenac chronic users commonly presents elevated aminotransferase values, despite the low rates of hospitalization due to clinical liver events. (116) The assessment of the hepatic safety of celecoxib versus three traditional NSAID showed that diclofenac presented the most hepatobiliary and serious hepatic adverse effects, although in low percentages. (117)

1.3.2) Diclofenac-membrane interactions

Some studies on diclofenac's interaction with membranes are already available in the literature. In general, these works have focused on the diclofenac location within the membrane and on its effect in some membrane biophysical properties, such as membrane fluidity (Table 2).

Firstly, the assessment of the drug partition in egg yolk phosphatidylcholine (EPC) was performed at pH 7.4 by 3 different techniques. All revealed concordant results, resulting in a K_p of around 1200 ± 100 M^{-1}. At pH 3.0 the obtained K_p was of 26000 ± 3000 M^{-1}, which is explained by the fact that the drug's is below its pK_a (3.97) and hence it is in its protonated form. The relevance of the drug ionization state is also notable for zeta-potential

measurements, where increasing concentrations of diclofenac resulted in a decrease of the membrane potential at pH 7.4, but induce no alterations at pH 3.0. (84)

The investigation of molecular interactions between diclofenac and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) liposomes using several spectroscopic and thermodynamic techniques revealed that the drug locates preferentially in the polar headgroups of the phospholipids, indicating a closer proximity to the phosphate region. (118) Diclofenac's location at the membrane surface was also studied by Ferreira *et al.*, who proposed that the drug was exposed to electrostatic adsorption with the zwitterionic headgroups of phosphatidylcholine, and sustained these results with the zeta-potential evidence mentioned previously. (84) Suwalsky *et al.* used both DMPC and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) liposomes as membrane model systems for similar purposes and only verified relevant interactions of diclofenac with DMPC. This work supported that the lipid hydration allows the insertion of diclofenac into the bilayer which leads to perturbations on DMPC structure. (99) So, gathered evidence indicates that the drug's amphiphilic nature results in electrostatic interactions of its negatively charged group with the positively charged phosphatidylcholine terminal, while its non-polar side remains in the neighbourhood of the hydrophobic acyl region of the lipid.

Table 2 – Summary of diclofenac-membrane studies (selected models, applied techniques and main study conclusions).

Model	Techniques	Main Conclusions	Ref.
EPC	Spectrophotometry Fluorescence anisotropy Zeta-potential	- Higher partition at low pH - ↓ Zeta-potential at pH 7.4 and no alterations at pH 3.0 - Drug location at membrane surface - ↑ of membrane rigidity	(84)
DMPC, DMPE, human erythrocytes	X-ray diffraction, SEM	- ↑ of membrane rigidity at lipid fluid phase of model membranes - Alterations of erythrocytes morphology	(99)
DMPC	FTIR-ATR, DSC, ITC, FRET	- Drug located near polar headgroups of phospholipids.	(118)
Mouse splenocytes	Fluorescence anisotropy	- No evidence of diclofenac-induced membrane perturbation	(119)

The conclusions retrieved from the drug location assays contributed for the comprehension of results concerning the alteration of the membrane fluidity. Ferreira *et al.*

observed membrane properties alterations in the headgroups region and an increase of rigidity of EPC, which is clarified by the interaction of the drug's carboxyl groups. (84) Similar results were seen by Suwalsky *et al.* when assessing the influence of diclofenac in the fluid phase of DMPC. (99) Another study showed that diclofenac lowers the lipid main phase transition temperature (T_m) probably due to the modification of the lipid headgroups' hydration that results in changes of this region's packing density. (118) A work that resourced to splenocyte membranes also studied NSAID influence in membrane structure, but under the utilized conditions there was no evidence of diclofenac-induced membrane alteration. (119)

Aiming to contribute for the understanding of diclofenac's perturbations in cell membranes, human erythrocytes were also used as a membrane model systems. SEM visualizations revealed morphological alterations with increasing concentrations of diclofenac. Presumably, this diclofenac action can contribute for its toxicity mechanism by reducing blood flow, losing oxygen and ultimately causing tissue damage. (99)

1.3.3) Project aim

There is ongoing research that aims at launching safe NSAID. However, the accomplishment of such goal implies a deeper knowledge on the NSAID mode of action and, in that sense, evidence supports that the interplay between the drugs and lipid membranes is a mechanism that is worth exploring. Although some novel drugs are currently under development, understanding how classical NSAID work is one of the key factors for a successful analysis of novel molecules.

Diclofenac is one of the most consumed NSAID worldwide, yet it presents some concerning unwanted effects. The information currently gathered in the literature concerning diclofenac's interaction with membranes essentially describes its effect at physiological pH (solely K_p and zeta-potential have been determined at pH 3.0). Even though they provide important data on the drug location in the membrane and its influence on the lipid bilayer transition phase and order parameters, they do not fully explain diclofenac's behaviour in the organism. Every NSAID is found in acidic environments, including inflamed cells and the GI tract. Therefore, the study of the diclofenac's actions in such conditions is fundamental to better understand the causes of both its therapeutic and toxic effects.

For that reason, the goal of the present work is to study diclofenac's effect in membrane biophysical properties in 3 different pH conditions – physiological (7.4), inflamed cells (pH 5.0) and gastric mucosa environment (3.0). Since PC is the most abundant

phospholipid of the cell membranes, DMPC liposomes were chosen as membrane model system.

Specifically, this project intends to:

- Evaluate diclofenac's partition in DMPC liposomes by derivative spectrophotometry;
- Understand diclofenac's binding mechanism to the membrane surface through fluorescence measurements;
- Assess diclofenac's location within the phospholipid bilayer by fluorescence quenching;
- Characterize diclofenac influence on the lipid phase transition and fluidity by performing fluorescence anisotropy measurements;
- Quantify the effect of diclofenac on liposomes permeability through a membrane leakage assay;
- Discuss the influence of medium pH on the interaction of diclofenac with biological membranes;
- Discuss the relevance of diclofenac's effects on membrane biophysical properties for its therapeutic and toxic actions and for the rational development of safer drugs.

2. Materials and Methods

2.1) Reagents

Diclofenac sodium salt was purchased from Sigma-Aldrich (St. Louis, USA). 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was supplied by Avanti Polar-Lipids Inc. (Alabaster, USA) and L-α-phosphatidylcholine from egg yolk (EPC) by Sigma-Aldrich (St. Louis, USA). The fluorescence probes – TMA-DPH (1-(4-Trimethylammoniumphenyl)-6-Phenyl-1,3,5-Hexatrienep-Toluenesulfonate), DPH (1,6-Diphenyl-1,3,5-Hexatriene) and ANS (1-Anilinonaphthalene-8-Sulfonic Acid) – were supplied by Invitrogen probes (Paisley, UK). Sephadex G-25 Medium, 5(6)-Carboxyfluorescein and polidocanol were purchased from Sigma-Aldrich (St. Louis, USA). Dimethyl sulfoxide (DMSO) and Ludox (colloidal silica) were both obtained from Sigma-Aldrich (St. Louis, USA). Perchloric acid 70 % from Riedel-de Haën (Seezle, Germany), L-ascorbic acid and monopotassium phosphate from Sigma-Aldrich (St. Louis, USA) and ammonium molybdate from BDH Laboratory Supplies (Poole, UK) were used.

All experiments were performed under three pH conditions with adjusted ionic strength (0.1 M NaCl). Tris HCl buffer (pH 7.4, 0.01 M) was prepared from Trizma base supplied by Riedel-de Haën (Seezle, Germany), Acetate buffer (pH 5.0, 0.01 M) from acetic acid and Formate buffer (pH 3.0, 0.01 M) from formic acid, both purchased from Sigma-Aldrich (St. Louis, USA). In all cases, pH was adjusted with NaOH or HCl solutions.

2.2) Drug solution preparation

Diclofenac's solubility in water depends on its ionization state (pK_a = 3.97 (84)), being smaller at more acidic environments, when the drug molecule is protonated and neutral. Therefore, solubility tests were performed to determine the smallest amount of DMSO necessary to assure diclofenac's solubilisation with Formate buffer. These studies showed that DMSO 2% (V/V) was enough to obtain diclofenac solutions (0-40 μM) at pH 3.0. To maintain identical conditions for all assays, every sample was prepared with DMSO 2% (V/V) regardless the pH in study. Despite DMSO ability to alter liposomes properties, the amount used in these studies does not significantly modify the biophysical properties of DMPC liposomes. (120)

2.3) Liposomes preparation

Liposomes are round-shaped artificial vesicles formed by lipid bilayers that contain an aqueous compartment. Their lipid composition (consisting mainly of phospholipids), size and surface charge can vary. Liposomes are classified according to their number of bilayers in multilamellar vesicles (MLV) and unilamellar vesicles, being the latter categorized according to their size in small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and giant unilamellar vesicles (GUV) (Table 3). Liposomes are extensively used as membrane models, since they allow mimicking cellular membranes and are easily prepared through different methods, including sonication, extrusion, high-pressure homogenization and detergent dialysis. In this work, LUV were the chosen membrane model system due to their simple preparation method and well-characterized membrane properties, such as their size, stability and curvature. (121)

Table 3 – Liposome size classification.

VESICLE TYPE	DIAMETER SIZE
Small unilamellar vesicles (SUV)	20 – 100 nm
Large unilamellar vesicles (LUV)	> 100 nm
Giant unilamellar vesicles (GUV)	1 - 100 μm
Multilamellar vesicles (MLV)	> 500 nm

Here, LUV were prepared by the thin film hydration method (Figure 7). Firstly, DMPC was dissolved in a mixture of chloroform and methanol (3:2, V/V). The organic solvents were then evaporated under a nitrogen stream in a rotary evaporator (Buchi R-210 rotavapor equipped with a heating bath BuchiB-491) at 40 ºC for 20 minutes. The lipid film formed in a round bottom flask was hydrated with buffer for 30 minutes at 40 ºC. Throughout this time, the dispersion was vortexed generating MLV. Finally, to obtain LUV of a specific size, extrusion was performed in a Whitey extruder at 40 ºC using a membrane with a 100 nm diameter pore size (Whatman® Nuclepore Track-Etched Membrane). Vesicles were extruded 15 times to reach the desired size.

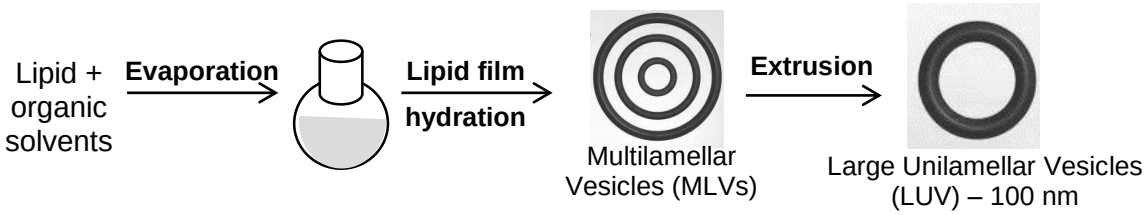


Figure 7 – Liposome preparation by the film hydration method

2.4) Drug partition

Determining the partition coefficient (K_p) is a simple method to initially understand the drug behaviour in the presence of a lipid membrane. This parameter provides information on how a given drug distributes between lipid and aqueous phases. Although it gives no indication on the solute location in the membrane, it supports the prediction of the drug's pharmacokinetics and pharmacodynamics features and helps to understand its toxic and therapeutic profile. (122)

To estimate the K_p, a partition equilibrium between the lipid and aqueous phases must be attained and the drug's concentration is determined in one or both the phases. (123) The K_p is in fact an equilibrium constant given by the ratio of the drug concentration in the lipid and aqueous phases (Equation 1):

$$K_{p,x} = \frac{[drug]_{lipid}}{[drug]_{water}} = \frac{\frac{n_{S,L}}{n_L+n_{S,L}}}{\frac{n_{S,A}}{n_A+n_{S,A}}} \approx \frac{\frac{n_{S,L}}{n_L}}{\frac{n_{S,A}}{n_A}} \qquad \text{Equation 1}$$

where n_A and n_L represent the moles of the aqueous and the lipid phases, respectively, and $n_{S,X}$ are the moles of solute in each phase. The expression is simplified due to negligible amounts of solute versus the amount of aqueous phase ($n_A >>> n_{S,A}$) and because the membrane should not be overloaded with solute ($n_L >>> n_{S,L}$). Also, the lipid and aqueous phases amounts are commonly represented by their volumes instead of moles. (123)

The K_p can be determined through several techniques involving or not the physical separation of free and "membrane-bound" molecules. Membrane filtration, centrifugation, equilibrium dialysis and chromatography are the main approaches used to directly determine K_p through the separation of lipid and aqueous phases and subsequent quantification of the solute amount in the lipid and/or aqueous phase(s). The main disadvantages of these strategies are the incomplete separation of phases and the equilibrium perturbation. Spectroscopic techniques, including UV-Vis absorption spectrophotometry, fluorescence spectroscopy and electron paramagnetic resonance, as well as titration calorimetry and zeta potential measurements, have been also used to

determine K_p without the separation of lipid and aqueous phases. In these cases, experimental measurements are performed with several lipid concentrations and the retrieved data are a combination of the free and bound molecules signals. These data usually follows a hyperbolic-like dependence on lipid concentration, which is fitted to an equation from which the K_p value is calculated. (122, 123)

2.4.1) Derivative spectrophotometry

In this work, the selected approach to determined K_p was derivative spectrophotometry, since methods without phases separation are less laborious and avoid the risk of disturbing the partition equilibrium. Spectrophotometry is quite sensitive, meaning there is no need to use high solute concentrations that could eventually lead to the drug insolubilization.

Considering the application of the Lambert-Beer law and the definition of K_p (Equation 1), the absorbance of a given sample containing a drug and both a lipid and aqueous phases can be described by:

$$Abs_T = Abs_w + \frac{(Abs_l - Abs_w)K_P[L]V_\varphi}{1 + K_P[L]V_\varphi} \qquad \text{Equation 2}$$

Where Abs_T, Abs_w and Abs_l correspond to the drug absorbance of the total, the aqueous and the lipid phases, respectively. [L] represents the lipid concentration (mol/L) and V_φ is the lipid molar volume (L/mol). (122)

The high background signals caused by the presence of LUV prevent the direct application of Equation 2 to determine K_p and thus, derivative spectophotometry is applied. This method allows the elimination of the background interference of the medium and the obtainment of spectra with a better resolution of overlapping bands through the calculation of the second or third derivatives of the absorption spectra with respect to the wavelength. In this case, the estimation of K_p is given by an equation similar to Equation 2:

$$D_T = D_w + \frac{(D_l - D_w)K_P[L]V_\varphi}{1 + K_P[L]V_\varphi} \qquad \text{Equation 3}$$

where D_T, D_w and D_l correspond to the derivative intensity obtained from the absorbance of the total amount of drug, the drug distributed on the aqueous phase and on the lipid phase, respectively. [L] represents the lipid concentration (mol/L) and V_φ is the lipid molar volume (L/mol). To determine diclofenac's K_p, samples with increasing concentrations of LUV (0-1000 μM) and a fixed concentration of drug (40 μM) were prepared and incubated for 30 min at 37 ºC to attain equilibrium. After incubation, samples were transferred to a 96-

well plate and their the absorbance spectrum was traced from 230 to 400 nm, also at 37 ºC, in a Synergy HT plate reader (Biotek Instruments, Inc.).

K_p calculator, an Excel software® developed by Magalhães *et al.* (122) and Origin Pro® were used to mathematically analyse the data and estimate the partition coefficient. *K_p calculator* avoids a time consuming process to obtain and evaluate all of the obtained spectra, allowing a quick and complete data analysis. This application automatically retrieves the absorbance spectra of the experimental results, along with the first three derivatives, when inserting the experimental data (absorbance values in a range of wavelengths for all samples). Also, it presents the maximum and minimum values of a given selected interval of the derivatives spectra, which are used for the fitting to Equation 3 to calculate K_p.

2.5) Drug membrane binding

A possible mechanism of drug interaction with the membrane is its binding to the phospholipid headgroups. This type of association can provide some indications of what is happening at the first contact of the drug with cell membranes. To study drug-membrane binding, an anionic fluorophore that is capable of sensing the polarity of bio-mimicking and biological environments was used. ANS is a fluorescent probe whose fluorescence quantum yield and wavelength of maximum emission depend on the surrounding microenvironment. (124) Although the probe does not fluoresce in water, its binding to DMPC liposomes strongly increases the fluorescent intensity. (125) So, a fluorescence signal decrease will be translated as an increase of the drug binding due to the competition between the drug and the probe for the liposome surface.

2.5.1) Steady-state fluorescence measurements

ANS stock solution was prepared in DMSO at high concentration so that the highest percentage of DMSO in the samples would not exceed the previously established 2%. Samples with a fixed concentration of DMPC (500 μM) and 3 concentrations of diclofenac (0, 40 and 80 μM) were prepared. Before adding the probe, samples were incubated at 37 ºC for 30 min. Then, steady-state fluorescence emission spectra were measured on a Jasco FP-6500 spectrofluorometer (Great Dunmow, UK) using the parameters indicated in Table 4. The measurements were executed at 37 ºC after consecutive additions of the probe in order to assess several ANS concentrations (0 – 65 μM).

Table 4 – Parameters used in steady-state fluorescence for the membrane-binding assay.

Probe	**ANS**
Temperature	37 ºC
λ_{ex}	377 nm
λ_{em}	400-600 nm
Gain	330 V
Slits	5 nm

The maximum fluorescence intensity registered for each sample with increasing concentrations of ANS was plotted against the respective ANS concentrations. Then, the fitting to Equation 4 was made to retrieve the constant binding (K), the cooperativity of the binding process (b) and the maximum fluorescence intensity (C_{max}).

$$[ANS]_B = C_{max}\frac{(K[ANS]_\infty)^b}{1+(K[ANS]_\infty)^b} \qquad \text{Equation 4}$$

$[ANS]_B$ represents the amount of bound-ANS and $[ANS]_\infty$ the total amount of ANS.

2.6) Drug membrane location

The determination of the drug location within the membrane is relevant to further understand the drug mode of action. Fluorescence quenching is an indirect method to evaluate this, since it allows the assessment of the drug location by the inclusion of a foreign compound (fluorescent probe) in the membrane. Importantly, for this type of study, steady-state fluorescence assays must be complemented with time-resolved lifetime measurements to fully understand the drug's quenching mechanism.

2.6.1) Fluorescent probes

There are a wide range of fluorescent probes that can be applied to study drug's membrane location. DPH is a popular probe of the membrane interior and it is assumed to be oriented parallel to the lipid hydrophobic chains (Figure 8), although it can reside in the centre of the lipid bilayer parallel to the surface. (126) TMA-DPH is a derivative of DPH that is anchored to the phospholipid polar headgroups due to the presence of a cationic group, so it locates in the interfacial region of phospholipid bilayers (Figure 8). (127)

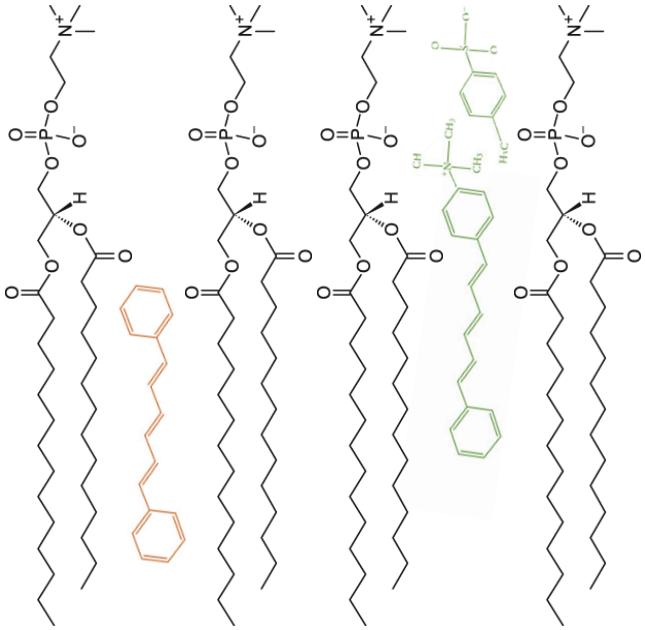


Figure 8 – Fluorescent probes location in DMPC bilayers (black). DPH (orange) is usually parallel to the lipid chains, while TMA-DPH (green) is near the interfacial region of phospholipid bilayers.

For all fluorescence experiments, TMA-DPH and DPH stock solutions were prepared in a chloroform:methanol mixture (3:2, V/V) and added to the organic solution of DMPC, at a proportion of 300:1 (lipid:probe), during LUV preparation.

2.6.2) Steady-state fluorescence quenching

Fluorescence quenching consists in the ability of a given molecule, termed quencher, to decrease the fluorescence intensity of a fluorophore. Quenching assays are useful to determine the quencher's location, since the phenomena takes place when the fluorophore and the quencher are close to each other. (128)

To study diclofenac's membrane location, samples with a fixed concentration of fluorophore labelled DMPC LUV (500 μM) and various concentrations of drug (0-100 μM) were prepared and incubated at 37 ºC for 30 minutes. After incubation, these samples were transferred to two 96-well plate (one for fluorescence measurements and another for absorbance readings) and the samples fluorescence intensity and absorbance spectra (respectively) were gathered on Cytation 3 imaging reader (Biotek Instruments, Inc.) using the experimental parameters described in Table 5.

Table 5 – Parameters used for steady-state fluorescence quenching assays.

Probe	**TMA-DPH**	**DPH**
	Fluorescence (T = 37 ºC)	
λ_{ex}	359 nm	357 nm
λ_{em}	429 nm	429 nm
Gain	70	
	Absorbance (T = 37 ºC)	
λ (start-end)	230-500 nm (1 nm interval)	

Importantly, to correctly interpret quenching results, the concentration of the quencher must be considered as the amount of the quencher present in the membrane (Equation 5) and the measure fluorescence intensities must be adjusted concerning the drug absorption (Equation 6).

The first correction is due to the fact that the fluorophores are not soluble in water, thus quenching occurs solely in the membrane. So, taken in consideration the quencher's partitioning between the lipid and aqueous phase (K_p), the quencher's concentration is given by:

$$[Q]_m = \frac{K_p[Q]}{1+(K_p-1)(V_\varphi[L])} \qquad \text{Equation 5}$$

Where the amount of lipid phase is estimated using the lipid's molar volume (V_φ) and its concentration ([L]) in the samples. [Q] represents the total concentration of the quencher in the sample. (128)

The corrected fluorescence values must be calculated to eliminate the apparent quenching that derives from drug absorption at the excitation wavelength of the fluorophore. This adjustment, presented in Equation 5, accounts for absorbance in absence (A_0) and presence of the drug (A_Q). (128)

$$I_{corr} = I * \left(\frac{A_Q}{A_0}\right) * \frac{1-10^{-A_0}}{1-10^{-A_Q}} \qquad \text{Equation 6}$$

2.6.3) Fluorescence quenching mechanisms

The quenching phenomena can occur due to different types of molecular interactions, namely energy transfer, molecular rearrangements and complex formation, which result in different types of quenching – preferably distinguished by lifetime measurements. (128) The most relevant types of quenching are presented in this section.

Dynamic or collisional quenching: collisional encounters occur between the fluorophore and the quencher. This phenomena is described by the Stern-Volmer equation:

$$\frac{I_0}{I} = 1 + k_q \tau_0 [Q]_m = 1 + K_D [Q]_m \quad \text{Equation 7}$$

I_0/I, representing the ratio of the initial fluorescent intensity and the fluorescence of the sample, is expected to be linearly dependent on the quencher membrane concentration ($[Q]_m$). K_D is the Stern-Volmer constant for this type of quenching, while k_q is the bimolecular quenching rate constant that indicates the accessibility of the fluorophores to the quencher, thus reflecting the efficiency of quenching. (128)

Static quenching: a non-fluorescent complex between the quencher and the fluorophore is formed, being this an important indication on the binding of the molecules. Importantly, this event takes place in the absence of a permanent change in the molecules. The Stern-Volmer constant for static quenching (K_S) is retrieved from Equation 8.

$$\frac{I_0}{I} = 1 + K_S [Q]_m \quad \text{Equation 8}$$

Like in the previous case, I_0/I has a linear relation with $[Q]_m$. Lifetime measurements can characterize each type of quenching as $\frac{\tau_0}{\tau} = 1$ is verified for static quenching, and $\frac{\tau_0}{\tau} = \frac{I_0}{I}$ is true for purely dynamic quenching mechanism. (128)

2.6.4) Deviations from linear Stern-Volmer plots

When there is a large extent of quenching, positive deviations from the Stern-Volmer equations can be verified. Two models can describe these deviations: combined dynamic and static quenching and the sphere of action model.

Combined dynamic and static quenching: both mechanisms are involved and instead of a linear relation for the fluorescence graphs, there is an upward curvature for high membrane concentrations of quencher. Here, the dynamic portion is determined by lifetime measurements and the quenching is described as (128):

$$\frac{I_0}{I} = (1 + K_D [Q]_m) * (1 + K_S [Q]_m) \quad \text{Equation 9}$$

Sphere of action model: there is an apparent static component because the quencher is adjacent to the fluorophore at the moment of excitation. The phenomena is described by:

$$\frac{I_0}{I} = (1 + K_D * [Q]_m) * e^{[Q]_m * V_{sphere}} \quad \text{Equation 10}$$

in which V_{sphere} is the volume of the sphere of action. (128)

2.6.5) Time-resolved lifetime measurements

Lifetime measurements are crucial to distinguish between different fluorescence quenching mechanisms. The lifetime indicates the time that the probes are available to interact with the environment. There are many types of lifetime measurement techniques, and here the time-resolved measurements were implemented. More specifically, frequency-domain measurements were executed. In these, an intensity-modulated light source excites the sample with a high frequency and its emission, which is intensity-modulated at the same frequency, is delayed in relation to the modulated excitation. That delay is measured as a phase angle shift between the excitation and emission and the peak-to-peak height provides a measure of the lifetime. This is possible because the emission's phase shift and modulation depend on the light modulation frequency and the relative values of lifetime. Experimentally, a wide range of frequencies is used to measure these two parameters, providing the frequency response of the sample. The output is the phase angle and the modulation plotted versus the selected frequencies; in exponential decays the first increases while the second decreases (Figure 9). (128)

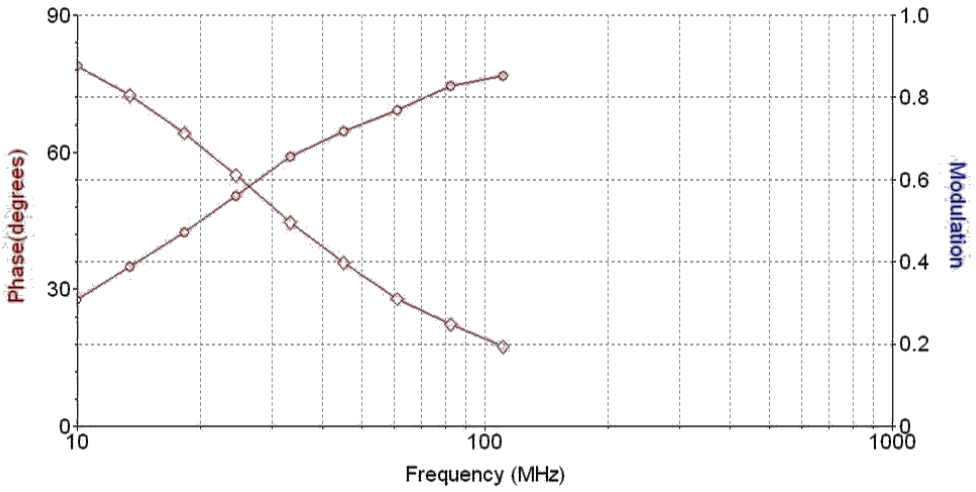


Figure 9 – Graphic output of a time-resolved frequency-domain lifetime experiment.

For this assay, samples with a fixed concentration of DMPC LUV (500 μM) labelled with TMA-DPH or DPH and various concentrations of drug (0-100 μM) were prepared and read, after an incubation period of 30 min at 37 ºC, on a Fluorolog Tau-3 Lifetime System. All measurements were made at 37 ºC using Ludox as a reference standard (τ = 0.00 ns). Table 6 displays the other experimental parameters used to determine the probes lifetime in the absence and presence of diclofenac.

Table 6 – Parameters used in time-resolved frequency-domain lifetime measurements.

Probe	**TMA-DPH**	**DPH**
λ_{ex}	359 nm	357 nm
λ_{em}	429 nm	429 nm
Slits	Manual = 0.7 mm; Excitation monochromator: Entrance = 7.0; Exit = 0.7 Emission monochromator: Entrance = 7.0; Exit = 7.0	
Modulation frequencies	20-160 MHz	10-150 MHz
Number of frequencies	10	
Integration time	10 seconds	

The resulting data for each sample was fitted to a 2-component model, from which 4 parameters are retrieved (f_1, f_2, τ_1 and τ_2), being afterwards the weighted average (τ) calculated. To extract information about the constant of the quenching component, K_D, $\tau 0/\tau - 1$ vs $[Q]_m$ was plotted and linearly fitted, being $\tau 0$ and τ the lifetime of the probes in the absence and presence of diclofenac, respectively.

2.7) Lipid phase transition

The lipid composition – degree of saturation, size of the fatty acid chains and cholesterol content – and temperature determine the physical state and organization of molecules within a phospholipid bilayer. With increasing temperatures, phospholipid bilayers present different physical states, from crystalline phases (highly ordered) to fluid phases (disordered) (Figure 10). Below the main phase transition temperature (T_m) the bilayer is in the gel phase, which consists in a solid ordered state. Above T_m, the bilayer is in a more disordered and fluid state, which is the fluid phase (Figure 10). This gel-to-liquid transition temperature will vary according to the bilayer hydrocarbon chains length and chemical structure and also on the structure of the lipid polar headgroups. Indeed, the presence of unsaturated acyl chains and of smaller acyl chains decreases T_m of phospholipid bilayers. (70)

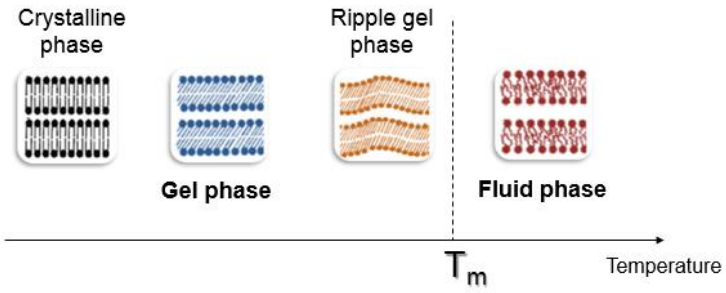


Figure 10 – Representation of a lipid phase transition, from the most organized phase (crystalline) to the fluid phase (above the main phase transition temperature, T_m), known to be the natural state of cell membranes.

The fluid phase generally represents the natural state of cell membranes; however, more rigid domains can be found in plasma membranes. (70, 129) In order to understand how diclofenac affects the lipid phase transition, steady-state fluorescence anisotropy was resourced to evaluate the diclofenac's effect on the DMPC main phase transition parameters, including T_m and cooperativity (B). The latter indicates how well the lipids cooperate when the phase transition occurs. This technique has proven to be adequate for the study of liposome membrane fluidity and to assess the interactions between lipid membranes and compounds with high membrane affinity. (130)

2.7.1) Fluorescence anisotropy

Fluorescence anisotropy enables the molecular dynamics study of fluorescent solutes. To do this, polarized light is used to excite the sample and subsequently its emission is likewise polarized. Anisotropy describes the extent of the emission polarization by providing the average angular displacement of the fluorophore (between the absorption and emission of a photon), which depends on its rate and extent of rotational diffusion during the excited state lifetime. In turn, the rotational diffusion is conditioned by the solvent viscosity and the shape of the rotating molecule; in this case, this parameter will depend on the lipid bilayer physical state. (128) Anisotropy (r) is given by the following equation:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} - 2I_{\perp}} \qquad \text{Equation 11}$$

$I_{\parallel}$ represents the intensity of the emission polarizer oriented parallel to the direction of the polarized excitation and $I_{\perp}$ is attributed to the observed intensity when the polarizer is perpendicular to the excitation. (128)

Similarly to fluorescence quenching studies, TMA-DPH or DPH labelled DMPC LUV (500 μM) in the absence and presence of diclofenac (0, 40 and 80 μM) were prepared. After the incubation period at 37 ºC, temperature-dependent scans were traced with data

collection for every 2 ºC. The used equipment was a Jasco FP-6500 spectrofluorometer (Great Dunmow, UK) – equipped with two polarizers in the paths for excitation and emission using the L-format method – coupled to a Jasco ADP 303T temperature controller (Great Dunmow, UK). Experimental parameters are listed in Table 7.

Table 7 – Parameters used in fluorescence anisotropy for the study of the lipid phase transition.

Probe	**TMA-DPH**	**DPH**
Temperature range	10 ºC – 40 ºC	
λ_{ex}	359 nm	357 nm
λ_{em}	429 nm	429 nm
Gain	500 V	
Slits	3 nm	

Data of anisotropy as a function of temperature were fitted according to:

$$r_s = r_{s1} + p_1 * T + \frac{r_{s2} - r_{s1} + p_2 * T - p_1 * T}{1 + 10^{B*\left(\frac{1}{T} - \frac{1}{Tm}\right)}} \qquad \text{Equation 12}$$

where r_s is the anisotropy, p_1 and p_2 are the slopes of the linear fits to the data before and after the phase transition region (respectively), and r_{s1} and r_{s2} are the corresponding 'y' intercepts. From here both B and T_m were retrieved.

2.8) Drug effect on membrane fluidity

Besides evaluating the influence of the drug on the lipid phase transition, it is also necessary to understand if the membrane fluidity is modified, at a specific physical state, with increasing concentrations of drug. Indeed, membrane fluidity comprises different types of mobility of membrane components, which are related to the order (phospholipids packing) and the microviscosity of phospholipids. The latter is related to the anisotropic movement of lipids (lateral and transverse diffusion). (131) Alterations in the membrane fluidity can influence proteins function and, thus, their monitoring can contribute to explain some of the NSAID therapeutic and toxic effects.

2.8.1) Fluorescence anisotropy

To study diclofenac's effect on membrane fluidity, samples with a fixed concentration of DMPC (500 μM) labelled with DPH or TMA-DPH and several drug concentrations (0- 100 μM) were prepared and the probes' anisotropy was measured at 10

ºC and 37 ºC after a previous incubation period of 30 min at the same temperature. The used equipment was a Jasco FP-6500 spectrofluorometer (Great Dunmow, UK) – equipped with two polarizers in the paths for excitation and emission using the L-format method – coupled to a Jasco ADP 303T temperature controller (Great Dunmow, UK). Experimental parameters are detailed in Table 8.

Table 8 – Parameters used in fluorescence anisotropy to assess membrane fluidity.

Probe	**TMA-DPH**	**DPH**
Temperature range	10 ºC and 37 ºC	
λ_{ex}	359 nm	357 nm
λ_{em}	429 nm	429 nm
Gain	400 - 500 V	
Slits	3 nm	

Anisotropy data can be influenced by the probe itself, as increasing concentrations of drug may alter the fluorophore excited state lifetime, resulting in variations on anisotropy values which do not reflect alterations on membrane microviscosity. (132) So, in order to eliminate the drug effect on the fluorophore excited state lifetime, anisotropy values are corrected according to Equation 13.

$$r' = \frac{\theta_0 + \tau_0}{\theta_0 + \tau} * r_{ss0} \qquad \text{Equation 13}$$

r' represent the corrected anisotropy values, while rss_0 is the experimental anisotropy value in the absence of drug. τ_0 and τ correspond to the lifetime of the fluorophore in the membrane in the absence and presence of the drug, respectively. θ_0 is the probe rotational correlation time in the absence of drug. (119)

The probes rotational correlation time is dependent on membrane lipid composition, temperature and pH and thus, it should be determined for the exact same conditions (lipid, temperature and pH) used in fluorescence anisotropy measurements. However, it was not possible to experimentally determine this parameter, since the required equipment to perform time dependent anisotropy measurements was not available. Therefore, reported values found in literature for both TMA-DPH and DPH were used. As it was not possible to find experimental values for lower pH than physiological pH, the same value was assumed for all pH studied in this work. Moreover, values reported for TMA-DPH and DPH labelled DMPC LUV at 15 ºC and 40 ºC were assumed to be similar to those obtained at 10 ºC and 37 ºC, respectively. The employed θ_0 for TMA-DPH and DPH at 10 and 37 ºC are presented in Table 9.

Table 9 – TMA-DPH and DPH rotational correlation times in DMPC bilayers. Values taken from (133).

	TMA-DPH	**DPH**
10ºC	~ 4.0 ns	~ 4.6 ns
37ºC	~ 1.5 ns	~ 2.0 ns

2.9) Drug effect on liposome permeability

The cellular membrane permeability is an important property that determines the function of the membrane and can disturb the equilibrium between the intracellular and extracellular environments when altered. Since NSAID are known to interact with lipid membranes, their association to bilayers may affect this property.

2.9.1) Carboxyfluorescein leakage assay

There are several methods to study the liposomal permeability and a variety of assays makes use of the self-quenching capacities of fluorophores, such as calcein and carboxyfluorescein. Basically, these fluorophores can be entrapped in liposomes at high concentration which are self-quenched. If the drug alters membrane permeability and leakage occurs, the fluorophore leaves the liposome and ends up diluted in the external aqueous environment, hence gaining fluorescence. The more drug-induced leakage, the more intense will the fluorescence signal be. (129) With carboxyfluorescein, the molecule chosen for this work, it has been verified that its encapsulation results in about 98% of self-quenching – the phenomena is explained by the formation of a dimer that is non-fluorescent. (134)

To assess the liposome permeability, EPC films (25 mM) were hydrated with a carboxyfluorescein aqueous solution (50 mM). In order to obtain the carboxyfluorescein-loaded liposomes, the free fluorophore was separated from the liposomes using a size-exclusion chromatography. The chromatographic column was prepared with Sephadex G-25 medium and hydrated with Tris HCl buffer (10 mM, pH 8.0) containing 300 mM of NaCl. (135) The separation was performed at room temperature.

After the separation of the liposomes and the free fluorophore, the liposomal fraction was collected and the most concentrated fraction was used to prepare samples with different concentrations of diclofenac (0 to 200 µM). After a 30 min incubation at 37 ºC, the samples' fluorescence intensity was read in a Cytation 3 imaging reader (Biotek Instruments, Inc.) also at 37 ºC using λ_{ex} = 490 nm and λ_{em} = 519 nm. (136) The addition of

polidocanol 10% (V/V), which destroys the liposomes, allowed to determine the maximum fluorescence intensity of the samples. Data were analysed in terms of leakage percentage though the following calculation:

$$\%\ Leakage = \frac{I_s - I_0}{I_T - I_0} * 100 \qquad \text{Equation 14}$$

Where I_0 is the initial fluorescence intensity (samples without drug), I_s is the sample fluorescence and I_T is the signal obtained after polidocanol addition. (135)

Additionally, the determination of the phospholipid concentration of the liposomal fraction collected from size-exclusion chromatography was executed, since this chromatographic process highly dilutes the initial concentration of phospholipids. This determination was performed through a colorimetric method that quantifies inorganic phosphate. (135) Aliquots of the collected liposomal fractions were added to glass tubes and the solvent was completed evaporated at 120 ºC. Perchloric acid 70 % (0.4 mL) was added and the glass tubes were covered with glass marbles and kept at 180 ºC in bath of paraffin oil for 1 h. After cooling, 1 mL of water and 0.4 mL of ammonium molibdate 1.25 % (w/v) were added to the tubes and vortexed. The addition of 0.4 mL of 3 % ascorbic acid (w/v) and immediate vortexing followed. Next, the tubes were maintained in a water bath at 100 °C for 5 min and then immediately cooled in running water. Finally, absorbance was read at 797 nm and the phosphate concentration was calculated based on the standard curve made of 0.001 M of monopotassium phosphate solution. This curve was obtained by pipetting different volumes of the monopotassium phosphate solution into glass tubes, which were treated exactly the same way as the glass tubes containing the liposomal fractions aliquots.

3. Results and Discussion

3.1) Diclofenac's membrane partition

The partition coefficient (K_p) of diclofenac in DMPC liposomes, used as a membrane model system in this work, was determined through derivative spectrophotometry. An example of the gathered absorbance spectra and subsequent data analysis is shown in Figure 11. From the absorbance spectra of diclofenac experimentally obtained for increasing concentrations of DMPC LUV (Figure 11 – A), the third derivative spectra was calculated (Figure 11 – B). Using maximum values from that spectra (~286 nm), data was plotted against the respective DMPC concentration (Figure 11 – C, black dots) and the resulting curve was fitted to Equation 3 (Figure 11 – C, red line). From that fitting, K_p values were retrieved.

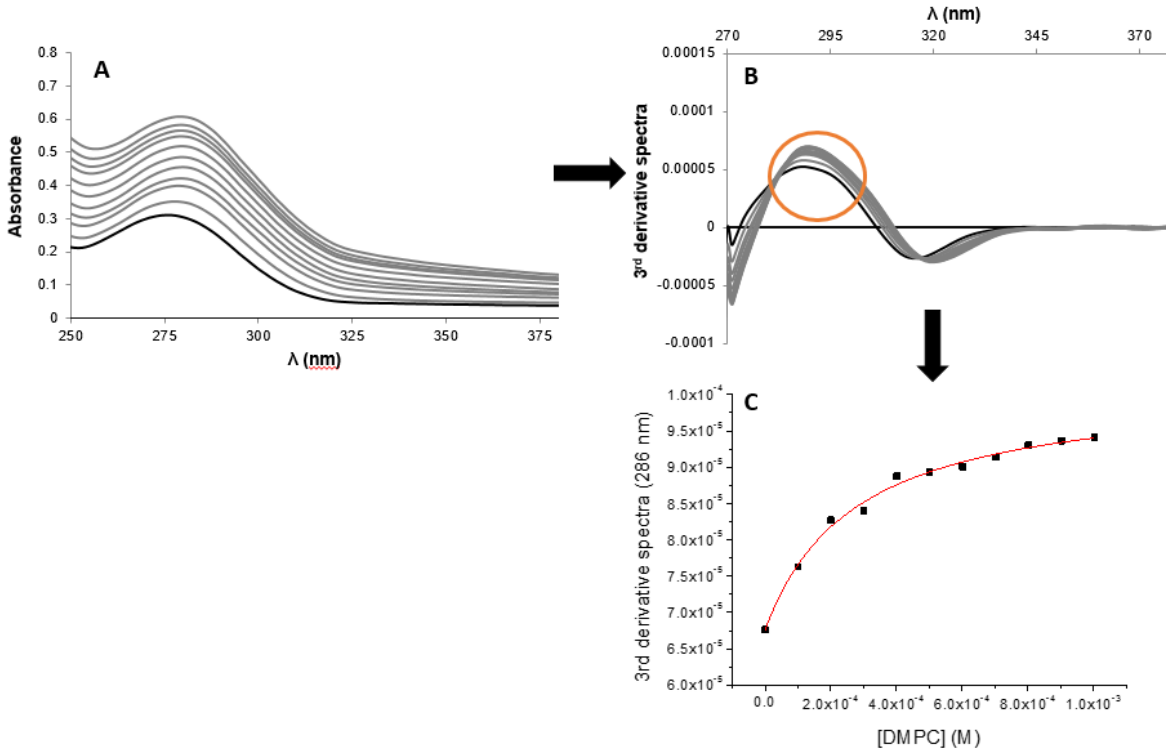


Figure 11 – Absorbance spectra (A) and third derivative spectra (B) of diclofenac with increasing concentrations of DMPC LUV at pH 5. The fitting of Equation 3 to maximum values of the third derivative spectra (orange circle indicates maximum region) versus respective DMPC concentration is displayed in curve C.

The existence of a spectral bathochromic shift indicates a change in the polarity of the environment. This shift can be observed both on the original absorbance spectra (Figure 11 – A) and on the third derivative spectra with increasing concentrations of DMPC LUV (Figure 11 – B), thereby proving that the drug is partitioning from the aqueous to the lipid phase. Additionally, the third derivative spectra show distinct isosbestic points (Figure 11 –

B), which confirms the complete elimination of the background signals arising from the light scattered by liposomes and also indicates the existence of drug molecules at both aqueous and lipid media.

Diclofenac's partition coefficients were estimated for the three pH conditions under analysis, as presented in Table 10. The K_p that results from the above mentioned fitting is in L/mol. However, in order to compare values with other studies in the literature, the K_p calculation in the logarithmic scale is required. The presented values of log(D) – pH-dependent property – derive from the application of the logarithm to the dimensionless K_p, which is calculated by considering the lipid molar volume (V_m) (Equation 15) – known to be approximately 0.66 L/mol for DMPC. (137)

$$\log(D) = log_{10}(\frac{K_p}{V_m}) \qquad \text{Equation 15}$$

From Table 10 it is possible to verify that diclofenac's K_p varies inversely with pH. This means that the drug will have a greater affinity to the liposomes in more acidic environments. The K_p at pH 3.0 is more than 4 times superior to the K_p at pH 7.4. Since the drug molecule has a pK_a of 3.97 (84), its carboxylic acid will be deprotonated and negatively charged at physiological pH. Contrastingly, a great percentage of drug molecules (~ 90 %) will have the carboxylic acid protonated at pH 3.0. Therefore, the higher K_p at lower pH reflects the greater affinity of the protonated and neutral form of diclofenac to partition in the DMPC LUV. Accordingly, at pH 5.0 a mixture of drug molecules in both ionization states coexist, so the overall K_p value comprises both partitioning of the protonated and deprotonated forms of diclofenac.

Table 10 – Partition coefficients (M^{-1} and logarithmic scale) of diclofenac at three pH conditions and 37 ºC determined by derivative spectrophotometry. The displayed values are the mean and standard-deviation of at least three independent assays.

pH	K_p (M^{-1})	Log(D)
7.4	2036 ± 142	3.49 ± 0.03
5.0	3665 ± 561	3.74 ± 0.07
3.0	8958 ± 1520	4.13 ± 0.07

In fact, the dependence of the K_p on the ionization state of the drug molecules was already demonstrated for other NSAID. Taking indomethacin and acemetacin as examples, since both have pK_a close to 4, partitioning studies also reported a higher partition coefficient at pH 5.0 than at pH 7.4. (79, 80) The same applies to the particular case of

diclofenac. Its partition coefficients at pH 7.4 and 3.0 determined by Ferreira *et al.* showed the same tendency for the drug's affinity to the liposomes. Although results are comparable for physiological pH (K_p = 1100 ± 100), the higher partition at pH 3.0 (K_p = 26000 ± 3000) is probably explained by the use of a more fluid membrane model (EPC LUV). (84) Moreover, diclofenac's partition was first estimated from the conventional octanol/water system and the reported log(P) value was 4.40. (138) The partition coefficient measured in the octanol/water system only takes into account hydrophobic interactions and does not consider any electrostatic interactions and/or hydrogen bonds that may occur between compounds and membranes. (139) Therefore, and as expected, this log(P) is close to log(D) gathered at pH 3.0, since the majority of molecules are protonated and hydrophobic interactions will drive the partitioning of diclofenac with DMPC bilayers. In contrast, electrostatic interactions may be greatly relevant for higher pH, where charged molecules are present, justifying the discrepancy of the reported log(P) with the experimentally obtained log(D).

3.2) Membrane binding of diclofenac

Membrane-binding assays were performed to obtain evidence on diclofenac possible interaction with the membrane's surface. A fluorescence titration technique, using the anionic probe ANS, was performed in the absence and presence of the drug to evaluate a competition mechanism between diclofenac and ANS for the same binding sites at the DMPC bilayer surface. The measured fluorescence intensities, related to the amount of ANS electrostatically bound to the phospholipids headgroups, were plotted against ANS concentrations resulting in the binding isotherms presented in Figure 12.

Visually, it is possible to perceive that the drug has practically no influence of the probe binding to the liposome surface at physiological pH (Figure 12 – A). However, a decrease of the fluorescence intensity dependent on diclofenac concentrations is noticeable at pH 5.0 (Figure 12 – B), immediately indicating that the drug is influencing the probe-membrane binding. That tendency is even more accentuated at pH 3.0 (Figure 12 – C), which corroborates diclofenac's greater affinity to the membrane in more acidic environments.

The fitting of the curves displayed in Figure 12 to Equation 4 allowed the estimation of the binding constant (K), the binding process cooperativity (b) and the maximum fluorescence intensity (C_{max}). These values are presented in Table 11 for all studied conditions. No relevant differences were found in any parameters at pH 7.4, thereby confirming that the deprotonated form of diclofenac, at least in the studied concentrations,

does not compete with ANS to bind to the membrane surface. On the other hand, C_{max} decreases for pH 5.0 and even more for pH 3.0 in the presence of the drug. This proves that, with increasing concentrations of diclofenac, there are less ANS molecules binding to the membrane. So, ANS and the drug are competing for the same binding sites at the membrane surface. Despite hindering the access of ANS to the membrane by a competition mechanism, diclofenac does not alter the binding rate of ANS seen that no major changes in K and b were observed.

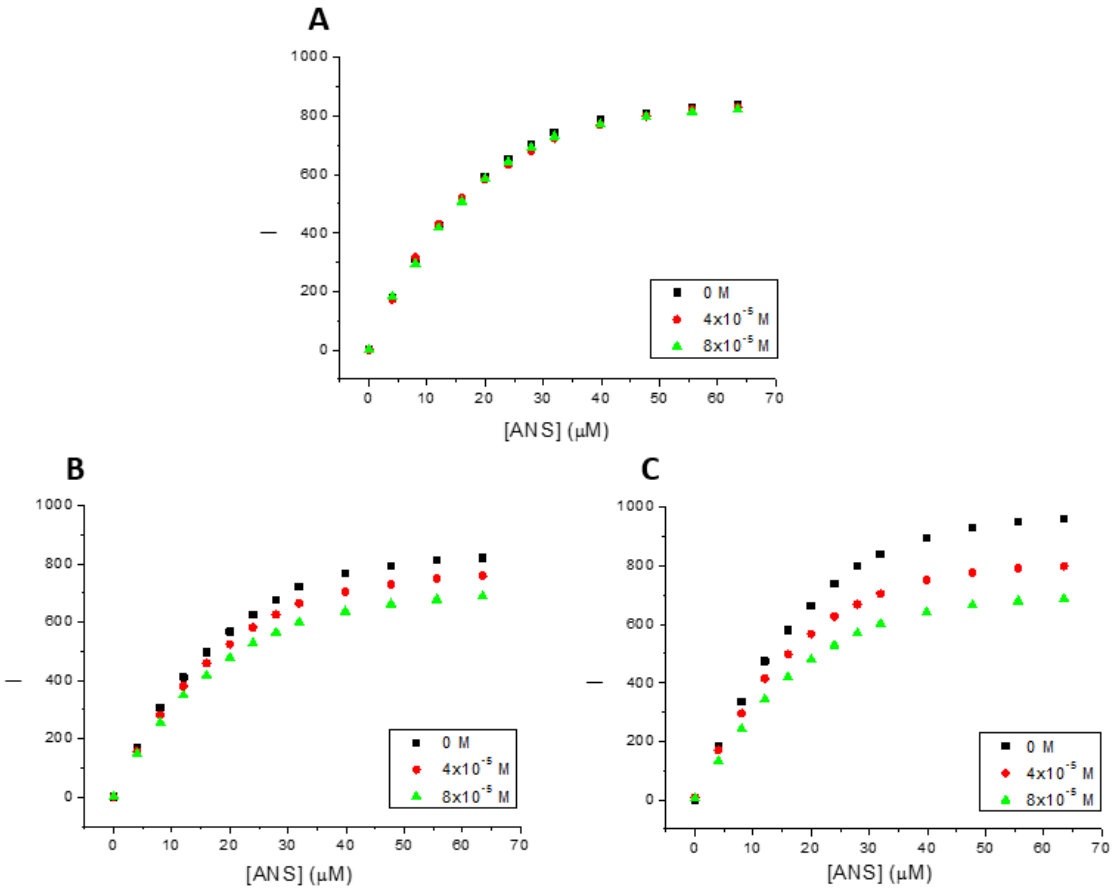


Figure 12 – ANS membrane binding isotherms (fluorescence intensity vs [ANS]), obtained with three drug concentrations (0 μM – black squares, 40 μM – red dots, 80 μM – green triangles) and for three pH conditions – pH 7.4 (A), pH 5.0 (B) and pH 3.0 (C) – at 37 ºC.

Since ANS is a negatively charged probe, it establishes electrostatic interactions with the bilayer headgroups. (140) The replacement of the probe by diclofenac at the liposome surface could be firstly assumed to occur by the same type of interactions. However, at pH 7.4, in which diclofenac molecules are deprotonated and negatively charged, no competition was verified. This result may be related to the lower K_p and subsequent smaller membrane concentrations obtained at this pH. Diclofenac-membrane binding is much more relevant for lower pH, when the drug molecules become protonated

and neutral. Thus, hydrophobic interactions and/or hydrogen bonds may drive the drug-membrane binding and diclofenac may locate within the headgroup region of phosphatidylcholines, thereby preventing ANS-membrane binding.

Table 11 – ANS membrane-binding parameters – binding constant (K), cooperativity (b) and maximum fluorescence intensity (C_{max}) – obtained with increasing concentrations of diclofenac, under three pH conditions and at 37 ºC. Values are presented as mean and standard deviation of two independent assays.

pH	[Diclofenac] (M)	$[Diclofenac]_m$ (M)	K	b	C_{max}
7.4	0	0	0.073 ± 0.001	1.33 ± 0.01	936 ± 43
	4.0×10^{-4}	4.9×10^{-2}	0.068 ± 0.003	1.25 ± 0.01	976 ± 6
	8.0×10^{-4}	9.7×10^{-2}	0.074 ± 0.002	1.33 ± 0.01	938 ± 17
5.0	0	0	0.069 ± 0.001	1.29 ± 0.05	1005 ± 50
	4.0×10^{-4}	6.6×10^{-2}	0.069 ± 0.003	1.25 ± 0.02	916 ± 34
	8.0×10^{-4}	1.3×10^{-1}	0.067 ± 0.001	1.27 ± 0.05	857 ± 59
3.0	0	0	0.08 ± 0.01	1.40 ± 0.06	1049 ± 86
	4×10^{-4}	9.0×10^{-2}	0.076 ± 0.004	1.34 ± 0.03	880 ± 59
	8×10^{-4}	1.8×10^{-1}	0.074 ± 0.006	1.36 ± 0.01	747 ± 63

No previous studies in the literature have performed a similar assay with diclofenac. Nevertheless, the assessment of diclofenac binding to human polymorphonuclear neutrophils (PMN) by Perianin *et al.* has effectively shown its ability to compete for the cell's high-affinity binding sites of the chemotactic factor N-formyl-methionyl-leucyl-phenylalanine (FMLP), demonstrating diclofenac's tendency to bind to PMN (141) and therefore providing evidence for the drug's affinity to cellular membranes. Also, some works have investigated diclofenac's interaction with mitochondria and have proven its ability to induce mitochondrial permeabilization transition (142), which can hint at a possible binding between diclofenac and the mitochondria membrane.

In this context, nimesulide binding to a mitochondrial membrane model revealed that this NSAID has affinity for mitochondrial membranes. (103) Additionally, isothermal titration calorimetry was used to study the binding of indomethacin and acemetacin with EPC liposomes, allowing to verify that the membrane's biophysical properties were modified when saturation with the drugs was attained – this apparently takes places at a lipid/drug ratio of 12:1 for acemetacin and 17:1 for indomethacin. (143)

3.3) Membrane location of diclofenac

To assess the membrane location of diclofenac, the drug's quenching ability was studied by performing fluorescence quenching assays with the fluorescent probes TMA-DPH and DPH that have well-known membrane location (Figure 8). In order to identify the type of quenching mechanism occurring between diclofenac and each probe, experiments were complemented with time-resolved lifetime measurements. An example of the quenching and lifetime results is displayed in Figure 13 for each probe.

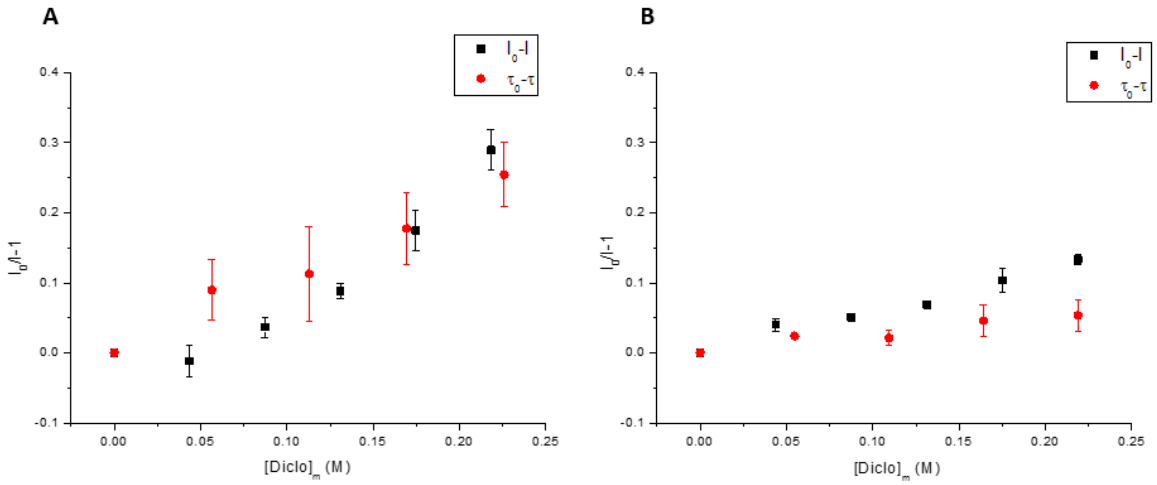


Figure 13 – Quenching and lifetime results obtained with TMA-DPH (A) and DPH (B) for pH 3.0 at 37ºC. Black squares represent data retrieved from fluorescence quenching assays (I_0/I -1) and red dots represent lifetime data ($\tau_0/\tau - 1$).

From the information obtained with both assays it is possible to conclude on how the probe is quenched. In the case of DPH, a straightforward analysis was made since the Stern-Volmer constant (K_{sv}), retrieved from the linear fitting of the data gathered in fluorescence quenching assays, differed from the constant estimated for the dynamic quenching (K_D), obtained by time-resolved lifetime data (Figure 13 – B). Thus, it was verified that the quenching phenomena resulted from the contribution of both dynamic and static quenching. The static component was calculated as being the difference between K_{sv} and K_D.

For TMA-DPH, fluorescence quenching data did not present a linear profile (Figure 13 – A). Thus, its analysis would firstly appear to be adequate by considering the two models that describe positive deviations from the linear Stern-Volmer plots (Equations 9 and 10). Yet, neither the combined dynamic and static quenching nor the sphere of action models described the non-linear profile of the curves. In fact, a closer look shows that there is, in fact, a negative deviation for low diclofenac concentrations (0 – 0.10 M) and not a positive deviation for higher concentrations, since a linear profile is obtained for diclofenac

concentrations over 0.10 M. This negative deviation may be related to the rigidifying effect of diclofenac verified in TMA-DPH labelled DMPC liposomes, at those same concentrations and at 37 ºC, that was later observed with the evaluation of diclofenac's effects on membrane fluidity by fluorescence anisotropy measurements (section 3.5). Indeed, an increase of membrane order at the region where TMA-DPH inserts within the membrane leads to an increment of the probe fluorescence intensity, which overcomes the quenching ability of diclofenac at low concentrations, leading to an overall decrease of I_0/I. Since, with exception to low concentrations, fluorescence quenching and time-resolved lifetime data obtained with TMA-DPH labelled liposomes are nearly overlapping (Figure 13 – A) for all studied conditions (data not shown), a purely dynamic quenching mechanism was assumed for the interaction of diclofenac with this probe. Hence, K_{sv} were considered to be equal to K_D values. The quenching constants obtained by fitting the curves to the adequate quenching model are presented in Table 12.

Importantly, the drug location is assessed by analysing the drug's extent of quenching with each probe, which is given by the bimolecular quenching rate constant (K_q). This value provides the efficiency of quenching and, since it is obtained from K_{sv} divided by τ_0, it allows to compare the extent of quenching for different probes by eliminating the influence of the intrinsic lifetime of each probe. K_q values are presented in Table 12. The typical values of K_q for diffusion-controlled quenching are near $1x10^{10}$ $M^{-1}s^{-1}$. However, smaller values can be obtained when steric shielding of the fluorophore occurs or if the drug has a low quenching efficiency. Contrastingly, higher values may be obtained if some type of binding interaction between the drug and the probe occurs. (128)

Table 12 – Quenching constant (K_{sv}), with respective static (K_S) and dynamic (K_D) components estimated after selecting the adequate quenching model, and bimolecular quenching rate constant (K_q). Values of K_{sv} and K_D correspond to linear fitting slopes of fluorescence quenching and lifetime assays and respective associated errors. For TMA-DPH K_{sv} values (*) were assumed to be equal to K_D.

Probe	**Quenching Model**	**pH**	**K_{sv} (M^{-1})**	K_S (M^{-1})	K_D (M^{-1})	**K_q x 10^8 ($M^{-1}s^{-1}$)**
TMA-DPH	Dynamic quenching	7.4	1.1 ± 0.1*	-	1.1 ± 0.1	2.54 ± 0.03
		5.0	1.15 ± 0.05*	-	1.15 ± 0.05	2.58 ± 0.02
		3.0	1.1 ± 0.1*	-	1.1 ± 0.1	2.29 ± 0.02
DPH	Dynamic and static quenching	7.4	1.28 ± 0.06	0.6 ± 0.1	0.65 ± 0.09	1.52 ± 0.01
		5.0	0.46 ± 0.07	0.26 ± 0.07	0.20 ± 0.02	0.55 ± 0.01
		3.0	0.57 ± 0.05	0.34 ± 0.06	0.23 ± 0.04	0.66 ± 0.01

The analysis of Table 12 and Figure 14, where the quenching data obtained with both probes at the three pH conditions are presented, shows that diclofenac has a low quenching efficiency. Indeed, K_q values obtained for both probes are lower than the diffusion-controlled value confirming the previously stated. Although diclofenac possesses two relevant chemical groups that are effective quenchers (chlorine and aromatic amine) (128), the drug molecule orientation in the bilayer may limit its ability to quench the fluorescent probes. This is a possible explanation for the small extent of quenching hereby obtained for diclofenac.

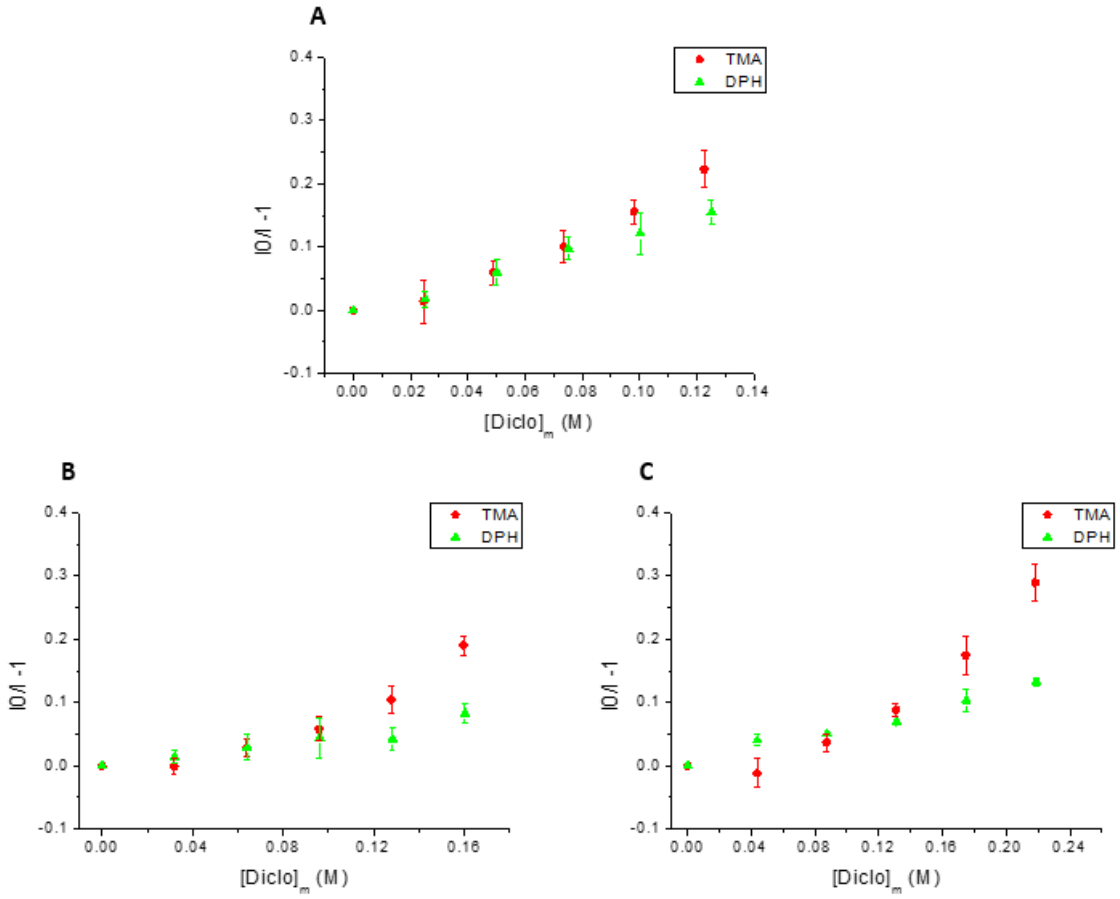


Figure 14 – Fluorescence quenching ($I_0/I - 1$) plots for TMA-DPH (red dots) and DPH (green triangles) as a function of diclofenac's membrane concentration obtained at three pH conditions – pH 7.4 (A), pH 5.0 (B) and pH 3.0 (C) – at 37ºC.

K_q values are suitable for the direct comparison of the diclofenac's quenching efficiency for both probes and demonstrate that the extent of quenching is clearly higher for TMA-DPH (Table 12). Therefore, this experiment provides evidence that diclofenac may be located near the polar headgroup region of the phospholipid bilayer, since it exerts a greater quenching effect in TMA-DPH, which is known to anchor to phospholipids headgroups. This finding is in agreement with the results obtained when studying the diclofenac-membrane

binding (section 3.2), which showed the ability of diclofenac to occupy the binding sites of ANS at the membrane surface. Nevertheless, the drug's small quenching of DPH also indicates that part of the molecules reaches the lipid acyl chains, where DPH is usually located. Interestingly, the quenching efficacy of TMA-DPH does not depend on the drug's ionization state or medium pH (Table 12), which indicates that both protonated and deprotonated molecules will rather locate at the headgroup region of phospholipids.

Previous experimental studies coherently pointed out that diclofenac locates nearby the polar headgroups of the membrane bilayer and suggested that interactions occurred by electrostatic adsorption with the zwitterionic headgroups of phosphatidylcholine. In fact, this electrostatic adsorption may drive the diclofenac-membrane interaction when the drug is deprotonated and negatively charged but when the drug molecules are protonated and neutral hydrophobic interactions and/or hydrogen bonds must take place. The non-polar side of the drug molecule is thought to remain in the neighbourhood of the hydrophobic acyl chains of the phospholipids. (84, 99, 118)

Both ibuprofen and naproxen were found to locate preferentially in the phosphate region (118) and clonixin's membrane location assessment with several fatty acid probes similarly indicates that the drug is positioned in the external part of the membrane. (83) It has been reported that NSAID with neutral charge will tend to establish hydrophobic interactions with the phosphoslipids' acyl chains and thereby insert deeper into the bilayer, while negatively charged NSAID preferentially establish electrostatic interaction with the phospholipids' headgroup region. (144) However, the assumption that a neutral charged NSAID inserts deeper into the bilayer was not verified here with diclofenac.

3.4) Diclofenac's effects on lipid phase transition

The diclofenac's effect on the main phase transition parameters of DMPC LUV was evaluated by fluorescence anisotropy measurements in a range of temperatures (10-40 ºC). The output of these assays was data with a sigmoidal profile in which the initial portion with higher anisotropy values (smaller probe rotation) correspond to the lipid's gel phase and the final portion to its fluid phase; the abrupt drop of anisotropy corresponds to the occurrence of the phase transition (Figure 15). The red line in Figure 15 represents the fitting of the data to Equation 12, from which both the temperature (T_m) and the cooperativity (B) of the lipid main phase transition were retrieved.

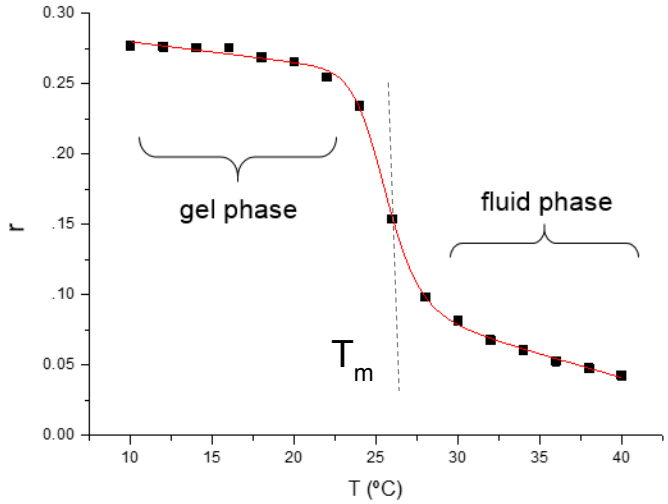


Figure 15 – Fluorescence anisotropy of TMA-DPH in DMPC liposomes as a function of temperature at pH 7.4. The red continuous line is the best fitted curve to Equation 12.

First and foremost, close values for DMPC T_m reported in the literature (24 ºC) (145) were obtained (Table 13). Although values in the absence of the drug are concordant with both probes at pH 7.4 and pH 5.0 (~24.7 ºC), superior T_m were obtained at pH 3.0. Such increase may be related to the partial protonation of the phosphate groups of DMPC at pH 3.0, which alters the overall electrostatic properties of DMPC bilayers and may lead to an increase of DMPC packing density, thus explaining the superior T_m obtained for pH 3.0. Indeed, zwitterionic/cationic binary lipid mixtures, for instance DMPC/DMTAP (dimirystoyl-trimethylammonium-propane), present higher T_m values than that obtained with DMPC alone explained by a rearrangement of lipids packing by the presence of cationic lipids. (146)

From a straightforward observation of the sigmoidal profiles obtained for TMA-DPH labelled DMPC LUV in the absence and presence of diclofenac in different concentrations and under the three pH conditions in analysis (Figure 16), it is evident that the drug's effect is much more relevant for lower pH. The gathered T_m and B values for all the studied conditions (Table 13) confirm such observation. These values show that both the temperature and cooperativity of the lipid main phase transition decrease with increasing concentrations of diclofenac. This trend is much more noticeable as the pH lowers, reflecting the greater affinity (higher K_p) of diclofenac to the phospholipid bilayer at lower pH and the subsequent higher membrane concentration of drug at these pH. So, the analysis of Table 13 allows to conclude that diclofenac's effect on the DMPC phase transition parameters is not only dependent on the drug's membrane concentration, but also on its ionization state. Indeed, regarding TMA-DPH results, the effect of 9.0 x 10^{-2} M of the protonated form of diclofenac (pH 3.0) is much more pronounced than the effect of a similar

membrane concentration (9.7 x 10^{-2} M) of diclofenac at pH 7.4. Thus, these two molecular forms must interact with DMPC bilayers differently.

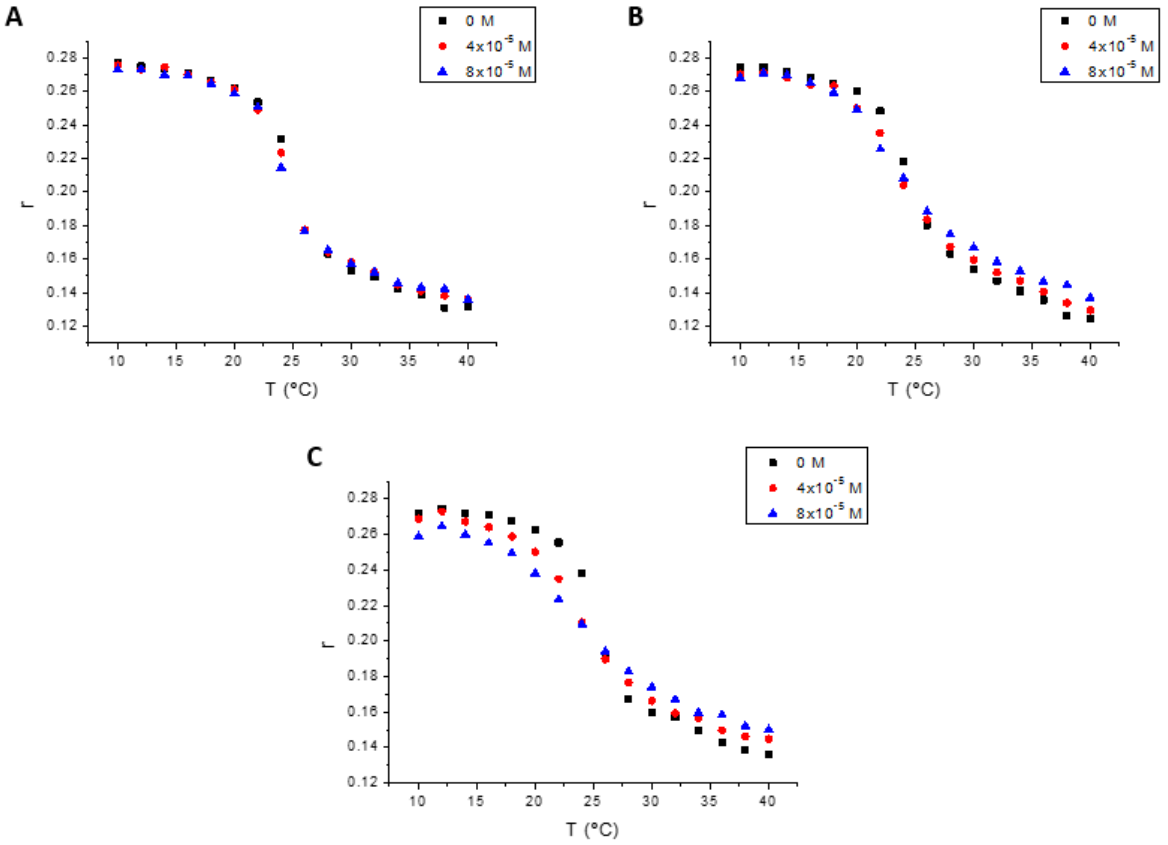


Figure 16 – Steady-state anisotropy of TMA-DPH labelled DMPC LUV as a function of temperature under three diclofenac concentrations (0 µM – black squares, 40 µM – red dots, 80 µM – blue triangles) at three pH conditions (7.4 – A, 5.0 – B, 3.0 – C).

Importantly, for high drug concentrations at pH 3.0 (Figure 16 – C), the sigmoid profile gets broader and this is related to the notorious decrease of the lipid's cooperativity in the phase transition (Table 13). This event is also evident when looking at T_m values which, unlike what occurs in other conditions, are higher for 8.0 x 10^{-5} M of diclofenac than for 4.0 x 10^{-4} M at pH 3.0 for both probes. Thus, the T_m in these conditions is not necessarily a value, but a range of values in which the phase transition is occurring – the onset temperature at which the phase transition starts occurring is in fact lower as the drug concentration increases (accordingly to the observed tendency with other pH) but the T_m value retrieved from the fitting corresponds to an intermediate value of this temperature range.

Furthermore, comparing the results obtained with the two probes, it possible to see that, although tendencies are similar, there is a more pronounced effect when TMA-DPH is

labelling the DMPC LUV. This is explained, firstly, by the fact the phospholipid bilayers have a fluidity gradient in which their core is more disordered and therefore less subjected to alterations. (147) Since TMA-DPH locates near the phospholipids headgroups, which consist in the area with higher level of organization, the drug presence leads to a greater perturbation. Additionally, considering the previous results obtained for membrane location, diclofenac has shown to influence TMA-DPH in a greater extent which also justifies a greater effect on the membrane biophysical properties in this region.

Table 13 – DMPC phase transition parameters (T_m and B) in the absence and presence of diclofenac under three pH conditions and for 2 different probes (TMA-DPH and DPH). The presented values correspond to means and standard-deviations of at least two independent assays.

Probe	pH	[Diclofenac] (M)	$[Diclofenac]_m$ (M)	T_m	B
TMA-DPH	7.4	0	0	24.71 ± 0.02	358 ± 15
		4.0×10^{-4}	4.9×10^{-2}	24.49 ± 0.06	284 ± 34
		8.0×10^{-4}	9.7×10^{-2}	24.13 ± 0.05	223 ± 22
	5.0	0	0	24.7 ± 0.1	295 ± 34
		4.0×10^{-4}	6.6×10^{-2}	23.6 ± 0.3	132 ± 8
		8.0×10^{-4}	1.3×10^{-1}	23.1 ± 0.3	86 ± 2
	3.0	0	0	25.3 ± 0.5	265 ± 43
		4×10^{-4}	9.0×10^{-2}	23.4 ± 0.1	91 ± 1
		8×10^{-4}	1.8×10^{-1}	23.92 ± 0.09	80 ± 7
DPH	7.4	0	0	24.9 ± 0.1	389 ± 16
		4.0×10^{-4}	4.9×10^{-2}	24.79 ± 0.04	321 ± 32
		8.0×10^{-4}	9.7×10^{-2}	24.47 ± 0.07	273 ± 17
	5.0	0	0	24.73 ± 0.03	317 ± 39
		4.0×10^{-4}	6.6×10^{-2}	23.7 ± 0.2	147 ± 16
		8.0×10^{-4}	1.3×10^{-1}	23.20 ± 0.09	91 ± 5
	3.0	0	0	26.04 ± 0.04	288 ± 5
		4×10^{-4}	9.0×10^{-2}	24.68 ± 0.01	104 ± 7
		8×10^{-4}	1.8×10^{-1}	24.9 ± 0.8	71 ± 3

A work that used DSC to assess diclofenac effect on the DMPC phase transition also showed that the drug lowers its T_m and the lipid's cooperativity – in this case, this was indirectly seen by the broadening of the lipid phase transition peak. Furthermore, it indicates

that diclofenac is likely to induce a higher reorganization in the lipid membrane, in comparison to other NSAID, since a stronger shift in the T_m was observed. (118)

Studies with other NSAID, namely meloxicam, tenoxicam, piroxicam and lornoxicam, have proved their ability to lower T_m and broaden the phase transition of DPPC in neutral and acidic environments (148). Similar results were obtained with acemetacin and indomethacin (86), being in agreement with the actions reported herein for diclofenac.

3.5) Diclofenac's effect on membrane fluidity

The influence of diclofenac on DMPC fluidity was assessed by steady-state fluorescence anisotropy using TMA-DPH and DPH as fluorescent probes. Importantly, experimental anisotropy values (r_{ss}) were corrected (r') to eliminate the drug influence on the probes excited lifetime state (Equation 13). Indeed, the difference between r_{ss} and r' describes the real variation of anisotropy caused by the drug. Hence, results are presented as r_{ss}-r' (Figure 17 – left side) and also expressed according to their fluidizing effect (Figure 17 – right side), calculated from Equation 16.

$$\%\, fluiding\ effect = \frac{r_{ss} - r'}{r_0'} * 100 \qquad \text{Equation 16}$$

Since lower anisotropy values reflect higher membrane fluidity, it is possible to verify for both probe a general tendency to fluidify the membrane in the gel phase (Figure 17 – A and C). Results with TMA-DPH showed a marked concentration-dependent fluidizing effect at both acidic pH in the gel phase (Figure 17 – A) and at pH 3.0 in the fluid phase (Figure 17 – B). Interestingly, a slight rigidifying effect occurs at 37 ºC for the lowest concentrations of diclofenac. Relatively different tendencies were observed with DPH-labelled DMPC LUV. While in the gel phase, the drug has a more pronounced fluidizing effect at pH 3.0 (Figure 17 – C), a rigidizing effect was observed for pH 3.0 and 5.0 at 37 ºC (Figure 17 – D). When comparing results obtained with both probes, diclofenac clearly exerts a more pronounced fluidizing effect for TMA-DPH labelled liposomes. Once again, the headgroup region of DMPC bilayers are more organized and thus, the drug insertion in this region will greatly disturb its fluidity. Contrastingly, DPH is located between the acyl chains of DMPC which is a more disorganized region of the bilayer than the headgroup region. The opposite effect of diclofenac on membrane fluidity verified in the gel and fluid phase may be related to the initial organization the bilayer. Indeed, at the gel phase the acyl chains have a higher packing density than at the fluid phase. Therefore, the drug penetration may disorganize the acyl chains at the gel phase, while it may lead to some rearrangement of the lipid acyl chains at the fluid phase, thereby increasing the bilayer rigidity.

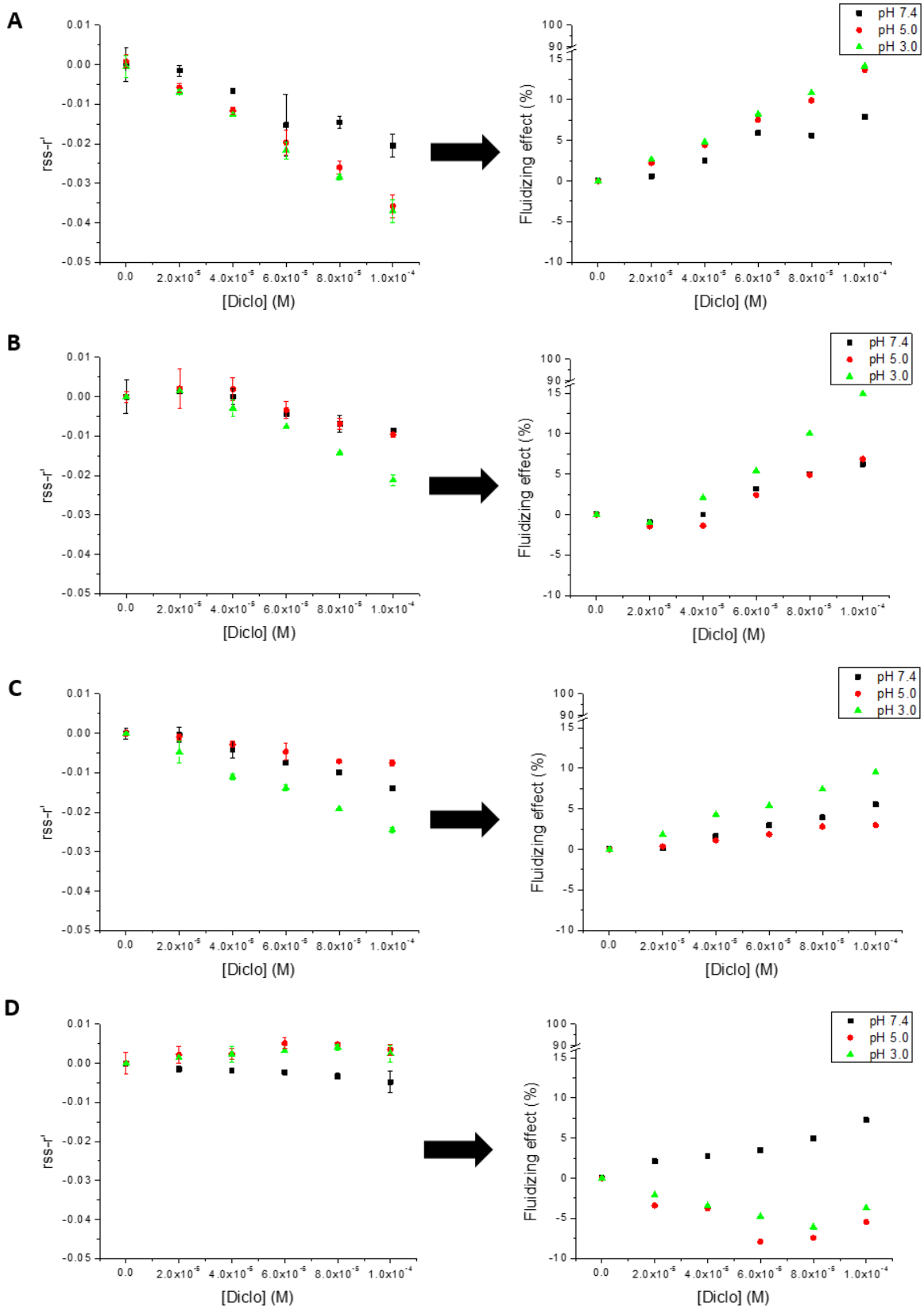


Figure 17 – Corrected steady-state anisotropy values (r_{ss}-r' – left side) of TMA-DPH (A and B) or DPH (C and D) labelled DMPC LUV with increasing concentrations of diclofenac for three pH conditions (pH 7.4 – black squares, pH 5.0 – red circles, pH 3.0 green triangles) and at 10 ºC (A and C) and 37 ºC (B and D). At the right side, the respective fluidizing effect as a function of diclofenac concentration is presented.

A similar study has been performed with diclofenac and reported an increase of the rigidity in the phospholipids headgroups of EPC. (84) The same behaviour was observed in this study for low concentrations of diclofenac, while higher concentrations caused a fluidizing effect on the DMPC headgroups region. This discrepancy may be due to the difference on the phospholipids used to prepare the membrane models. Indeed, EPC form bilayers with greater fluidity than DMPC due to the presence of unsaturated acyl chains. So, the drug insertion may organize EPC bilayers, while disordering DMPC bilayers with higher initial order. Yet, both works highlight that diclofenac has a stronger action in the headgroups of phospholipids.

Concerning works with other NSAID, an increase of membrane fluidity in a concentration dependent manner has been verified with nimesulide, indomethacin, acemetacin (132), meloxicam, lornoxicam (149) and clonixin (83). Altogether, these studies provide evidence that NSAID can insert in lipid bilayers, alter their dynamics and possibly modify the activity of membrane-bound proteins.

3.6) Diclofenac's effect on membrane permeability

A leakage assay using carboxyfluorescein as a fluorophore was performed to assess diclofenac's capacity of altering membrane permeability. When carboxyfluorescein is at high concentrations inside the inner aqueous compartment of LUV, its fluorescence is self-quenched. If the drug disrupts the bilayer permeability, the dye will be released and diluted, and its fluorescence intensity will greatly increase.

In this context, the first step was the separation of carboxyfluorescein-loaded liposomes using a chromatographic column (Figure 18). Since this was performed at room temperature, the use of DMPC in these conditions was a hindrance for the success of the experiment, since this lipid (T_m ~ 24 ºC) is in the gel phase or even in the phase transition temperature at room temperature. For that reason, EPC liposomes were chosen as membrane model system, because EPC has a lower T_m and the same polar headgroup of DMPC.

Carboxyfluorescein has four pK_a values, meaning that it can be found in five different ionization states according to the medium pH. These intrinsic structural changes of the molecule alter its spectral response, hence the fluorophore presents different emission and absorbance spectra under different pH. (136) Although this assay was performed under the three pH conditions selected for this project, the analysis of the results at acidic pH (5.0 and 3.0) was not possible since the fluorophore suffered molecular alterations and the disruption of membrane permeability did not lead to an increase on carboxyfluorescein fluorescence

intensity. Consequently, the results presented herein only refer to experiments performed at physiological pH.

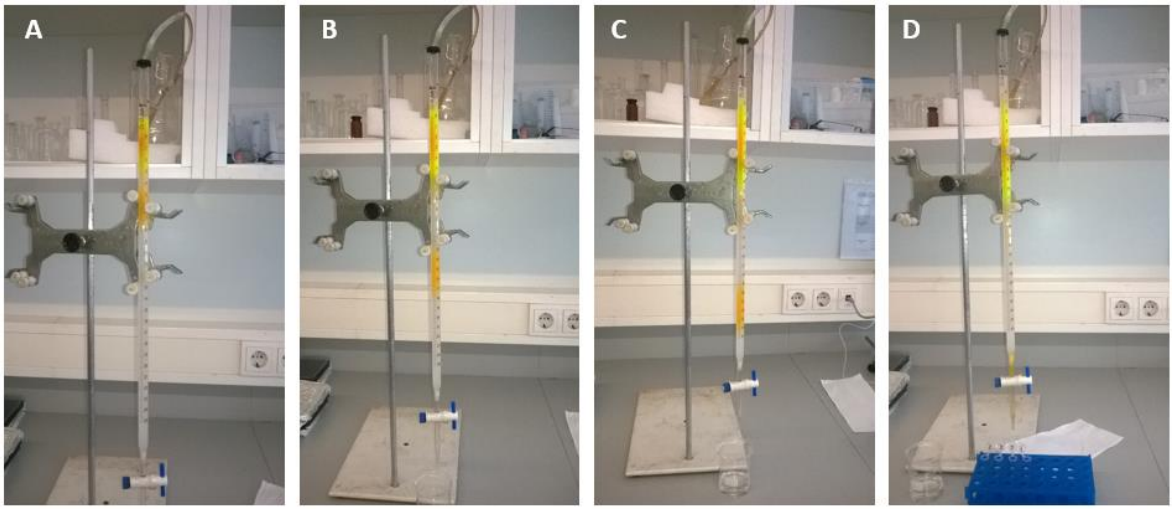


Figure 18 - Carboxyfluorescein-loaded EPC LUV separation through size-exclusion chromatography. A, B and C show the progression of the lipid separation from the free carboxyfluorescein. D corresponds to the collection of the lipid fraction.

After obtaining carboxyfluorescein-loaded EPC LUVs, different concentrations of diclofenac were added and after an incubation period, the fluorescence intensity was measured. Experimental data were then adjusted to Equation 14 and the percentage of leakage versus the drug/lipid ratio is presented in Figure 19.

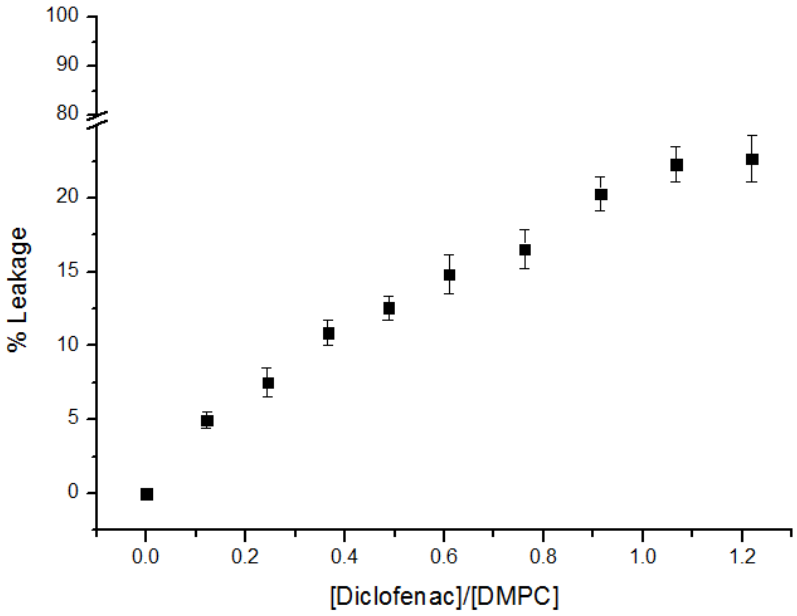


Figure 19 – Leakage percentage versus the drug/lipid ratio ([Diclofenac]/[DMPC]). Presented data corresponds to the mean value and standard deviation of two experimental replicates.

Firstly, a diclofenac effect on membrane permeability was verified since the percentage of leakage increases with higher drug/lipid ratios (Figure 19). This effect seems to be directly dependent of the drug/lipid ratio until approximately 1.0; from there a saturation plateau is apparently reached. The maximum leakage attained with diclofenac in these conditions was around 20%, which is a considerable effect in terms of membrane permeability. Importantly, as the drug partition indicates a higher affinity to the membrane as the pH lowers, it can be predicted that this effect would be more significant for pH 5.0 and 3.0. In order to obtain results for acidic conditions, a non pH-dependent fluorophore must be used.

There are related works in the literature that evaluated NSAID-induced permeability through an identical assay, using calcein as a fluorophore. In 2004, the assessment of several NSAID showed an increase of PC liposomes permeability and the authors suggest that this effect was related to their cytotoxicity. (98) Despite the attainment of very high leakage percentages, the drug/lipid ratio was much higher than 1, which contrasts to the drug/lipid ratios used in this work. The study of the effect of indomethacin on phospholipids (dilinoleoyl-phosphatidylcholine, DLPC) revealed low releases of calcein (< 5 %). However, as the focus of the study was the evaluation of membrane damage in the presence of bile contents, the addition of bile acids significantly enhanced the drug effect on membrane permeability. (150) A similar result to the one obtained with diclofenac in this work was found for nimesulide – 15% leakage at 1:1, with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) as a model membrane. (103)

Although there are no similar studies in the literature for diclofenac, this drug has been previously considered to increase the intestinal mucosa permeability (151) and to induce the mitochondria permeability transition (142). In fact, the study of diclofenac effect on membrane permeability is very relevant because evidence points for these mechanisms as possible causes for NSAID-induced enteropathy, since these drugs are able to increase intestinal permeability. (152)

4. Conclusion

The present work focused on assessing, from a biophysical perspective, diclofenac's interactions with biological membranes, in order to better understand the drugs' COX-independent mechanisms of action and toxicity.

In concordance to what had been verified with many other NSAID, diclofenac's ionization state revealed to be one of the major influencing factors of the drug behaviour in membranes. In fact, this was not only revealed by the higher drug partition in liposomes with lower pH, but by other experiments that studied alterations in membrane properties, including lipid phase transition and membrane fluidity, in which the drug effect was pH-dependent and repeatedly more pronounced at pH 3.0.

Additionally, the assessment of diclofenac's membrane location through fluorescence quenching studies and supported by the membrane binding assay contributed for the elucidation of results concerning the modifications of the membrane biophysical properties. Membrane location results were concordant with previous studies performed at physiological pH, indicating that diclofenac remains near the phospholipid headgroups, regardless its ionization state, possibly establishing hydrophobic interactions and/or hydrogen bonds when in the protonated state and electrostatic interactions when negatively charged molecules are present. Importantly, gathered evidence reveals the ability of the drug to disorganize the phospholipid bilayer, since diclofenac is able to decrease the temperature and cooperativity of the phospholipid main phase transition; to fluidize the membrane headgroup region when present in high concentrations; and to increase membrane permeability at physiological pH.

In the first place, the pH-dependency of diclofenac can be related to drug's therapeutic effect in inflamed cells (pH~5.0), as well as to the frequently verified diclofenac-induced gastric complications, since the gastric mucosa possesses a pH gradient between 1.2 (gastric lumen) and 7.4 (epithelial cells). Indeed, COX is a well-known target of conventional NSAID and being a membrane protein – located in the endoplasmic reticulum or nuclear envelope – it can be indirectly inhibited by diclofenac through modifications of membrane lipids properties that modulate the proteins' activity and function.

Regarding the most relevant adverse effects of diclofenac – GI and CV – the presented findings support some hypothesis to explain its toxicity. Lichtenberger *et al.* suggested the existence of a gastric surface-active phospholipids protective layer, which could be disrupted by the action of NSAID. In this line of thought, results obtained for diclofenac can corroborate this theory, since the reported modifications of the phospholipid's biophysical properties by diclofenac may facilitate the entrance of noxious

agents in the gastric mucosa, contributing for the occurrence of gastric adverse effects. Furthermore, the increased intestinal permeability was already identified as a NSAID-induced enteropathy cause, and this mechanism may be related to cell damage resulting from energy depletion. (152) In this sense, findings of increased membrane permeability induced by diclofenac can contribute to the explanation of its GI toxicity. Finally, the verified diclofenac-induced membrane disturbances can also be related to the occurrence of CV adverse effects. There is increasing evidence that the development of CV diseases, including hypertension, atherosclerosis and thrombosis, is associated with alterations on lipid composition and structure of membranes. (72) Since erythrocytes have been shown to be altered in hypertensive subjects – in terms of lipid composition and levels of signalling proteins (153), it would be plausible to relate diclofenac's membrane effects as a cause of erythrocytes morphological alterations described by Suwalsky et al. (99), which in turn may contribute or increase patient susceptibility to CV adverse effects.

In sum, this experimental study has provided insights on diclofenac's effects at the membrane level that contribute for the understanding of its biological actions. Importantly, the comprehensive description of traditional NSAID effects on membrane biophysical properties may accelerate the development of safer NSAID. The comparison of novel drugs' effects on membranes with those caused by traditional NSAID may distinguish the drugs with less impact on membranes, indicating the best candidates for clinical use. Also, the addition of *in vitro* studies with model membranes as a preliminary step during drug development will decrease its economic and biological costs by reducing the number of molecules reaching *in vivo* evaluations. The *in vitro* study of NSAID-membrane interactions was already on the basis of the rational development of PC-NSAID which appear to be better tolerated in the GI tract than traditional NSAID. (20, 22, 23) Therefore, the study of NSAID-membrane interactions may contribute for the development and commercialization of effective and safer anti-inflammatory drugs in the near future, thereby improving the quality of life of NSAID-chronic users.

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