Samuel Coelho Cabral

SYNTESIS AND CHARACTHERIZATION OF CELECOXIB-LOADED NANOMICELLAR TOPICAL FORMULATION ON DIABETIC RETINOPATHY

Dissertação para obtenção do grau de Mestre em Tecnologias do Medicamento sob a Orientação da Professora Doutora Maria Eugénia Pina e da Doutora Paula Ferreira, apresentada à Faculdade de Farmácia da Universidade de Coimbra

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ABSTRACT

One of the most challenging areas of pharmaceutical technology is development of ocular drug delivery systems. This is due to the complex anatomy and physiology of the eye. This complexity leads to many of the developed systems being ineffective, especially, when the aim is to treat posterior segment diseases. Diabetic retinopathy (DR) and diabetic macular edema (DME) are two of these diseases and are a leading cause of blindness in working-age populations. These two disorders are caused by an exaggerated expression of vascular endothelial growth factor (VEGF), in parallel with the induction of inflammatory processes. Currently DR and DME treatments are invasive therapies, that have demonstrated several associated side effects. Thus, it is necessary to develop new, safer therapeutic approaches. Celecoxib is a non-steroidal anti-inflammatory drug (NSAIDs) with anti-VEGF and antiproliferative effects. It has been shown to be a promising alternative for the treatment of DR and DME.

The purpose of this study was to develop a clear topical nanomicellar formulation (NMF) that presents a small nanomicellar size and a high entrapment efficiency of celecoxib. The topical application of NMF in the conjunctival sac, allows the nanomicelles to cross the sclera channels and reach the posterior ocular tissues (retina/choroid), releasing celecoxib in both.

NMFs were prepared by blending of the following polymeric surfactants: D-α-tocopherol polyethylene glycol-1000 succinate (Vitamin E TPGS) and Pluronic F-127 (PF 127) using the solvent evaporation method. NMFs were analyzed for size, polydispersity index (PDI), zeta potential, entrapment efficiency (EE%), drug loading (DL%) and optical clarity.

The results show that NMFs presented a size ranging from 44 to 49 nm, an acceptable PDI (0.090-0.150) and a negative zeta potentials. The celecoxib-loaded NMF showed EE%, DL % and optical clarity excellent. The formulation F presented the smallest size (44.17±0.12) nm and hight EE% (92.02%).

Keywords: Diabetic retinopathy; Diabetic macular edema; Celecoxib; Vitamin ETPGS; Pluronic F-127; Topical nanomicellar formulation; Ocular drug delivery; Posterior segment tissues;

RESUMO

Uma das áreas mais desafiadoras da tecnologia farmacêutica é o desenvolvimento de sistemas liberação de fármacos oftálmicos. Este facto deve-se à complexa anatomia e fisiologia do globo ocular. Essa complexidade leva a que muitos dos sistemas desenvolvidos sejam ineficazes, principalmente quando o objetivo é o tratamento de doenças do segmento posterior do globo ocular. A retinopatia diabética (DR) e o edema macular diabético (DME), são duas dessas doenças responsáveis pela maioria dos casos de cegueira na população ativa. Estes dois distúrbios são causados por uma expressão exagerada do fator de crescimento endotelial vascular (VEGF), em paralelo com a indução de processos inflamatórios. Atualmente, os tratamentos da DR e do DME são terapias invasivas, que têm demonstrado vários efeitos colaterais associados. Assim, é necessário desenvolver novas abordagens terapêuticas, mais seguras. O celecoxib é um anti-inflamatório não-esteroide (NSAIDs) com efeitos anti-VEGF e antiproliferativos. Este fármaco tem demonstrado ser uma alternativa promissora para o tratamento da DR e do DME.

O objetivo deste estudo foi desenvolver uma formulação nanomicelar (NMF), tópica, límpida que apresentasse um tamanho nanomicelar pequeno e uma elevada eficiência de aprisionamento de celecoxib. A aplicação tópica de NMF no saco conjuntival, permite que as nanomicelas atravessem os canais da esclera e alcancem os tecidos oculares posteriores (retina/coróide), libertando neles o celecoxib.

As NMFs foram preparadas pela mistura dos seguintes surfactantes poliméricos: succinato de D-α-tocoferol polietilenoglicol-1000 (Vitamin E TPGS) e Pluronic F-127 (PF 127), utilizando o método de evaporação do solvente. As NMFs foram analisadas quanto ao tamanho, índice de polidispersão (PDI), potencial zeta, eficiência do aprisionamento (EE%), encapsulamento do fármaco (DL%) e clareza óptica.

Os resultados mostram que as NMFs apresentaram um tamanho que varia de 44 a 49 nm, um PDI aceitável (0,090-0,150) e um potencial zeta negativo. As NMF carregadas com celecoxib mostraram excelentes EE %, DL% e clareza óptica. A formulação F apresentou o tamanho menor (44,17 ± 0,12) nm e a eficiência de aprisionamento mais elevada (EE% 92,02%).

Palavras-chave: Retinopatia diabética; Edema macular diabético; Celecoxib; Vitamina ETPGS; Pluronic F-127; Formulação nanomicellar tópica; liberação de fármacos oftálmicos; Tecidos do segmento posterior;

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ABBREVIATIONS

AS – Anterior segment

ABS – ATP-binding cassette

Abs – Absorvance

AGE`s – Advanced glycation end products

Akt – Protein kinase B

ARPE-19 – Human retinal pigment epithelium cells

BAB – Blood-aqueous barriers

BRB – Blood-retinal barriers

CEB – Corneal epithelial barrier

CMC – Critical micellar concentration

CNV – Choroidal neovascularization

Cox-2 – Cyclooxygenase-2

CSME – Clinically significant macular edema

DLS – Dynamic light scattering

DM – Diabetes mellitus

DME – Diabetic macular edema

DR – Diabetic retinopathy

EDL – Electric double layer

FDA – Food and drug administration

FFR – Fast field reversal

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hep-2 cells – Human epithelial cells

HLB – Hydrophobic lipophilic balance

HUVECs – Human umbilical vein endothelial cells

ICAM – Intercellular adhesion molecule

IC50 – Half maxima inhibitory concentration

LD50 – Median lethal dose

MD – Macular degeneration

MDR – Multi-drug resistance

MNs – Mixed nanomicelles

MRPs – Multi-drug resistance proteins

NPDR – Non-Proliferative Diabetic Retinopathy

NMFs – Nanomicellar formulations

NSAID – Non-steroid anti-inflammatory

ODD – Ocular drug deliver

PALS – Phase analysis light scattering

PDR – Proliferative Diabetic Retinopathy

PEG – Polyethylene glycol

PEO – Poly (oxyethylene)

PF127 – Pluronic F-127PlGF – Placental growth factor

PPO – Poly (oxypropylene)

PS – Posterior segment

P-gp – P-glycoprotein

QELS – Quasi-elastic light scattering

RF\6A – Choroidal endothelial cells

RPE – Retinal pigment epithelium

SFR – Photon correlation spectroscopy

TKRs – Tyrosine kinase receptors

TNF – Tumor necrosis factor

VCAM – Vascular cell adhesion molecule

VEGF – Vascular endothelial growth factor

Vitamin E TPGS – D-α-tocopheryl polyethylene glycol 1000 succinate

ZP – Zeta potential

ZSN – ZetaSitzer Nano

Z–AVE – Z–Average

1. INTRODUCTION

Ophthalmic drug delivery is one of the most interesting and challenging endeavors facing the pharmaceutical scientist. The anatomy, physiology, and biochemistry of the eye render this organ highly impervious to foreign substances [1]. The eye is divided in the anterior and posterior segments, that are constituted by many static and dynamic barriers, that are responsible for the low effectiveness of conventional systems (eye drops, suspensions and ointments) [2]. These formulations mainly target the diseases in the anterior segment, since it cannot reach the posterior segment of the eye. The diseases of posterior segment can be treated with intravenous, intravitreal or periocular drugs administration [1]. The intravenous route may not be able to delivery drugs to posterior ocular tissues in therapeutic concentrations due to various ocular barriers. On the other hand local ocular drug delivery strategies (intravitreal and periocular) are associated with drug administration related side effects [3]. In this situation, the objective of pharmaceutical scientists is develop new ocular drug deliver (ODD) systems, that allow prolonged and localized release of the drug, in order to improve the therapeutic efficacy and patient compliance, and minimize side effects.

The human eye is affected by several diseases, in this work we will give importance to one of the most worrisome, diabetic retinopathy (DR) and the consequent diabetic macular edema (DME). DR and DME are common complications in patients with diabetes and may have a sudden and debilitating impact on visual acuity, eventually leading to blindness [4]. Advanced stages of DR are characterized by the growth of abnormal retinal blood vessels, and DME occur after breakdown of the blood-retinal barrier, resulting in leaking blood vessels and fluid accumulation in the macula area. Current agents therapeutics to treat DR and DME include steroid implants and anti-VEGF injections. Although, they are effective in preventing the progression of the disease, are not very accepted by patients, because they are invasive treatments that may result in side effects [2].

Celecoxib is a sulfonamide non-steroid anti-inflammatory (NSAID) and selective cyclooxygenase-2 (COX-2) inhibitor. It is a lipophilic compound (log P = 3.5), with poorly aqueous solubility [5, 6]. It has been investigated for anti-vascular endothelial growth factor (anti-VEGF) and anti-proliferative effects for the treatment of various diseases, including in ocular disorders such as DR and DME [7]. The celecoxib has been shown inhibit VEGF secretion/expression [8] and proliferation [9] in human retinal pigment epithelium cells (ARPE-19) at concentrations much lower than celecoxib concentrations with cytotoxic effects in these cells. Therefore, the celecoxib can be an alternative therapeutic treatment of

DR and DME.

Intravitreal and periocular routes, can provide significantly higher local levels of celecoxib to the retina [10]. However, the ideal would be to develop an ODD system that is minimal invasive. For this, in this work, we intend to develop a celecoxib-loaded nanomicellar formulation (NMF) that allows to increase the aqueous solubility and to obtain high levels of drug in the posterior ocular tissues (retina/choroid).

The mixed nanomicelles can easily encapsulate and solubilize hydrophobic drugs, besides it has a nanoscale size which allows them to cross some ocular barriers [3].

When applied in the form of a topical eye drop, mixed nanomicelles can easily cross the sclera channels, reach the posterior ocular tissues and release drug, by lipid–lipid interactions between nanomicellar core and lipid bilayer of Bruch`s membrane and retinal pigment epithelium (RPE) [11].

To obtain the NMFs, we selected two polymeric surfactants approved by FDA (D-α-tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS) and Pluronic F-127 (PF 127)) [12]. Vitamin E TPGS is a amphiphilic polymer. It has a hydrophobic lipophilic balance (HLB) value of 13,0 and critical micellar concentration (CMC) of 0.02 wt % [13]. PF 127, is a copolymer with a HLB value of 22 and a low critical micellar concentration (CMC) of $2.8x10^{-6}$ [14]. The combination of Vitamin ETPGS with PF 127 allows to lower the CMC of the mixed nanomicelles and consequently increase their stability [15]. In the pharmaceutical industry, Vitamin E TPGS and PF 127 are employed to solubilize hydrophobic drugs, modulate drug pharmacokinetics, improve bioavailability and inhibit multi-drug efflux pumps (P-glycoprotein, P-gp) [12, 16, 17]. The inhibition of P-gp by Vitamin E TPGS and PF 127 monomers, after disruption of micelles, may help achieve higher drug accumulation into the posterior ocular lipid tissues.

2. PROPOSED OBJECTIVES AND DISSERTATION ORGANIZATION

The work developed in this thesis aimed at achieving the following specific objectives:

I. Synthesis of clear topical nanomicellar formulations with different ratio of Vitamin E TPGS/PF127;

II. Characterization of clear topical celecoxib-loaded nanomicellar formulations by size, PDI, zeta potential, drug-loading, entrapment efficiency and appearance;

III. Selection of the most appropriate formulation for the ODD system targeted for the treatment of Diabetic Retinopathy.

In this context, the thesis is divided into 7 chapters:

- In the first chapter a brief introduction is made that contextualizes the work;
- In the chapter 2 are carried out a description of the specific objectives and a dissertation presentation;
- In the chapter 3 is carried out a brief review about the following topics: human eye, diabetic retinopathy, celecoxib, nanomicellar topical formulations and conjunctival/scleral pathway, that served as support to the experimental work;
- In the chapter 4 is made the presentation and the description of the materials and methods used in experimental work;
- The chapter 5 is dedicated to on experimental work of dissertation, the obtained results and respective discussion;
- The chapter 6 is dedicated to the conclusions of the work, and finally the future research perspectives are outlined (chapter 7).

3. GENERAL CONSIDERATIONS

3.1. Eye

3.1.1. Anatomy and physiology of the eye

The eye ball has a specific and robust anatomy, physiology and biochemistry, which allows to become highly impermeable foreign substances. Its composition involves three layers: connective, vascular and neural tissues. The connective tissue consists of transparent cornea connected to white sclera through the limbus. The vascular tissue is composed of the choroid as well as two ciliary bodies in the middle connected by the iris in front of the globe. The neural tissue constitutes the retina, which has the function of transmitting the electrical impulse to the brain through the optic nerve [18, 19].

The iris divides the eye into anterior and posterior segments, occupying one-third and two-third of ocular tissues, respectively [20]. The anterior segment consists of the cornea, iris, ciliary body, crystalline, aqueous humor, and conjunctive. The posterior segment contains the vitreous humor, retina, choroid, sclera and optic nerve (fig. 1) [2, 20].

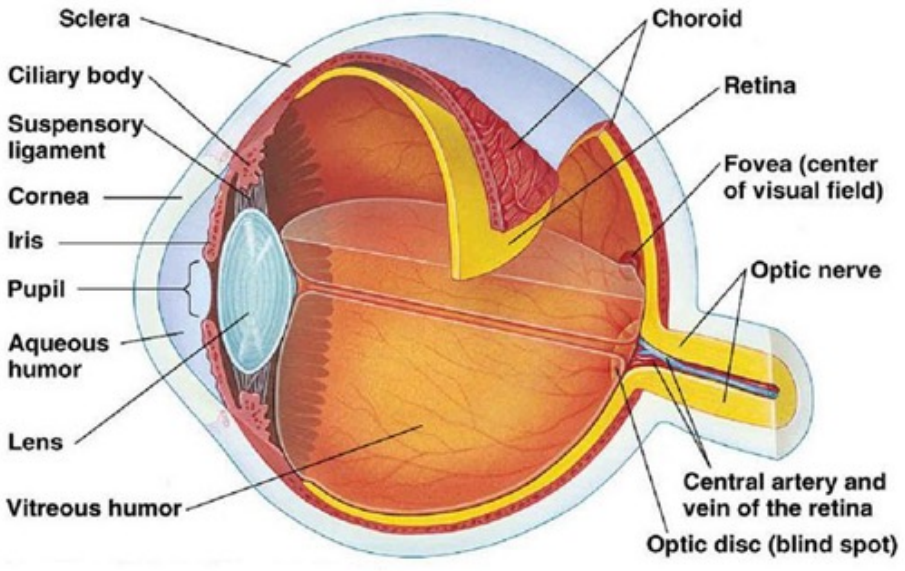


Figure 1- Eye anatomy.

Impeccable functionality of the visual cells is largely dependent upon integrity of the cells/ tissues in posterior and anterior segments of the eye, where selective restrictiveness of the ocular tissues membranes and barriers control the shutting of solutes to maintain the ocular homeostasis. All the biological membranes and barriers selectively regulate cross of locally and/or systemically administered drugs and blood-borne molecules to the anterior and posterior compartments of the eye. The Figure 2 represents a schematic perfect function of the ocular biological membranes and barriers. They can be considered as very robust control machines responsible for the harmonized functioning of the eye. They can be static barriers (e.g., difference layers of the cornea, sclera, iris/ciliary body through blood-aqueous barriers (BAB) and retina through blood-retinal barriers (BRB), dynamic barriers (e.g., precorneal factors, choroidal and conjunctival blood flow and lymphatic clearance), and efflux pumps such as multi-drug resistance (MDR), known as P-glycoprotein (P-gp) and multi-drug resistance proteins (MRPs) [20].

Precorneal factors, which are natural and mechanic barriers, are responsible for this low bioavailability, and these are: solution drainage, blinking, tear film, tear turn over and induced lacrimation [21]. Tear film, which composition and amount are determinants of a healthy ocular surface, offers the first resistance against installed topical pharmaceuticals, due to its high turnover rate. The mucin present in the tear films plays a protective role by forming a hydrophilic layer that moves over the glycocalyx of the ocular surface and clears debris and pathogens [22]. The anatomic volume of cul-de-sac (~ 30μL) and the human tear volume (~7μL), in association with the rapid restoration time (~ 2-3 min), contribute to the elimination of applied eye drops in the ocular mucosa [21].

Cornea is the outermost avascular and transparent part of the eye consisting of three membranes, namely, the epithelium, the inner stroma and the endothelium [23]. The corneal epithelium is naturally lipoid and represents a resistance to permeation of topical administered hydrophilic drugs. The stroma, is made up extracellular matrix and consists of a lamellar arrangement of collagen fibrils. The highly hydrated structure of the stroma represents a barrier to permeation of lipophilic drug molecules. Endothelium is the innermost monolayer of hexagonal-shaped cells. Although the endothelium is a separating barrier between the stroma and aqueous humor, it helps on maintenance of the aqueous humor and corneal transparency due to its selective carrier-mediated transport and secretory function [24].

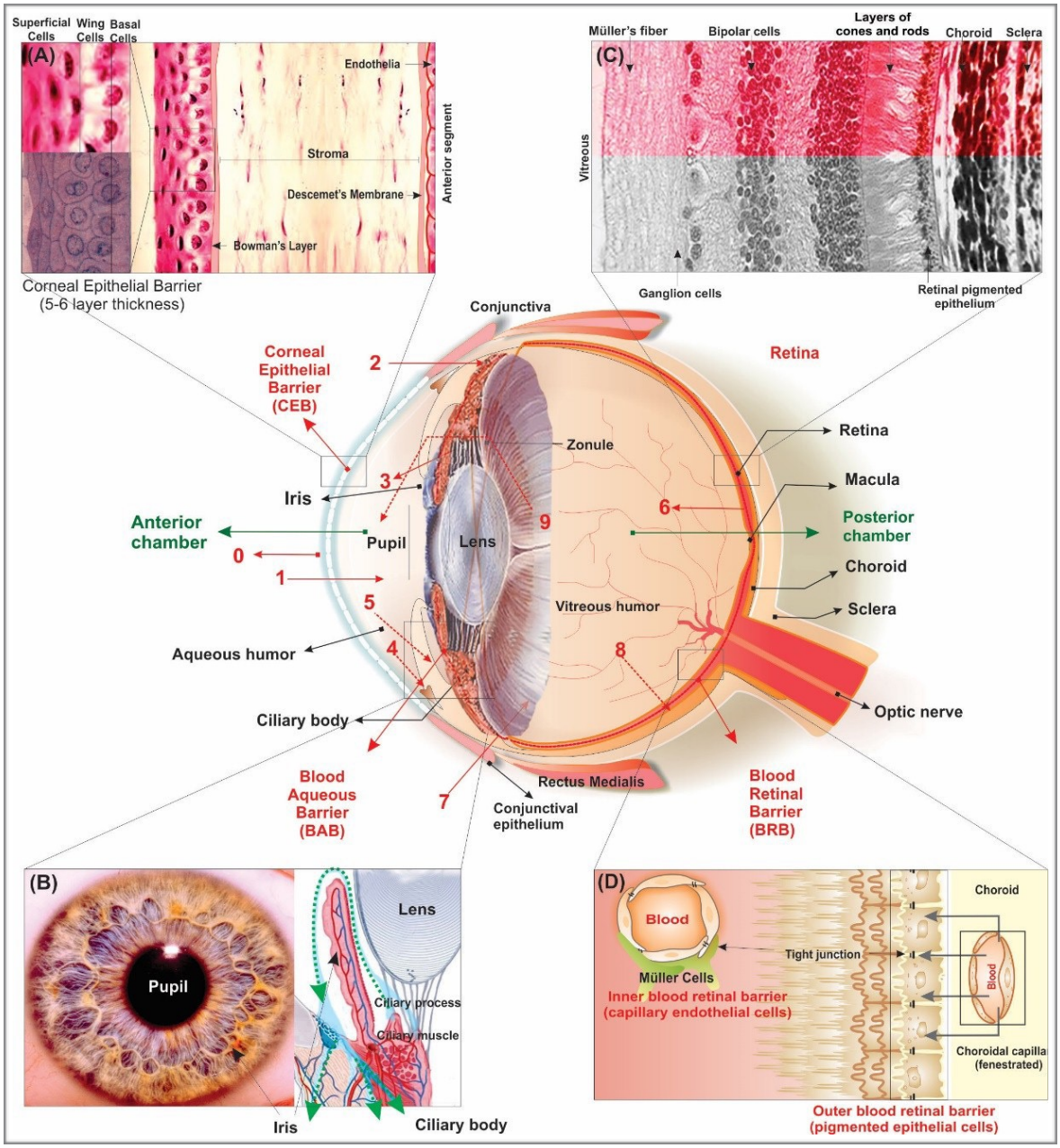


Figure 2 - Schematic demonstration of the anatomy and the biological membranes and barriers of the eye. Panels represent: the corneal epithelial barrier (CEB), the BAB, the biostructures of the retina, and the BRB. The numbers represent: 0- Tear film; 1- Cornea; 2- Conjunctival/scleral route; 3- Iris blood vessels; 4- Aqueous humor outflow; 5- Drug wash-out (BAB function); 6- The systemically administered drugs must cross the BRB; 7- Intravitreal delivery directly into the vitreous; 8- Drugs wash-out by the retinal blood vessels; 9- Drugs within the vitreous can be diffused into the anterior chamber [20].

Conjunctiva is a thin transparent mucous epithelial barrier, lines the inside of the eyelids (palpebral conjunctiva), and covers the anterior one-third of the eyeball (bulbar conjunctiva). The joining area between palpebral and bulbar conjunctiva is referred to as the fornix (forniceal conjunctiva). The conjunctiva is composed of two layers: an outer epithelium and its underlying stroma. The epithelium is covered with microvilli and consists of stratified epithelial cells with 5-15 layers. The epithelial cells at the apical side connect with each other by tight junctions that play a role in permeability barrier. Conjunctival epithelial tight junctions can further retard passive movements of hydrophilic molecules. The stroma (containing structural and cellular elements, including nerves, lymphatics and blood capillaries) loosely attaches to the underlying sclera [25]. The lymphatics and blood capillaries can drug wash-out for the systemic circulation, thereby lowering ocular bioavailability. The molecules that escape the conjunctival vasculature permeate through the sclera and choroid to reach the neural retina and photoreceptor cells [26]. The conjunctiva contributes to the formation of the tear film by way of secreting substantial electrolytes, fluid, and mucin [22].

Sclera is the outermost layer of the eye, performs many key functions. Apart from offering protection to the interior ocular structures, it determines the final shape and size of the eye, serves as the anchor for extra ocular muscles and provides support for the ciliary muscle, which subserves lenticular accommodation. It also provide channels for blood vessels and nerves serving intraocular structures and allows the exchange of fluids, including aqueous humor entering the choroid via the uveoscleral pathway [27]. The sclera, mainly, consists of collagen fibers and proteoglycans embedded in an extracellular matrix and it is more permeable than the cornea. The sclera permeability is independent of lipophilicity unlike the corneal and conjunctival layers, but mainly depends on the molecular ray [12].

Choroid is a highly vascularized and pigmented structure localized between the *lamina fusca* of the sclera and the RPE, extending anteriorly from *ora serrata* to the optic nerve posteriorly. The choroid provides the blood supply to the retina. It is composed of the choriocapillaris, the basal membrane of which forms the outer part of the 5-laminar structure of Bruch´s membrane, the middle layer of medium-sized vessels (Sattler´s layer), the outer layer of large vessels (Haller´s layer) and the supra-choroid, limited externally by the *lamina fusca* [28]. The choroid is a significant barrier, since higher choroidal blood flow can also eliminate a considerable fraction of the drug before it reaches the neural retina [29].

Blood-aqueous barrier (BAB), consists of two discrete cell layers located in anterior segment of the eye (the tight capillary endothelium of the iris/ciliary blood vessels and the no pigmented ciliary epithelium) [30, 31]. The iris acts as a variable aperture to control the light that enters the eye and the ciliary body epithelium is responsible for secretion of aqueous

humor into the posterior chamber of the eye. It´s extends posteriorly from the iris root to the retina, forming a ring around globe. The ciliary body is highly vascularized by the choroidal capillaries, which are fenestrated and leaky [32]. The choroidal capillaries are continuous, contain tight junctional complexes and prevent the entry of solutes into the intraocular environment such as the aqueous humor [29].

Blood-retinal barrier (BRB), is composed of two types of cells, the RPE and the retinal capillary endothelial cells, known as the outer and inner blood-retinal barrier, respectively [33]. The RPE located between the neural retina and the choroid, is a monolayer of highly specialized cells. RPE aids in biochemical functions by selective transport between photoreceptors and choriocapillaries. Furthermore, its maintains the visual system by uptake and conversion of retinoid [26]. The inner BRB located within the inner layers of the neuroretina (ie, ganglion nerve cell layer), is formed by tight junctions between nonfenestrated capillary endothelia covered by astrocytes and Muller cell foot projections [30]. Tight junctions are viewed as controllers of the paracellular transport limiting the selective diffusion of ions and small solutes through the space between neighboring cells, there are part of the the apical junctional complex, that also includes the adherents junctions and the gap junctions [30]. These junctional complexes restrict intracellular permeation of the therapeutic agents from blood into the posterior segment [26].

Efflux pumps identified on ocular tissues include P-gp and/or multi-drug resistance associated proteins (MRPs). P-gp is the product of the multi-drug resistance 1 (MDR1) gene, an ATP-binding cassette (ABS) superfamily of proteins [34, 35]. In eye, the P-gp is predominantly located in the apical membranes of corneal epithelium [36], retinal pigment epithelium and capillary endothelium [37]. It protects cells from cytotoxic compounds by actively transporting them out of the cell against a concentration gradient, thereby reducing intracellular levels below their effective and/or toxic concentrations [34, 35]. P-gp has an affinity to efflux lipophilic compounds in normal as well as in cancerous cells, possibly leading to emergence of drug resistance. MRPs works in a similar manner but effluxes organic anions and conjugated compounds [29].

3.1.2. Main routes of drug administration in the eye

The main routes of drug administration in the eye are: topical, oral/systemic, intravitreal, intracameral, subtenon and periocular (subconjunctival, peribulbar and retrobulbar) administrations (fig. 3 and table 1).

Topical administration, mostly in the form of eye drops, is employed to treat anterior segment disease. However, due to the precorneal factores and corneal barriers less than 5% of the administered drug is absorbed by the tissues, being necessary several administrations to maintain the therapeutic levels [27].

Drugs administered *systemically* reach the chorioretinal tissue through the bloodstream. However, their entry is greatly limited by the blood-aqueous and the blood-retinal barriers.To maintain therapeutic concentrations, a large amount of drug is required, that may lead to side effects [38].

The *periocular route* includes subconjuntival, subtenon, retrobulbar and peribulbar administration. It is safer than intravitreal route, but less effective in providing drugs to retinal tissues, due to choroidal and conjuntival blood circulation [39].

Unlike periocular injection, *intravitreal injection* offers distinct advantages as the molecules are directly inserted into de vitreous. However, multiple injections may be required, as a result of the limited half-life of many compounds in the vitreous, that can increase the risk of cataracts, retinal detachment, hemorrhage and endophthalmitis [40].

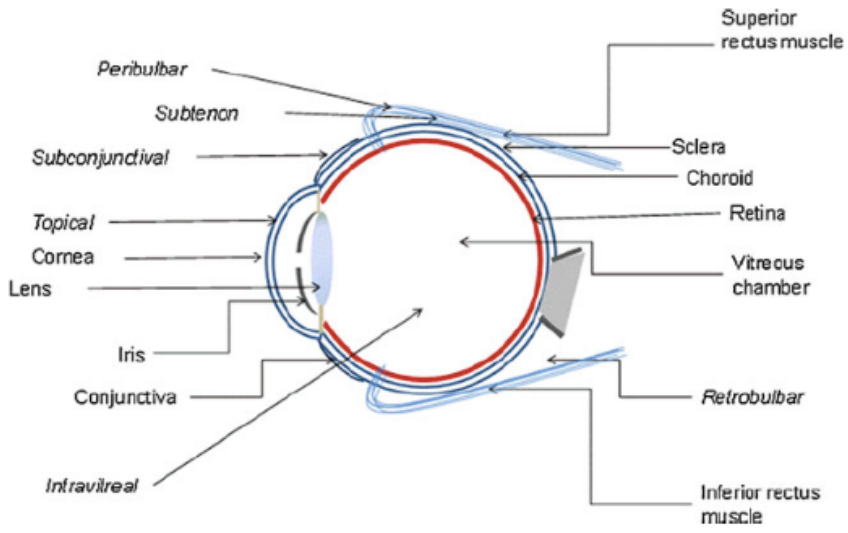


Figure 3 - Routes of drug administration to eye [27].

Table 1- Summary of routes of administration, benefits, challenges and applications in the treatment of disease [27].

Route of administration	Benefits	Challenges	Applications in treatment of disease
Topical	• Hight patient compliance • Sel-administration • Noninvasive	• Higer tear dilution and turnover rate • Cornea acts as barrier • Efflux pumps • BA<5%	• Keratitis • Uveitis • Conjuntivitis • Scleritis, • Episcleritis • Blepharitis
Oral/systemic	• Patient compliance • Noninvasive	• BAB and BRB • High dose causes toxicity • BA<2%	• Scleritis, • Episcleritis • CMV retinitis • PU
Intravitreal	• Direct delivery to the vitreous and retina • Sustain drug levels • Evades BRB	• Retinal detachement • Hemorrhage • Cataract • Endophthalmitis • Patient incomplience	• BRVO • AMD • CRVO • CME • UME
Intracameral	• Delivery to the anterior and posterior segment	• TASS • TECDS	• Anesthesia • Prevention of endophthalmitis • inflamation
Subconjunctival	• Anterior and posterior segment • Site for depot formulations	• Conjuntival and choroidal circulation	• Glaucoma • CMV retinitis • AMD • PU
Subtenon	• High vitreal drug levels • Relatively noninvasive • Fewer complications unlike intravitreal delivery	• RPE • Chemosis • Subconjunctival hemorrhage	• DME • AMD • RVO • Uveitis
Retrobulbar	• Administer high local doses • More efective than peribulbar • Low IOP influence	• Retrobulbar hemorrhage • Globe perforation • Respiratory arrest	• Anesthesia

AMD - Age-Related Macular Degeneration, BA - Bioavailability, BAB - Blood-Aqueous-barrier, BRB - Blood-Retinal-Barrier, BRVO - Branched Retinal Vein Occlusion, CME - Cystoid Macular Edema, CMV - Cytomegalovirus, CRVO - Central Retinal Vein Occlusion, IOP - Intraocular pressure, PU - Posterior Uveitis, RVO - Retinal Vein Occlusion, TASS - Toxic anterior segment syndrome, TECDS - Toxic endothelial cell destruction syndrome, UME - Uveitis Macular Edema.

3.1.3. Recent developments in ocular drug delivery (ODD)

Table 2 - Summary of ODD technologies in development as well as some clinical trials and the respective diseases referring to these new systems.

ODD Technology	Description	NCT	Phase	Last Update	Indications
Punctal plug	• Non-invasive • Deliver in AS • Drug loaded polymeric medical device	NCT02014142	II	Jan. 16, 2017	• Ocular hipertention
Polymeric implants	• Invasive • Typically biodegradable • Deliver in PS • Drug containing polymeric construct	NCT02556424	III	Sep.13, 2016	• Inflamatory macular edema
Refillable implants	• Invasive • Non-degradable • Deliver in PS • Refillable drug contains reservoir	NCT02510794	II	Oct. 3, 2016	• MD
Encapsulated cells implants	• Invasive • Non-degradable • Deliver in PS • Cell containing reservoir	NCT01530659 NCT02862938 NCT00447954	II II II	Oct. 26, 2016 Sep. 14, 2016 Nov. 15, 2016	• Retinitis pigmentosa • Glaucoma • MD
Topical inserts	• Non-invasive • Non-degradable • Deliver in AS • Drug containing soft elastomers	NCT01915940	II	Aug. 8, 2016	• Glaucoma
Gene therapy	• Invasive • Deliver in PS • Viral vector based delivery system	NCT02407678 NCT03066258	II I	May 2, 2017 May 30, 2017	• CH • MD
Nanotechnology (liposomes, cyclodextrins, microemulsions, etc)	• Topically or injectable • Biodegradable • Deliver in AS and PS	NCT02992392 NCT02908282	I I	Dec. 14, 2016 Nov. 18, 2016	• Dry eye • Dry eye

AS - Anterior Segment, CH - Choroideremia, DME - Diabetic Macular Edema, MD - Macular Degeneration, NCT - Patent Number, PS - Posterior Segment.

3.1.4. Eye diseases

The anterior segment tissues are directly exposed to the external environment and thus susceptible to different diseases [41]. These common diseases are: dry eye, pain, redness, discharge, allergic conjunctivitis (bacterial, viral, and fungal infections), blepharitis (inflammation of the eyelid) [42]. The main diseases that affect the posterior segment are: retinoblastoma, choroidal neovascularization (CNV, growth of the choroidal capillaries below the retinal epithelium) [41], and degenerative retinal diseases (formation of new blood vessels in the retina), such as: diabetic retinopathy, diabetic macular edema and age-related macular degeneration [43]. The inflammation of uvea is called as anterior or posterior uveitis, depending on if inflammation affects iris-ciliary body or choroid, respectively [42]. Glaucoma is also a disease that affect both segments, is characterized by ocular tension of anterior segment tissues and/or elevated intraocular pressure after degeneration of retinal ganglion cells [41].

3.2. Diabetic Retinopathy (DR) and Diabetic Macular Edema (DME)

3.2.1. Prevalence and characterization of DR and DME

DR is one of the most common complications of diabetes mellitus (DM) and the leading cause of blindness and visual dysfunction in working-age populations [4].

According to estimates of the World Health Organization, the number of people worldwide with DM will rise to 360 million by the year 2030; all of these individuals will be at risk of developing DR [44]. The prevalence rate for retinopathy for all adults with diabetes aged 40 and older in the worldwide has been estimated at 34,6% (93 million people) [45].

In Portugal according to the Annual Report of the National Diabetes Observatory, in 2015 the estimated prevalence of DM in the Portuguese population aged 20-79 (7.7 million individuals) was 13.3%, that is, more than 1 million of Portuguese people [46]. About the prevalence of DR in Portugal there are few studies, only the RETINODIAB, which consisted of the evaluation of the DR screening program in the Lisbon and Vale do Tejo Health Region, implemented between July 2009 and December 2014, assessed the prevalence in this target-population (52739). Overall, DR was detected in 8584 patients (16.3%) [47].

DR is a progressive condition with microvascular alterations that lead to retinal ischemia, retinal permeability, retinal neovascularization, vitreous hemorrhage and DME [48].

The DR is classified in 5 stages (table 3), being 3 of low risk (without apparent retinopathy, slight non-proliferative DR and moderate non-proliferative DR) and 2 of higher risk (severe non-proliferative DR and proliferative DR (fig. 4 - B, C respectively) [49].

DME represents the major cause of moderate visual loss from DR. The main molecular mechanism underlying DME is the disruption of the BRB by phosphorylation of the junctional proteins. DME is an important sign that is assessed separately from the stages of retinopathy, because it can develop at any stage of DR [50]. Edema (fig. 4 - D) develops clinically in the macular region and localizes, histologically, between retinal photoreceptors.The presence of DME does not always cause visual decline. Vision loss is determined by the localization, duration, and severity of DME [50].

Clinically significant macular edema (CSME) is a subset of the DME stage and is defined as retinal edema localized close to, on the fovea or of large enough size anywhere in the macular region [51]. This definition is used to determine a patient's risk of moderate visual loss. Presence of CSME increases risk of moderate visual loss over a 2-year period. DME involving the fovea is most associated with visual acuity decline [50].

Table 3 - Classification of DR by severity [45, 48]

Disease severity level	**Defining features**
No apparent retinopathy	No microvascular lesions
Mild NPDR	Microaneurysms only
Moderate NPDR	Microaneurysms and other microvascular lesions, but not severe NPDR
Several NPDR	More than 20 intraretinal hemorrhages in four quadrants or venous beading in two or more quadrants, or intraretinal microvascular abnormalities in one or more quadrant but not PDR
PDR	One or both of the following; neovascularization and vitreous/preretinal hemorrhage
Clinically significant macular edema	Retinal thickening or hard exudates within 500 μm from centre of macula
NPDR- Non-Proliferative Diabetic Retinopathy, PDR- Proliferative Diabetic Retinopathy.	

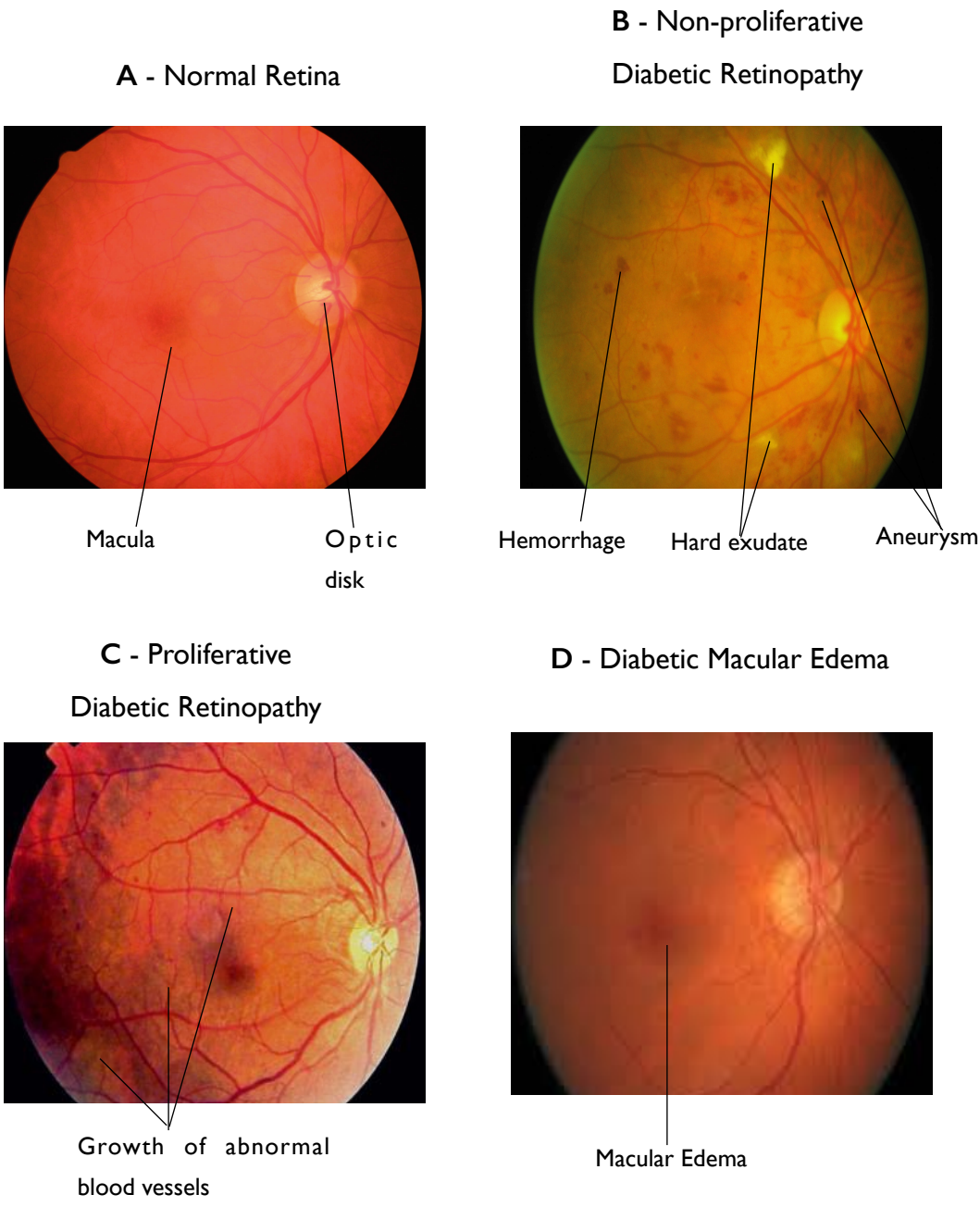


Figure 4 - Schematic representation of normal retina (**A**), non-proliferative DR (**B**), proliferative DR (**C**) and DME (**D**).

3.2.2. Pathophysiology of DR and DME

The main risk factors for DR, include duration of diabetes, chronic and sustained hyperglycemia, hyperlipidemia and hypertension [52]. The mechanisms by which these risk factors causes tissue injury and disease progression in the retina are not fully understood. However, studies have shown that DR is a multi-factorial disease involving multiple biochemical mechanisms, including aldose reductase pathway, oxidative stress, activation of protein kinase C, formation of advanced glycation end products (AGEs) and increasing the flux of glucose to sorbitol through the polyol and hexosamine pathways [53, 54]. These mechanisms have in common induced retinal inflammation and up regulation of VEGF, which are interlinked [55].

3.2.2.1. Role of inflammation in DR

The inflammation is a process that involves multiple mediators such as pro-inflammatory cytokines (tumor necrosis factor (TNF)-α and interleukins), chemokines (CCL2, CCL5), adhesion molecules (E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1), that initiate the interaction between leukocytes and the endothelium and guide directional leukocyte migration towards infected or injured tissue [53]. Leukocytes are the cell class responsible for innate immunity (neutrophils, monocytes, eosinophils, basophils, and mast cells) and adaptive immunity (T lymphocytes, B lymphocytes, and natural killer cells), effectively protect the host from infectious pathogens but also induce tissue injury in certain diseases. Among these cells, neutrophils and monocytes are very likely to induce microvascular injury because they have high probability to attach to the vessel wall and block blood flow due to their large population and size [53]. This leukostasis has been found to be significantly increased in retinas of diabetic animals, and might contribute to the capillary non perfusion, angiogenesis and vascular permeability in DR [56].

3.2.2.2. VEGF a central role in development of DR and DME

VEGF family in humans consist of several proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) [57, 58]. All of them act by binding with specific receptors that

are typical tyrosine kinase receptors (TKRs) [59]. VEGF-A is a main factor for physiological and pathological angiogenesis [60]. It is a heparin-binding homodimeric glycoprotein of 36-46 kDa weight [61]. VEGF-A is responsible for increased vascular leakage in the initial stages of DR and neovascularization, both in the DME and PDR [62]. In the initial stages, the up regulation of VEGF-A may lead alterations in the inner BRB, that normally, at the retinal capillary level is composed of pericytes that cover the vessels outside, endothelial layer, and basement membrane in between these cells. These alterations may be selective loss or drop-out of pericytes, disfunction and apoptosis of retinal capillary endothelial cells and thickening of the basement membrane [53]. The pericytes are capillary wall cells essencial for vasoregulation (dilation and capilar contraction) and vascular stability [33]. The endothelial cells of normal retinal capillaries have each other tight junctions that prevent the passage of fluids, macro molecules, proteins and lipids for the extra-vascular space [55]. The loss of pericytes and the endothelial disfunction contributes to the rupture of the internal BRB, for the appearance of capilar dilations, formation of micro-aneurysms, capillary occlusion and ischemia. These changes in the internal BRB, provoke the release of cytokines, increase of VEGF, stimulate angiogenesis and consequent increase of vascular permeability by the opening of thigh junctions [50]. In the DME and later stages of DR, the VEGF-A up regulates adhesion molecules (E-selectin, ICAM-1, VCAM-1 and COX-2), which traps the attracted leukocytes blocking the vessel lumen resulting in capillary non-perfusion. Leukostasis and capillary non-perfusion resulting in retinal hypoxia. Hypoxia is a more potent stimulator of VEGF up regulation and it is the initiating factor in the development of retinal new vessels and increase vascular permeability seen in PDR and DME. The increased VEGF up regulation reinitiates the cycle and initiate the development of macular edema and retinal neovascularization [50, 63].

3.2.3. Currently ophthalmic therapeutic options to treat DR and DME

Present ocular treatment used to treat DR revolves around four major strategies: anti-VEGF agents, steroids implants, retinal laser photocoagulation and surgical intervention [52].

As described above the VEGF-A play a central role in angiogenesis and is the main factor for development of DR. In the light of this notion, several anti-VEGF agentes were introduced on the market. These agents, not only inhibit vessel growth and neovascularization, but also induce regression of pathological micro-vessels, stabilize normal vessels, and prevent leakage

and concomitant inflammatory response [63]. They are the first choice for treatment of CSME and PDR. Intravitreal injection of anti-VEGF allows obtain high concentrations in the vitreous but have several limitations, such as: the limited duration of its therapeutic effect, high frequency and the risk associated with their routes of administration and are more expensive [64].

Intravitreal steroids implants have been reported to generate favorable results in the treatment of DR and EMD. Glucocorticoids are a very versatile group of drugs with a broad spectrum of action (anti-inflammatory, anti-apoptotic, anti-edematous and anti-angiogenic effects). However, they are associated with the appearance side effects, such as: cataracts, glaucoma and endophthalmitis [52].

Laser photocoagulation seal or destroy abnormal leaking blood vessels. It is generally indicated in PDR and CSME.This intervention prevents further deterioration of vision if applied sufficiently early in the progression of the disease but does not usually restore lost vision [51].

Surgical treatment is used in advanced cases of PDR, with vitreous hemorrhage, tractional retinal detachment, and extensive fibrous membranes [52]. It is also used to remove the premacular posterior hyaloid from patients with persistent diffuse macular edema [65]. However, the visual outcome after vitrectomy is unpredictable, significant postoperative complications may occur, including the formation of cataracts, recurrent vitreous hemorrhage, retinal detachment and neovascular glaucoma [66].

Table 4 - Summary of current ophthalmic therapeutic options for DR and DME [52].

Category	**Treatment options**
Anti-VEGF agent	• Ranibizumab (Lucentis) • Aflibercept (Eylea)
Steroid implants	• Dexamethasone sustained-release intravitreal implant (Ozurdex) • Fluocinolone acetonide implant (Retisert)
Laser photocoagulation	• Pattern scan laser (Pascal) • Subthreshold diode micropulse laser (SDM) • Retinal rejuvenation therapy (2RT) • Selective retina therapy (SRT)
Surgical treatment	• Transconjunctival sutureless 23- or 25-gauge vitrectomy

3.3. Celecoxib

3.3.1. Characterization of celecoxib

Celecoxib (fig. 5) is a sulfonamide NSAID and selective cyclooxygenase-2 (COX-2) inhibitor approved for the treatment of rheumatoid arthritis, osteoarthritis, juvenile rheumatoid arthritis, ankylosing spondylitis, acute pain, primary dysmenorrhea, and familial adenomatous polyposis [67].

Celecoxib is more selective 300 times for COX-2 than COX-1 [68]. COX-2 is the inducible isoform of COX and predominately involved in inflammatory responses, while COX-1 is constitutively expressed and involved in daily housekeeping functions [69]. Consequently, prolonged selective inhibition of COX-2 may be preferable [70].

Celecoxib belongs to the class 2 of the Biopharmaceutical Classification System, which includes poorly soluble and highly permeable drugs [5]. It is a lipophilic compound (log P = 3.5) [6] with aqueous solubility of 3-7μg/ml at pH 7 and 40°C [5]. Generally, compounds of this nature show dissolution rate limited absorption in vivo. Therefore, improving the solubility of such a drug is expected to enhance bioavailability and hence therapeutic potency. So far, celecoxib has been loaded in lipossomas [71], microparticles [8], nanoparticles [72], microemulsion-based gel for transdermal delivery [73] and self-microemulsifying drug delivery systems [74] for various medical applications.

Treatment with celecoxib has been shown to act on multiple targets and pathways in cancer cells, including proliferation, apoptosis, angiogenesis, invasion and tumor-induced immune suppression via COX-2 dependent and independent mechanisms [75].

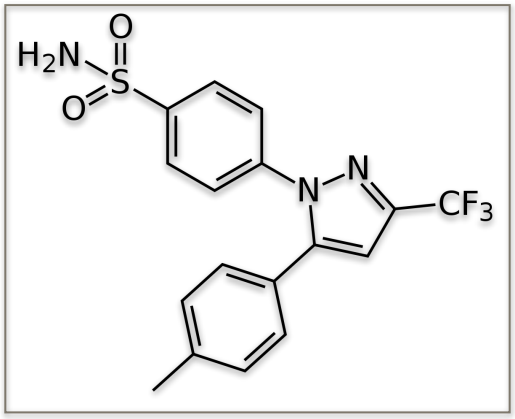


Figure 5 - Chemical structure of Celecoxib.

3.3.2. VEGF inhibition by celecoxib

Celecoxib has VEGF inhibitory effects as demonstrated in several anticancer studies using different cell types, such as: non-small lung [76], human breast [77], human colon [78] and human prostate [79] cancer cells. Through inhibition of Cox-2, celecoxib can significantly reduce the levels of prostaglandins in various tissues [56].

Diabetes induced inflammation leads to the induction of COX-2 expression [53]. The expression of COX-2 is elevated in diabetic subjects with proliferative retinopathy [80] and in experimental diabetic rat [81] and mouse [82, 83] models. COX-2 is the product of an immediate early gene that is rapidly inducible and tightly regulated. It is involved in the metabolism of arachidonic acid to prostaglandins [84]. Prostaglandins, in turn, stimulate the expression and secretion of VEGF in tumor cells [85], cultured retinal cells [86], and others human cultured tissues [87, 88]. Many cell types in the eye including the retinal pigment epithelial cells, retinal capillary pericytes, retinal microvascular endothelial cells, Müller cells, neurons and ganglion cells produce VEGF [89]. Thus, inhibition of the Cox-2 enzyme system is likely to reduce prostaglandin generation, VEGF expression and vascular abnormalities.

Ayalasomayajula and Kompella reported that oral celecoxib (50 mg/kg) inhibited retinal VEGF mRNA expression and decreased retinal vascular leakage in the diabetic rat models [90].

Amrite et al. [8] determined whether celecoxib inhibits VEGF expression in cultured human RPE cells (ARPE-19). After a 12-hour quiescence period, ARPE-19 cells were exposed to celecoxib ranging in concentration from 100pM to 10μM. Secreted VEGF was measured at the end of 12 hours of treatment. The results show a statistically significant inhibition of the VEGF mRNA expression in ARPE-19 cells with 0,001μM of celecoxib. There was a dose dependent decrease in VEGF mRNA expression as well as VEGF protein secretion with increasing concentrations of celecoxib (fig. 6). The inhibition of VEGF was not due to cytotoxicity, because the concentrations of celecoxib having cytotoxic effects on these cells are much higher.

Another study [91], investigate the anti-angiogenic effects of periocular administration of celecoxib, on the expression of VEGF in diabetic mouse models. The results show a positive correlation. The administration of celecoxib can significantly reduce ocular VEGF levels in diabetic mouse model.

This VEGF inhibitory effect of celecoxib has potential value in treating DR and other VEGF induced neovascular conditions of the eye.

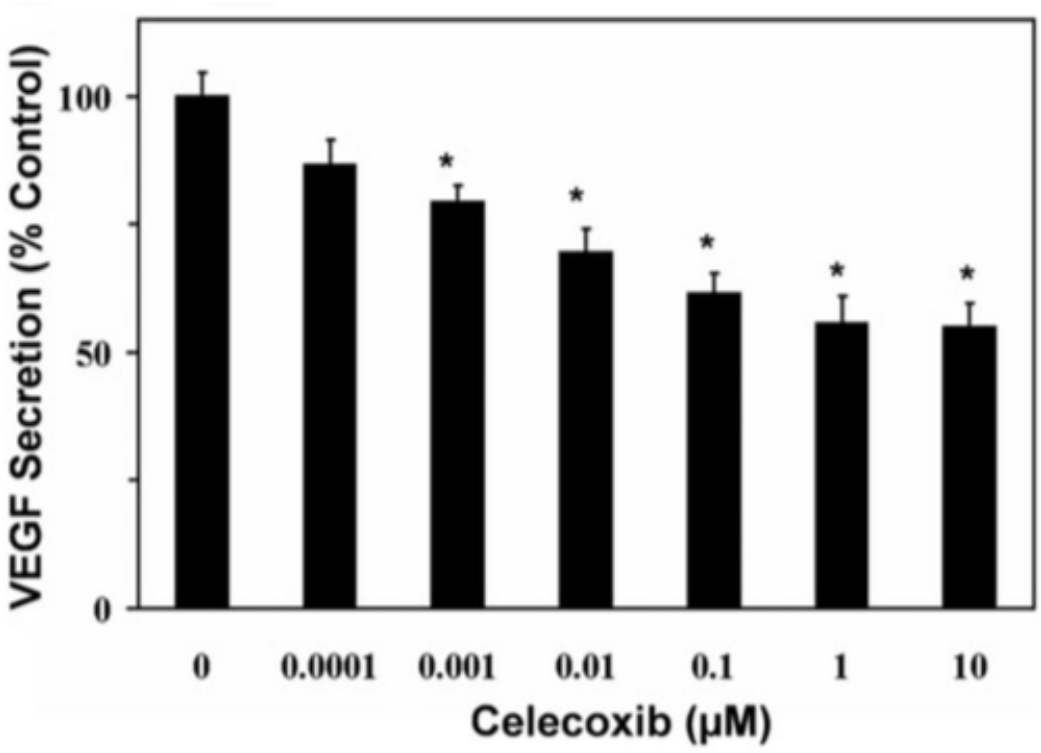


Figure 6 - Celecoxib inhibits VEGF secretion from ARPE-19 cells. Data are expressed as the mean ± SD of results in eight experiments. * Significantly different from the control [8].

3.3.3. Antiproliferative effects of celecoxib

When the RPE, endothelial cells and choroidal vasculature are under the influence of the pathologic growth factors, begin to proliferate and consequently develop proliferative diabetic retinopathy (PDR). Anti-proliferative drugs act on specific phase of the cell cycle, and target the proliferating cells, that could be a benefitial approach for the treatment of PDR [56].

The second function of celecoxib, is the potential to inhibit cell proliferation and stimulate apoptotic cell death at much lower concentrations than any other coxibs [92].

Celecoxib has been demonstrated to have antiproliferative effects on several cell types including, human umbilical vein endothelial cells (HUVECs) [93, 94], Hep-2 cells [95], human breast cancer cells [96] and ovarian cancer cells [97].These antiproliferative effects appear to

be Cox-independent since that, other selective COX-2 inhibitors have no effect on cell proliferation, apoptosis or cell cycle distribution [99].

The possible mechanisms (fig. 7) for the antiproliferative effect may be the inhibition of protein kinase B (Akt) signalied in these cells by celecoxib [9]. The Akt pathway is important for regulation of cell survival, growth, differentiation, metabolism, proliferation and angiogenesis, is present in all cells of higher eukaryotes and is highly conserved [98].

Amrite et al. [9] investigated the antiproliferative effects of celecoxib in adult retinal pigment epithelial cells (ARPE-19 cells) and the choroidal endothelial cells (RF\6A cells). The results indicated that celecoxib caused a dose-dependent antiproliferative effect in ARPE-19 and RF\6A cells (IC_{50} of 23 and 13 µM, respectively). The concentrations of celecoxib required for antiproliferative effects are lower than dose required for the cytotoxicity. These effects of celecoxib act by mechanisms independent of its Cox-2 inhibitory activity, because rofecoxib (another Cox-2 inhibitor) had no effects on the proliferation of the two cell types, and flurbiprofen (an inhibitor of Cox-1 and Cox-2) had weak antiproliferative effects on ARPE-19 cells, with IC_{50} of 90 µM.

This antiproliferative effect of celecoxib could be benefitial in the treatment of the proliferative stages of DR.

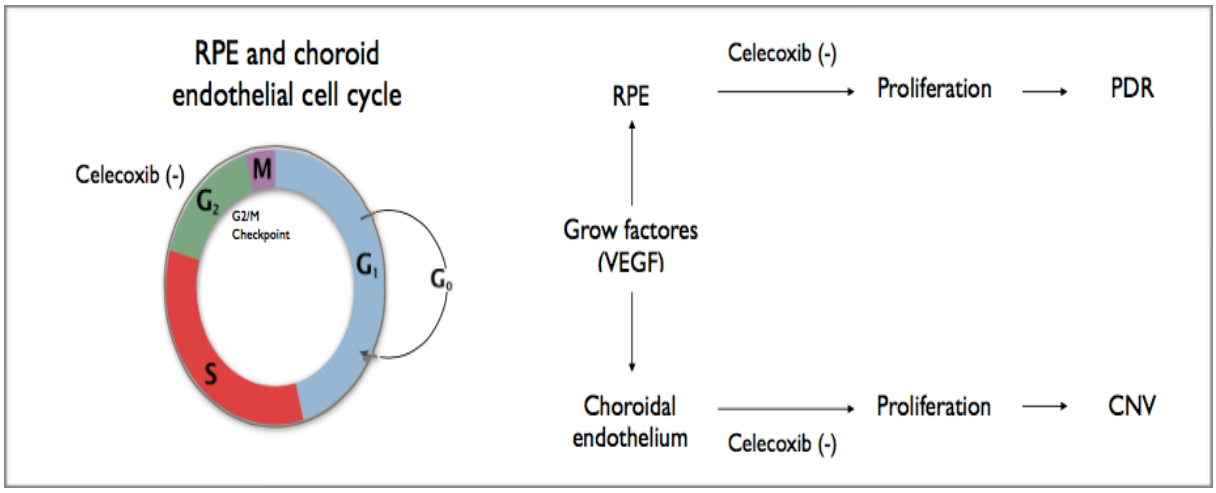


Figure 7 - Proposed mechanism for the use of celecoxib in choroidal neovascularization and RPE proliferation. The (-) indicates inhibition by celecoxib [9].

3.4. Nanomicellar topical formulation in ocular drug delivery

3.4.1. Nanomicelles

A molecule possessing both hydrophilic (polar) and hydrophobic (non polar) groups is know as an amphiphilic molecule. These molecules, when exposed to a suitable solvent guide to self-assemble and form normal or reverse nanomicelles depending on the type and degree of orientation. The hydrophilic portion orients towards the polar solvent whereas the hydrophobic portion of the molecule orients away from the solvent. Due to this guidance, the hydrophobic parts are clustered in the core while the hydrophilic portions are allied towards the outer surface to maximize contact with water. Those clustered aggregates are termed as normal nanomicelles. On the other hand, when amphiphilic molecules are exposed to a hydrophobic solvent, tend to form nanomicelles with hydrophobic region towards outside and the hydrophilic portions towards inside [3].

Normal nanomicelles can be utilized to encapsulated, solubilize and delivery hydrophobic drugs, while reverse nanomicelles can be applied to encapsulated and act as better candidates for delivery of hydrophilic drugs [100].

Nanomicelles are formed in various size (10 nm to 200 nm) and shapes (spherical, cylindrical and star-shaped, etc.) depending on the molecular weights of the core and corona forming blocks. The self-assembly take place above certain concentration, referred to as critical micelle concentration (CMC) [101] (fig.8).

Nanomicelles serve as excellent drug delivery systems owing to their ability to minimize drug degradation, reduced adverse side effects and improve drug bioavailability [100, 102]. Nanomicelles can be formed with surfactants or polymers [3]. General polymeric nanomicelles are more stable than surfactants nanomicelles, due to the low CMC [103].

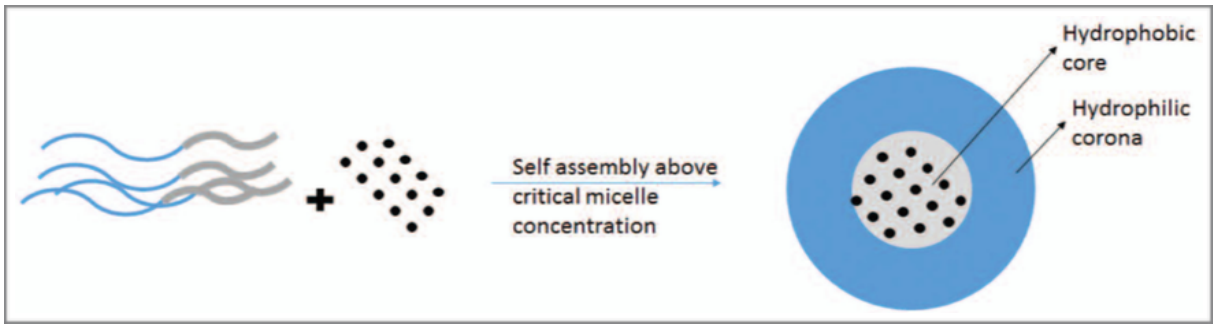


Figure 8 - Schematic illustration of formation of spherical micelle and drug encapsulation [101].

3.4.2. Methods of micelle preparation

In ocular drug delivery (ODD), nanomicelles offer unique advantages explained by their nanoscale size, aqueous clear/transparent drug formulation, encapsulation and solubilization hydrophobic drugs and enabling hight permeation through ocular barriers with minimal or no irritation [3].

A successful ODD system based on nanomicelles, should has the following key characteristics: stabilization, efficiently and reversible drug loading, prolonged retention and circulation time, biodegradability and/or biocompatibility [103].

There are several important atributes that must be deliberated to rational design nanomicelles for ODD system. Some of the important factors are site of action, polymer composition, drug loading, release rate, nanomicelle tissue interaction, size and surface charge. Hydrophobic drug is encapsulated in the micelle core during or after micelle formation depending on the preparation method. The process involves hydrophobic interactions and/or hydrogen bond formation between drug and polymer [104].

The most commonly used methods of micelles formation are: oil in water (O/A) emulsion, direct dissolution, solvent evaporation, lyophilization or freeze drying and dialysis method (fig. 9) [3]. The choice of the appropriated method is typically based on the extend of the solubility of a micelle-forming block co-polymer in aqueous medium [104].

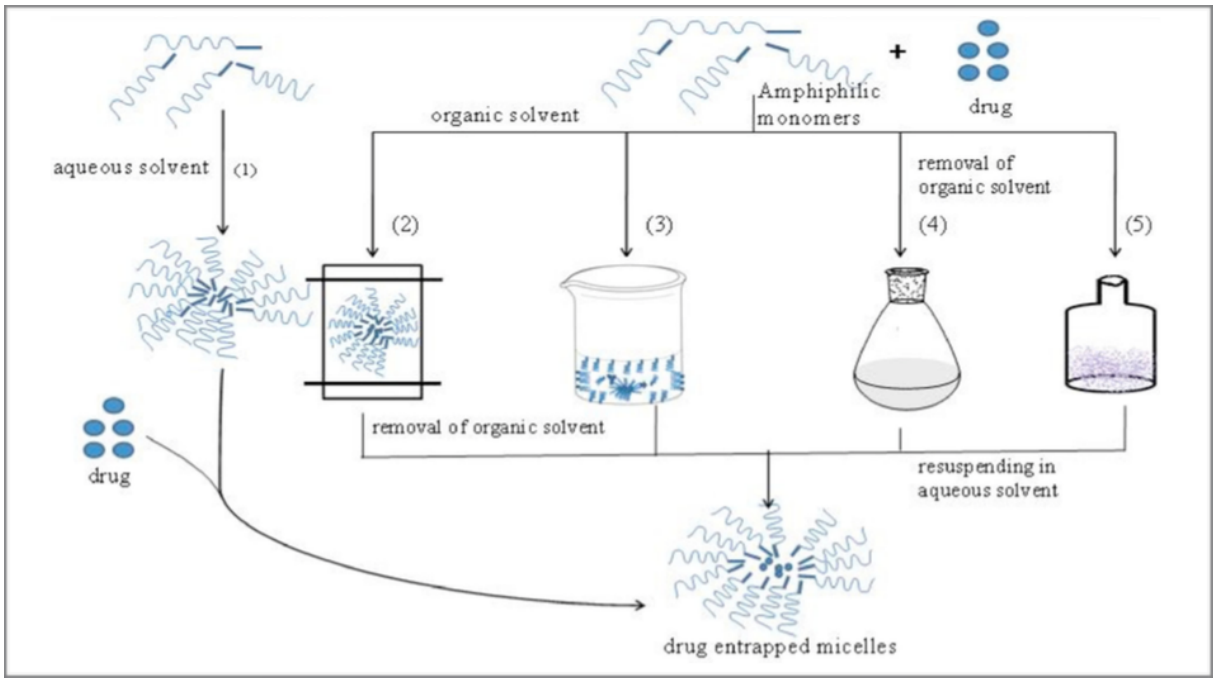


Figure 9 - Micelle preparation methods: (1) simple dissolution, (2) dialysis, (3) O/A emulsion, (4) solvent evaporation and (5) lyophilization or freeze drying [3].

O/A emulsion method consists in preparing an aqueous solution of the copolymer to which the solution of drug in the water-immiscible volatile solvent (e.g. chloroform) is added in order to prepare an oil in water emulsion followed by evaporation of volatile solvent. This method should be avoided when preparing the micelles intended for ocular delivery because it is almost impossible to completely remove the toxic chlorinated solvents during the evaporation process [104].

Direct dissolution is the simplest method of preparation and may be easy to scale up [101]. and it is frequently employed micelle preparation from copolymers with relatively hight water solubility. This technique involves the dissolving of the drug and the block copolymer directly in an aqueous media (distilled deionized water or buffer). The preparation may requires stirring, heating and/or sonication in order to load drug into nanomicelles. Micelles formation is initiated through dehydration of the core forming blocks. This method has been generally employed for moderately hydrophobic polymers such as poloxamers [105].

Dialysis method is, generally, employed for micelle preparation from amphiphilic copolymers with low water solubility [105], drug loading procedure is also useful for copolymers which require a common organic solvent to solubilize. Organic solvent such as dimethylsulfoxide, N,N-dimethyformamide, acetonitrile, tetrahydrofuran, acetone or dimethylacetamide are commonly selected. For this technique, the copolymer and the active agent are dissolved in a common organic solvent and micelle formation is stimulated by the addition of aqueous media (water) to the drug-copolymer mixture. The selection of the solvent significantly influences the physical and the drug encapsulation properties of the micelles [3]. However, to ensure the complete removal of the used organic solvent, the dialysis has to be extended over several days, which is quite inappropriate for industry [104].

In solvent evaporation method, both the copolymer and active agent are dissolved in a common solvent or mixture of two miscible solvents. A drug-copolymer film is then formed upon stirring and drying the mixture. Micelles are spontaneously formed when the film is reconstituted with warm water or buffer. The reconstituted samples may be sonicated or passed through a high-pressure extruder to prevent multimodal size distribution. The extent of drug encapsulation through this method is largely determined by the common solvent initially employed for dissolving both the copolymer and active agent. Phase separation may likely be prevented during the evaporation process when a solvent, which can solubilize both the copolymer and drug, is selected [3]. This method is suitable for the preparation of micelle for ocular drug delivery because the organic solvent can be practically completely removed [104].

Freeze drying is another method for formulating micelles that involves dissolution of both the copolymer and drug ia a mixture of aqueous and organic solvent which is followed by lyophilization. The freeze-dried mixture can be reconstituted to obtain drug loaded micelles. Dimethylacetamida and *tert*-butanol have ben generally employed as co-solventes because of their hight vapor pressure which offers rapid sublimation followed by lyophilization. The micelles produced by this method also demonstrate adequate self-life along with high water dispersibility [3].

The method chosen for formulating drug encapsulated micelles would significantly influence both the physiochemical properties of the micelle and drug encapsulation efficiency. Generally, methods like solvent evaporation and film hydration result in higher encapsulation efficiency than direct dissolution and dialysis methods [101, 104]. Nevertheless, the size and polydispersity indices of the formulations are impacted by the nature of organic phase, the order of addition and the concentration of the copolymer in the organic solvent [3].

3.4.3. Topical application of mixed nanomicelles for posterior segment drug delivery

When an eye drop is applied topically, there are two routes by which the drug can reach the posterior eye tissues, the corneal pathway (cornea – aqueous humor – lens – vitreous humor – retina) and no corneal or conjunctival/sclera pathway (conjunctiva – sclera – choroid – retina) (fig. 10) [41]. The first pathway is often unsuccessful for hydrophobic molecules such as celecoxib, since the hydrophilic stroma becomes the rate-limiting barrier for trans-corneal absorption. Moreover, fluids in the anterior and posterior segments flow in opposite directions, hindering the passage of molecules from the aqueous humor to lens and through to the vitreous humor, thus making this an unfavorable pathway. The second pathway offers a viable strategy to reach the posterior ocular tissues, for hydrophilic molecules by passive diffusion through the scleral aqueous channels/pores [11]. Aqueous solubility of the molecule is an important determinant for its transport across sclera but also for its ability to evade the conjunctival blood and lymph vessel-mediated washout [13]. But again, celecoxib, being a hydrophobic drug, will encounter aqueous sclera as a potential barrier for permeation through the conjunctival/scleral route.

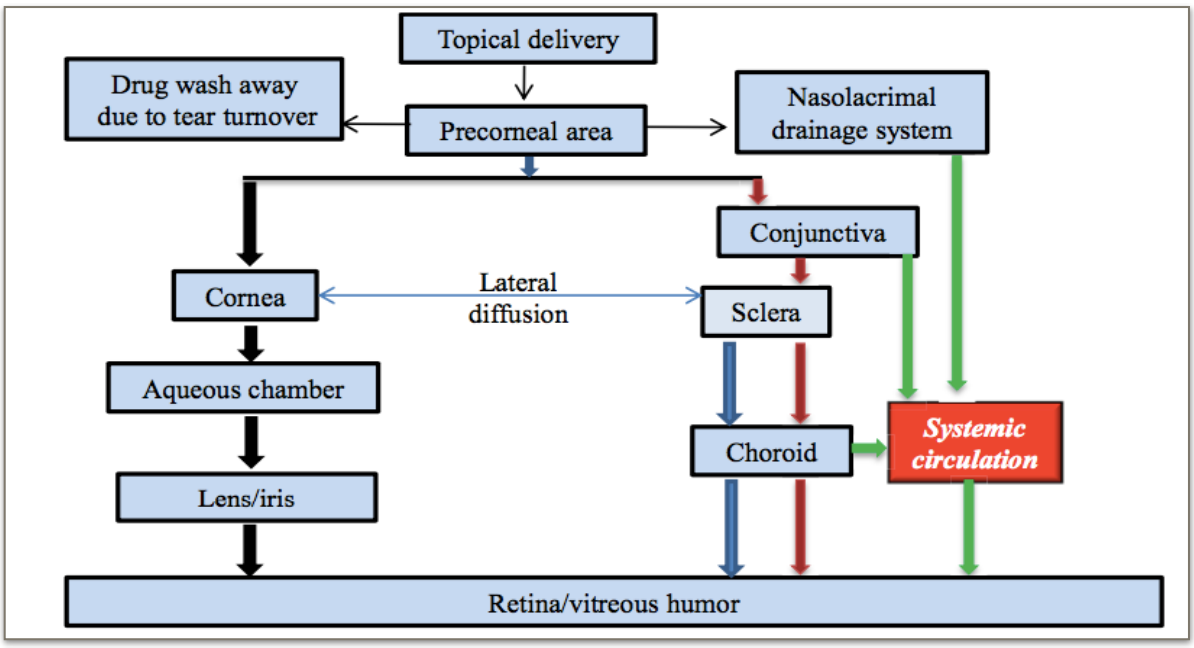


Figure 10 - General ocular penetration routes for topically applied drugs [41].

Mixed nanomicelles are a combination of different types of polymers for increase the solubility and stability of the micelles [106]. They may be an alternative to increase the permeation of celecoxib to reach posterior ocular tissues (retina-choroid). Due to its extremely small size, can penetrate through the conjunctival/scleral pathway after topical application [107]. The higher conjunctival/scleral surface area allows lateral diffusion of such nanomicelles to reach the posterior segment of the eye [103]. Moreover, they are spherical structures with hydrophobic core and hydrophilic corona, that can encapsulate hydrophobic drugs in the core, and thus solubilize the drug. In addition, the hydrophilic corona can minimize drug wash out into systemic circulation by the conjuntival/choroidal blood circulation and lymphatics [107]. Mixed nanomicelles have been used to solubilize several hydrophobic drugs for various applications (table 5).

Table 5 - Summary of the mixed nanomicelles developed with different polymers, as well as the therapeutic agent and nanomicellar size, respectively.

Polymer/Surfactant	Therapeutic agent	Nanomicellar size (nm)	References
Pluronic P105/PF 127	Docetaxel	23	[109]
Pluronic P123/ Vitamin E TPGS	Quercetin	18,43	[110]
Pluronic P105/ Vitamin E TPGS	Camptothecin	—	[108]
mPEG-PCL / Vitamin E TPGS	Resveratrol	26,4	[111]
PF 127/ Vitamin E TPGS	Resveratrol	20	[112]
PF 127/ Vitamin E TPGS	Folic acid	173	[113]
PF 127/ Vitamin E TPGS	Gambogic	17,4	[114]
Soluplus/PF 127	Apigenin	178,5	[115]
Soluplus/Vitamin E TPGS	Dioscin	67,15	[116]
PF 127/plasdone S630	Biochanin A	25,17	[117]
mPEG-PCL - methoxy poly(ethylene glycol)-b-poly-caprolactone, PF 127 - Pluronic 127, Vitamin E TPGS - D-α-tocopheryl polyethylene glycol 1000 succinate.			

Mixed nanomicelles cross the sclera channels and reaching posterior ocular lipid tissues, such as Bruch's membrane and retinal pigment epithelium (RPE), releasing in them the celecoxib by nanomicellar reversal mechanism. The lipid bilayer of RPE and Bruch's membrane establishes lipid-lipid interactions with nanomicellar core, causing celecoxib release into the cells. Furthermore, celecoxib permeation into the hydrophilic vitreous humor is prevented because of the hydrophobic nature of celecoxib, where it gets deposited into the highly lipophilic tissues (RPE, retina) [3, 103]. A hypothetical diagrammatic representation of mixed nanomicelles translocating from topical drop to posterior segment ocular tissues is depicted in figure 11.

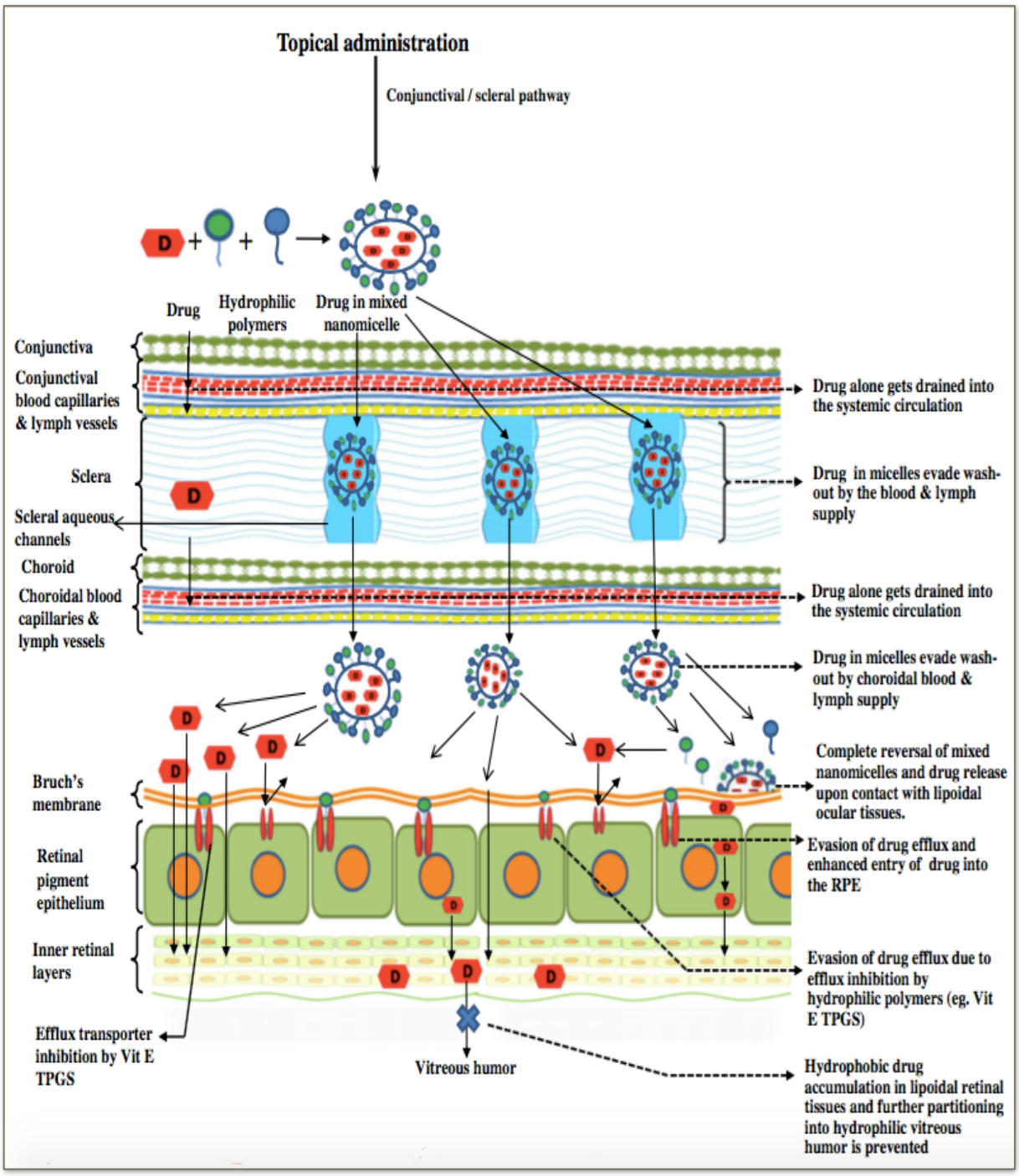


Figure 11 - Schematic representation of posterior ocular tissues drug delivery for drugs entrapped in mixed nanomicelles (utilization of scleral aqueous pores and evasion of conjuntival/choroidal blood vessels and lymphatics) (3).

In table 6 three studies with aqueous mixed nanomicellar topical eye drops are described. They used two biocompatible, biodegradable and amphiphilic polymeric surfactants (Vitamin E TPGS Octoxynol-40) with different hydrophilic lipophilic balance (HLB) values, to encapsulate voclosporin, dexamethasone and rapamycin in the core of nanomicelles. They developed formulations clear/transparent and free flowing aqueous solutions. Drug levels were evaluated in anterior and posterior ocular tissues with single and once daily drop instillation in rabbit animal model. The Cmax of voclosporin, dexamethasone and rapamycin, in retina/choroid after 1h single drop administration was of 48, 50 and 370 ng/g of tissue, respectively. Minimal and/or non detectable drug levels in aqueous humor, lens and vitreous humor [12, 101, 118].

The mixed nanomicelles approach established a platform technology to delivery therapeutics non-invasively to retina/choroid with topical drop instillation [3].

Table 6 - Nanomicellar topical aqueous drop formulation for back-of-eye delivery of Rapamycin, Dexamethasone and Voclosporin

Drug	Rapamycin	Voclosporin	Dexamethasone
Size (nm)	10.84	—	10.46
PDI	0.05	—	86
Surface charge	-789	—	-2.26
Absorbance at 400nm	41	—	0.04
Cmax in retina/ choroid after 1h (ng/g of tissue)	370	48	50
Vitreous humor (ng/g of tissue)	Non detected	2	Non detected
References	13	101	118

3.5. D-α-tocopheryl polyethylene glycol 1000 succinate (Vitamin E TPGS)

Vitamin E TPGS (fig.12) is a water-soluble derivative of natural Vitamin E, which is formed by esterification of Vitamin E succinate with polyethylene glycol (PEG) 1000 [17, 119]. PEG is the most frequently utilized hydrophilic segment due to its excellent water solubility and biocompatibility. It is usually chosen to forms the micellar shell, because its molecular weight is higher than the hydrophobic core forming molecule. This results in micelles having critical micellar concentrations in the micromolar range, and generally smaller than 100 nm [120].

Vitamin E TPGS is a nonionic surfactant with an average molecular weight of 1513 $g.mol^{-1}$, an amphiphilic structure of lipophilic alkyl tail and hydrophilic polar head, with a HLB value of 13.2 and a relatively low CMC of 0.02% w/w. It is a waxy solid (m.p. ~ 37-41°C) and completely soluble in water. It has also miscible with oils, such as soybean oil and medium chain triglyceride, others surfactants and co-solventes such as propylene and polyethylene glycols. It is stable at pH 4.5-7.5 and less than 10% hydrolyzed when kept for 3 months in neutral aqueous buffer. The Vitamin E TPGS safety has been reported at the oral LD50 is >7 g/kg for young adult rats of both sexes [17, 119].

Vitamin E TPGS can solubilize a variety of both water-soluble and water-insoluble compounds, such as cyclosporines, taxanes, steroids, antibiotics, between others [119].

Vitamin E TPGS could act as an inhibitor of P-gp. It has capacity of inhibiting P-gp activity, higher than others nonionic surfactants such as Tween 80, Pluronics and Cremophor EL [119]. It has been used in several formulations/applications, such as: fabricating nano suspensions [121], self-microemulsifying [122] and solid dispersion/tablet [123]; adjuvant for vaccine system [124]; nutrition supplement [125]; formulate nanoparticles [126], micelles [12] and liposomes [127]; based prodrug [128].

In ODD, Vitamin E TPGS is frequently used, with several functions, such as: vehicle for hydrophobic drugs, to improve the ocular permeability and to prolong ocular retention, (table 7).

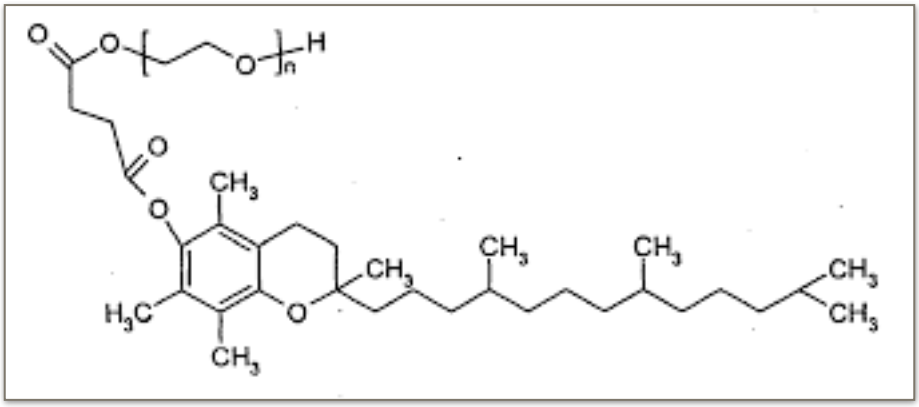


Figure 12 - Chemical structure of D-α-tocopheryl polyethylene glycol succinate 1000 (Vitamin E TPGS) [129].

Table 7 - Description of some ODD systems that containing Vitamin E TPGS in formulation.

ODD system	Drug	Disease	References
Solution	Riboflabin	Keratoconus	[130]
In situ gel systems	Curcumin	DR	[131]
Polymeric nanoparticles	Drozolamide	Glaucoma	[132]
Nanoparticles Intravitreal injection	Dexamethasone	Posterior segmenet diseases	[133]
Nanomicellar topical aqueous drop	Acyclovir	Herpetic keratitis	[134]
Nanomicellar topical aqueous drop	Rapamycin	Posterior segmenet diseases	[12]
Nanomicellar topical aqueous drop	Dexamethasone	Posterior segmenet diseases	[118]

3.6. Pluronic F-127 (PF127)

Pluronic F-127 (fig. 13) (PF127, also know as a poloxamer 407) is a copolymer of poly (oxyethylene106)-poly (oxypropylene70)-poly (oxyethylene106), (PEO106-PPO70-PEO106). It is a copolymer with a weight of 4000 $g.mol^{-1}$ and a low CMC of $2.8x10^{-6}$ [15]. These PEO/ PPO ratio is of 2:1 (which contributes to its hydrophilicity), and it is non-toxic, with low viscosity below 4 °C and which forms a semi-solid gel at body temperature. PF127 is more soluble in could water than in hot water due to the increased solvation and hydrogen linkages at low temperatures [16].

PF127 when dispersed in aqueous medium, at low concentrations form micelles, with a central hydrophobic core (PPO) and a hydrophilic corona (PEO), witch allows it to solubilize hydrophobic solutes [135]. It also allows an enhancement of mucosal absorption by inhibition of P-gp activity [16].

PF127 is biocompatible with cells and body fluids, and weak immunogenic properties. It has been approved by FDA and widely used in topical, ocular, nasal and rectal drug delivery systems [136].

In ODD, PF127 is frequently used, with several functions, such as: vehicle for hydrophobic drugs, to improve the ocular permeability and to controle release of drug in ocular tissues (table 8).

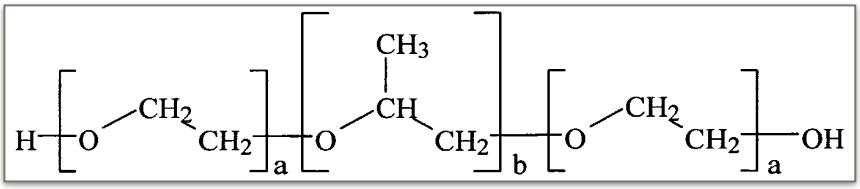


Figure 13 - Chemical structure of Pluronic F-127 (PF 127): (a) - 106 and (b) - 70.

Table 8 - Representative ODD systems that containing PF127 in formulation.

ODD system	Drug	Disease	References
Cubosome	Timolol Maleate	Glaucoma	[137]
Nanostructured lipid carriers (NLC)	Ibuprofen	Eye inflamation	[138]
In situ gel formulations	Ketorolac Tromethamine	Conjuntivitis	[139]
Micelles	Melatonina	Ocular hypertention	[140]
Nanoparticules	Fluocinolone acetonide	Posterior uveitis	[141]
In situ gel formulations	Curcumin	DR	[142]
Nanocrystal	Brinzolamide	Glaucoma	[143]
Cationic liposomal in situ gels	Vitamin A palmitate	Dry eye	[144]

4. Materials and Methods

4.1. Materials

In this experimental work were used the following ray-materials: Celecoxib, Vitamin E TPGS, Pluronic F 127, Chloroform, Ethanol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution and distilled deionized water.

Celecoxib ($C_{17}H_{14}F_3N_3O_2S$, Mw = 381.36 $g.mol^{-1}$) is a white crystalline powder with low aqueous solubility and hight solubility in organic solvents, such as ethanol and chloroform. It was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Vitamin E TPGS ($C_{33}O_5H_{54}(CH_2CH_2O)n$, Mw = 1513 $g.mol^{-1}$) is white water-soluble waxy solid with low melting point (range 37 - 41). It was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Pluronic F 127 ($HO(C_2H_4O)_{106}(C_3H_6O)_{70}(C_2H_4O)_{106}H$, Mw = 4000 $g.mol^{-1}$) is a white crystalline powder with hight aqueous solubility. It was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Chloroform ($CHCl_2$, Mw = 119.38 $g.mol^{-1}$) is a colorless liquid, dense and with sweet-smelling. It was obtained from Fisher Scientific UK.

Ethanol (C_2H_6O, Mw = 46.06844 $g.mol^{-1}$) is a volatile, flammable, colorless liquid with a slight characteristic odor. It was obtained from Fisher Scientific UK.

HEPES buffer solution ($C_8H_{18}N_2O_4S$, Mw = 238.3012 $g.mol^{-1}$) is a zwitterionic organic chemical buffering agent. It was obtained from Fisher Scientific UK.

Distilled deionized water (H_2O) is a clear, colorless, odorless and tasteless liquid. It was obtained by reverse osmosis (Millipore ELIX 10)..

4.2. Equipment

In this experimental work was used the following equipment:

- ZetaSizer Nano ZS (ZSN) (Malvern);
- UV-Vis spectrophotometer (V-S 30/SSO Jasco);
- Scale (model AS 220/C/2 of Radwag);
- Rotary evaporator (model 9300.0-200 rpm);

4.3. Preparation of celecoxib-loaded NMFs

The celecoxib-loaded NMFs were prepared by a solvent evaporation method.

The formulations were prepared in two steps: 1. preparation of basic formulation and 2. rehydration.

In step one:

- Vitamin E TPGS and PF 127 were correctly weighed at different ratios (3:7, 5:5 and 7:3) and dissolved separately in 10 ml of chloroform (total of the polymers blend was 90 mg);
- Vitamin E TPGS and PF 127 solutions were mixed together to obtain a clear homogeneous solution;
- Celecoxib (10 mg) was accurately weighed and dissolved separately in 10 ml of chloroform;
- The celecoxib solution was added to the mixed polymer solution in a bottom glass flask. The mixture was well stirred, which resulted in a clear solution;
- Solvent was evaporated by rotary evaporation at 40°C to obtain a thin film around the glass flask. The flask was kept open overnight at room temperature to remove any residual solvent;

In step two:

- This thin film was then hydrated with 4 ml of the HEPES buffer solution. Sonication was applied for 20 min. Addition of HEPES spontaneously generated celecoxib-loaded mixed nanomicelles;
- The formulations were filtered through a 0,2-µm filter to remove any foreign particulate matter;
- Three blank formulations were also prepared following a similar procedure described above without celecoxib.

In table 9 are described all prepared formulations with/without celecoxib at different ratios of PF 127:Vitamin E TPGS.

Table 9 - Description of the prepared formulations.

Formulations	A	B	C	D	E	F
Ratio of PF127:Vit E TPGS	7:3	5:5	3:7	7:3	5:5	3:7
Weight (mg) PF127:Vit E TPGS	63:27	45:45	27:63	63:27	45:45	27:63
Celecoxib (mg)	—	—	—	10	10	10

4.4. Characterization of NMFs

4.4.1. Size, polydispersity index (PDI) and zeta potential (ZP)

The particle size, polydispersity index and zeta potential were investigated by dynamic light scattering using a ZetaSizer Nano ZS (ZSN) (Malvern).

As the particles are constantly in motion, the intensity of light dispersed by the particles appears to fluctuate. The ZetaSizer Nano (ZSN) system measures the rate of intensity fluctuation and then uses this to calculate the size of particles. The signal intensity of the light scattered by a particle decreases with time and ZSN system measures several signals with time scales in order of nanoseconds. The similarity of two intensity signals is called correlation. Two consecutive signals are strongly correlated and two signals separated in time scale will be less correlated till no correlation occurs.

Z-Average (Z-AVE) is the intensity weighted harmonic mean size of the hydrodynamic diameter distribution. The Z-AVE increases as the particle size increases. Therefore, it provides a reliable measure of the average size of a particle size distribution measured by DLS [144].

The zeta potential (ZP) also termed as electrokinetic potential, is the potential at the slipping/shear plane of a colloid particle moving under electric field. The electric potential of a surface is the amount of work that needs to be done to bring a unit positive charge from infinity to the surface without any acceleration. The ZP reflects the potential difference between the electric double layer (EDL) of electrophoretically mobile particles and the layer of dispersant around them at the slipping plane. During a zeta potential measurement, the

electrophoretic mobility is quantifying by measuring the small frequency shift of the light of a coherent laser source scattered by the charged nanoparticles that are moving in an external electric field. The shift in frequency is measured by a Doppler interferometer. The ZSN from Malvern uses the phase analysis light scattering (PALS) technique, which consist in the sequence of two different mobility measurements [145]. First, the fast field reversal (FFR) mode is applied. It consists in quickly reversing the external electric field, allowing to measure the true particle mobility without the interference of electroosmotic phenomena. In this way an average value of the zeta potential is calculated. Then, the slow field reversal (SFR) mode is applied, which consists in slowly reversing the field, reducing electrode polarization. It allows calculating a distribution of zeta potential values [144].

Dynamic Light Scattering (DLS), also known as Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering (QELS), is a non-invasive, well-established technique for measuring the size and respective distribution of molecules and particles typically in the submicron region. Typical applications of dynamic light scattering are the characterization of particles and emulsions or molecules, which have been dispersed or dissolved in a liquid [146].

DLS is not capable to handle concentrated samples. The solution should be diluted as far as possible to ensure that there are enough particles to spread light enough for analysis but try to avoid the formation of aggregates and multiple scattering. Thus, to determine the average hydrodynamic size, 1 ml of each sample was diluted in 1 ml of HEPES buffer solution. and to determine the ZP, 1 ml of each sample was diluted twice 1 ml of HEPES buffer solution.

4.4.2. Entrapment Efficiency and Drug-Loading

Entrapment efficiency (EE%) is the ratio of mass of drug entrapped into the carrier system to the total drug added. Drug-loading (DL%) is the ratio of drug to the mass of total carrier system (all excipients taken together) [108].

To determine the EE% and DL% of celecoxib in the mixed micelles, we proceeded to the following steps: first, we constructed the calibration curve of celecoxib, second we quantified the encapsulated celecoxib in the nanomicelles and finally we calculated the EE% and DL%.

4.4.2.1. Calibration Curve of celecoxib

Ultraviolet/visible spectrophotometry is one of the analytical methods most commonly used in analytical determinations in several areas. It is applied to determinations of organic and inorganic compounds, as for example in the determination and identification of the active principle in drugs.

Absorption in the ultraviolet/visible region depends on the number and arrangement of the electrons in the molecules or the absorbing ions. As a consequence, the absorption peak can be correlated with the type of binding that exists in the species being studied.

The most important aspect in quantum computation is the determination of the amount of light absorbed by the sample, which is based on the transmittance (T) or absorbance (A) measure. This measure is described by Beer-Lambert's law, which gives the relation between the intensity of the monochromatic light incident on the solution (I_0) and the intensity of the transmitted monochromatic light (I).

$$A = \varepsilon c b = \log_{10} \frac{I_0}{I} \qquad (1)$$

Where, c is the concentration of analyte (moles per liter, M), b is the optical path (expressed in centimeters, cm) and ε is the molar absorptivity of the analyte (expressed as M-1cm-1).

The Lambert-Beer law shows that the Absorbance (Abs) of a solution is proportional (linear relation) to the concentration of solute (c).

From the proportionality between absorbance and concentration it is possible to obtain a calibration curve, that relates the Abs and the Concentration of the sample.

To obtain the calibration curve of celecoxib, were performed the following procedures: Celecoxib (10mg) was accurately weighed and dissolved in chloroform and volume was made up to 100 ml to form a stock solution (100µg/ml). The stock solution was further diluted with chloroform to get a working standard solution of 10µg/ml which was then scanned in UV-Vis range. This screening process showed an absorption maximum at 257nm. Aliquots of 2, 4, 6, 8, and 10 ml of working standard solution corresponding to 2-10µg were placed in a series of 10 ml volumetric flasks and the volume completed with chloroform [147]. The absorption measurements of these solutions were carried out against chloroform as blank at 257nm. A calibration curve of Celecoxib was plotted.

4.4.2.2. Quantification of the encapsulated celecoxib

To quantify the encapsulated celecoxib in the mixed nanomicelles, first the drug-loaded micelles were diluted in 100 ml of ethanol to disrupt them and release encapsulated celecoxib. After wards, 1 ml of these solutions was diluted in 10 ml of ethanol and the UV absorbance was measured using UV-Vis spectrophotometer (UV-Vis spectrophotometer, V-S 30/SSO Jasco) at a wavelength of 257 nm. Finally the amount of encapsulated celecoxib was calculated using the calibration curve.

4.4.2.3. Calculation of the EE% and DL%.

Initially we used the concentration equation to obtain the celecoxib mass corresponding to the concentration obtained through the regression equation.

$$Ccel = \frac{Mcel}{Vs} \quad (2)$$

Where, Ccel is the concentration of celecoxib in solution, Mcel is the mass of the celecoxib encapsulated in micelles and Vs is the volume of the solution of micelles disrupt.

We then use the following equations to calculate the EE% and DL% of celecoxib in NMFs:

$$EE\ \% = \frac{\text{Mass of the celecoxib encapsulated in micelles}}{\text{Mass of the celecoxib initially added}} \times 100 \quad (3)$$

$$DL\ \% = \frac{\text{Mass of the celecoxib encapsulated in micelles}}{\text{Mass of celecoxib added + mass of polymers added}} \times 100 \quad (4)$$

4.4.3. Optical Clarity

One of the major objectives of this study was to prepare an aqueous clear solution of celecoxib.

Micelle size, typically smaller than the smallest wavelength of a visible light radiation (about 350 nm), denotes clarity of the solution. Optical clarity refers to 90% or greater transmission of light at 400 nm wavelength through a 1.0 cm path length. In general, light scattering occurs when any particle interferes with the visible light wavelengths [13, 101].

The optical clarity/appearance of prepared formulations was measured with UV-visible spectrophotometer (UV-Vis spectrophotometer, V-S 30/SSO Jasco). One millimeter of each sample was placed in a cuvette and absorbance was recorded (K=3). Distilled deionized water was used as blank/control.

4.4.4. Viscosity

Although, celecoxib solubility can be improved with aqueous NMF, however topical instillation of aqueous drops may not retain the formulation in the pre-corneal pocket for longer period of time. A large portion (>90%) of the topically applied formulations is lost due to tear drainage, induced lacrimation, and tear dilution. Reflex blinking and loss of excess drop instillation may lead to spill over. Therefore, to improve NMF retention in the pre-corneal pocket, viscosity enhancer with bioadhesive properties is advisable [13]. The addition of viscosity enhancer possibility that NMF stay in the pre-corneal pocket for a longer time, reduce tear wash-off and improve drug delivery [13]. A high viscosity was reported to have an impact on rate of tear drainage [148].

Examples of viscosity enhancers include hydroxy methyl cellulose, hydroxy ethyl cellulose, sodium carboxy methyl cellulose, hydroxy propyl methyl cellulose and polyalcohol [149], but previous studies report that povidone K90 is the most suitable for increasing the bioadhesion of NMFs [150].

A method possible to determine the viscosity of NMFs, briefly:

- The viscometer is full from one end with ~5 ml NMF with extreme care to avoid air bubble formation;
- The solution is aspirated from the other end;
- Time taken by the NMF to flow down under gravity is measured;

- The density of the NMF is determined;
- The viscosity of the NMF is determined by comparison with water, using the following equation:

$$\text{Viscosity(NMF)} = \frac{(\text{density(NMF)} \times \text{time(NMF)} \times \text{Viscosity(water)})}{(\text{density(water)} \times \text{time(water)})} \quad (5)$$

4.4.5. Osmolality and pH

Osmolarity and pH for the prepared NMF are critical and need to be maintained in the physiological range to avoid adverse effects of the formulations on the eye. In general, to adjust tonicity, agents such as glucose, xylitol, glycerol, boric acid or sodium chloride are used [158]. Tears have the tonicity equivalent to 0,9% solution of sodium chloride [151] which produces osmolality of ~305 mOsm/kg. Hypertonicity or hypotonicity of the topical drops may cause irritation to the eye. To maintain isotonic with tears, the formulations should be tested for tonicity and if necessary adjusted with sodium chloride solution to ~305mOsm/kg. The pH of the tears ranges between 6.5 to 7.6 [152]. Variation in the pH may have detrimental effects on the eye. Therefore, the pH of the formulations should be adjusted similar to the tear pH of ~6.8±0.1, for exemple, with 0.1 N sodium hydroxide or hydrochloric acid solution.

4.4.6. Critical micellar concentration

Surface adsorption of the polymer/surfactant (liquid / air interface) depends on the concentration of the monomers in the solution. At low concentrations, the polymer/ surfactant molecules distribute on the surface, being oriented parallel. With the increase of the concentration, the available area decreases in relation to the number of molecules and, consequently, a slight ordering of the same begins in relation to the surface. The orientation will depend on the nature of the surface whether hydrophilic or hydrophobic. In high concentration there is formation of a unidirectional layer; This concentration is known as critical micellar concentration (CMC), that is, CMC is the minimum polymer concentration

required to form micelles. The micelles, unlike the monomers, are dispersed throughout the solution, having no effect on the surface tension of the water [103].

Lower CMC value indicates increased stability of the micelle structure in aqueous solution [153]. For topically administered nanomicellar ophthalmic formulations, the CMC is a critical factor. It regulates the chances of premature drug release from the formulation due to constant tear dilution and nasolacrimal drainage [154, 155]. The applied formulation occupies the space in precorneal pocket by replacing lacrimal fluid. The total volume of tear that precorneal pocket can hold without overflowing is 10µl. The formulation applied will be continuously diluted by tears. This tear dilution may disrupt the micelles and release the drug at the site of application. To prevent the disruption of micelles by dilution a low CMC of the formulation is desired [108].

The low CMC value indicates that this combination of polymers, at very low concentrations, will be able to entrap the drug and can provide adequate solubility and stability after topical drop application [108].

The CMC of NMFs can be evaluated by several methods, such as: iodine UV spectroscopy method [109], laser dynamic light scattering (DLLS) (69), fluorescence probe technique (using pyrene as a hydrophobic probe) [108], static surface tension and differential refractive index [156].

4.4.7. Dilution stability studies

After topical drop instillation, NMFs may be rapidly diluted causing increase in size and release of the drug. In general, tears are produced at an average flow rate of 1.2 µL/min [156]. Any excessive tear production upon NMFs instillation should not have an impact on nanomicellar size. Therefore, it is necessary study the effect of dilution on the NMFs. To examine the effect of dilution on nanomicellar size, the NMFs are 100-fold dilution (above tear dilution that is expected in vivo). The concentration of monomers at these dilutions are well above the CMC. NMFs should remain inalterable, indicating high stability upon dilution [101].

4.5. Cytotoxicity studies

An important aspect of topical ODD is to evaluate drug delivery vehicles for biocompatibility. Formulation components are expected to be safe, reliable and effective for

its intended use. Also these components are to be biodegradable and biocompatible. In vitro toxicity evaluation is the primary requirement to determine the safety of the carrier systems and their ability to deliver therapeutic drug levels to ocular tissues [157].

For evaluate the cytotoxicity can be use in vitro models, derived from animal and human primary and immortalized cells. The exploitation of appropriate in vitro models is crucial for the development of new approaches to overcome ocular barriers. In comparison with in vivo and ex vivo models, in vitro cell-based models offer the advantage of being simple, quick to construct, relatively inexpensive and reproducible, while providing mechanistic understanding of the results [158]. In addition, in vitro models can be used to evaluate a number of combinations of experimental parameters, which is often not achievable with animal models (Table 10) [159].

Table 10 - Some examples of in vitro models for application in toxicity studies.

Models	Species	References
Corneal Epithelial	Primary rabbit (rPCECs)	[13, 118]
	Primary human	[160]
	Immortalized Clonetics	[161]
Conjuntival	Primary cow	[162]
Retinal Pigment Epithelium	Primary human (D407)	[13, 118]
	Immortalized human (ARPE-19)	[163]

The NMFs prepared applied in conjunctival sac. Therefore, after topical administration the NMFs will be in contact with conjuntival and corneal epithelial cells and following with retinal pigment epithelial cells. Thus, for correctly evaluate the cytotoxicity would be need, to test the effect of NMFs in vitro models of three layers mentioned above.

Previously studies [12, 101, 118], have used the cell proliferation and lactate dehydrogenase (LDH) assays, to determine cytotoxicity of NMFs (blank and drug-loaded).

4.5.1. Cell proliferation assay

Cell proliferation assay is based on the enzymatic cleavage of tetrazolium salt (WST-1) to a water-soluble formazan dye, which is detected by absorbance at 450 nm with a micro titer plate reader. The amount of formazan formed is directly proportional to the number of viable

cells [12, 118].

The following is the description of cell proliferation assay to determine the toxicity of MNFs on rPCEC and D407 cells.

After topical ophthalmic drop instillation into pre-corneal pocket, formulations/solutions are rapidly cleared (within 5 to 10 min) [155]. Therefore, to determine the cytotoxic effect of MNFs, 1 h of incubation period would be sufficient to evaluate any toxicity [11, 13, 118].

Briefly:

- 10,000 cells for well are placed into 96-well plates and exposed to 100 µL of placebo (blank) and drug-loaded MNFs for 1 h;
- MNFs (blank and drug-loaded) are prepared in serum-free cell culture media and filtered through 0,22-µm sterile nylon membrane filters under laminar flow;
- The serum-free cell culture medium (without drug) and 10% triton X-100 served as negative and positive controls, respectively;
- Viable cells percentage is calculated considering the medium as 100%;
- Premixed WST-1 cell proliferation reagent was added;
- The percent cytotoxicity of the placebo and drug-loaded MNFs are calculated.

Cytotoxicity is calculated according to Equation 5.

4.5.2. LDH Assay

LDH assay is the cell plasma membrane damage test.

The following is the description of LDH assay.

A 2-hour incubation time is sufficient because these formulations are to be delivered as topical eye drops into the precorneal pocket [13, 118]. The amount of LDH released in the culture media directly correlates with cytotoxicity.

Briefly:

- In vitro plasma membrane decay with blank and drug-loaded MNFs is conducted on rPCECs and quantitatively measured with Takara Aqueous Non-Radioactive LDH cytotoxicity detection Kit;
- The rPCECs are grown on 96-well plates;
- After 48 h of growth, rPCECs are added with 100 µL of serum-free cell culture medium;
- 100 µL of blank and drug-loaded MNFs in serum free culture media are added in each well and incubated for 2 h at 37°C;

- The cell culture medium and 10% triton X-100 served as negative and positive controls, respectively;
- After incubation, the plate is centrifuged at 250×*g* for 10 min in a dark condition;
- One hundred micrometers of supernatant is transferred to an optically clear 96-well flat bottom plate and added with a solution;
- This mixture is incubated for 15 min at room temperature in the dark;
- The amount of formazan formed is measured with a 96-well micro titer plate reader absorbance set at 490 nm.
- A rise in reading number indicates increased LDH release in the culture medium which directly correlates with the amount of formazan produced;
- Therefore, the amount of formazan produced is proportional to the number of plasma membrane damage cells.

Cytotoxicity is calculated according to Equation 6.

$$\% \text{ Cytotoxicity} = \frac{(\text{Observed experimental value} - \text{cell culture medium value})}{(\text{Triton X-100} - \text{cell culture medium value})} \times 100 \quad (6)$$

It is expected that % cytotoxicity of NMFs will be insignificant, this is, that the NMFs do not cause cell death and or damage to plasma membrane of rPCEC and D407, compared to the Triton X-100, which cause significante toxicity/membrane damage and release high amounts of LDH [118].

4.6. Ocular tissue distribution study

Animal experimentation plays an important role in the research and development of ODD systems [164]. For many years, live animals have been utilized to assess the effect of various ocular products to the eye [165]. Several animal models of DR, including the diabetic rat, mouse, pigs, zebrafish, monkeys, rabbits, dogs, cats and nonhuman primates [164].

The New Zealand white rabbits are more commonly models used for in vivo studies, because they are easily obtained, relatively inexpensive and have a well known anatomy, compared to other species, have large eyes [166].

Delivery of drug to the posterior ocular tissues is a major challenge. Diseases such as DR

and DME that affecting retina/choroid, require long-term drug delivery. We hypothesizes that conjunctival-scleral route, is more efective and safe for delivery celecoxib in posterior ocular tissues, but to prove this hypothesis, an in vivo ocular tissue distribution study is need.

Previous studies [11, 13, 118] have used the following procedures for ocular tissue distribution study of NMFs.

Briefly:

- Animals (New Zealand white rabbits) are anesthetized;
- Fifty microliters of drug-loaded MNFs is instilled into each conjuntival sac;
- After a period of 1 h, euthanasia is performed under deep anesthesia with an intravenous injection of sodium pentobarbital through the marginal ear vein;
- Following euthanasia, the ocular tissues (lens, iris-ciliary body, cornea, sclera, retina-choroid and vitreous humor) are extracted;

To determine the concentration of drug in the posterior ocular tissues the LC-MS/MS method is applied. LC-MS/MS is comprised of triple quadrupole mass spectrometer with electronspray ionization on a turbo ionspray source which is coupled to a liquid chromatography system. A reversed phase C8 column 5 μm 50×4.6 mm, is selected for the separation and analysis of drug with erythromycin as internal standard. The column temperature is maintained at 40°C. The mobile phase consisted of acetonitrile-water (80:20*v*/*v*) with 0.1% formic acid and is pumped at a flow rate of 0.25 mL/min. Multiple reaction monitoring mode is utilized to detect the compound of interest. A separate calibration curve (range of 3.5-100 ng/mL) is constructed for the back-of-the-eye tissue (retina/choroid, sclera) and vitreous humor.

5. RESULTS AND DISCUSSION

5.1. Preparation of celecoxib-loaded NMFs

Current mode of drug delivery to posterior segment of the eye, for treatment of DR is through of invasive routes like intravitreal injections and/or implants. These methods are also associated with serious complications and requires medical specialist to their administration [2]. Topical drops are the formulations with higher patient compliance for treatment of eye diseases; however, their main challenge is sub-therapeutic levels of drug that reaching the back-of-the-eye tissues [13].

To address these problems and replace the current existing route of drug administration to retina, we have developed a nanomicellar clear topical aqueous drop formulation that could deliver celecoxib non-invasively to the posterior segment tissues.

There are two potential pathways for a molecule to reach the back of the eye following topical administration: the corneal route and the trans-scleral or conjunctival route [167], trans-scleral may be explored as a predominant route for-back-of-the-eye delivery in comparison to corneal route. Aqueous solubility of the molecule is a determinant factor for its transport across sclera, but also for its ability to evade the conjunctival blood and lymph vessel-mediated washout [168]. Scleral aqueous pore diameter varies between 20 and 80 nm [169], which facilitates the diffusion of small hydrophilic molecules.

Celecoxib is a lipophilic molecule (log P = 3.5) with poor aqueous solubility (3-7µg\ml) [5]. In present study, we improves the celecoxib solubility using a clear aqueous NMFs. The mixed nanomicelles can encapsulated hydrophobic celecoxib inside the hydrophobic core. They have an hydrophilic corona that can be highly effective in utilizing scleral aqueous channels/pores and help permeation of celecoxib to reach the retina. Moreover, highly polar hydrophilic nanomicellar corona may minimize washout into the systemic circulation from the conjunctival and choroidal blood and lymph vessels thus overcoming dynamic barriers [13].

Use of single polymer to encapsulate a drug may result in clear aqueous micelles, but may cause poor formulation stability [11]. To improve the stability of micelles and lower their CMC, we selected a blend of two FDA-approved polymers, PF 127 and Vitamin E TPGS.

Efflux pumps, P-gp predominantly, present in BRB tissues can preventing the entry of celecoxib in these tissues [170]. Vitamin E TPGS and PF 127 are found to modulate P-gp

efflux transport via P-gp ATPase inhibition [171]. Upon disruption of mixed micelles and release of celecoxib into the back-of-the-eye lipid tissues, Vitamin E TPGS and PF 127 monomers may help to inhibit P-gp. Thereby, higher celecoxib accumulation into retina/ choroid may be achieved.

We successfully prepared six NMFs with a blend of Vitamin E TPGS / PF 127 at different ratios (3:7, 5:5 and 7:3), with or without celecoxib in HEPES buffer solution, using a solvent evaporation method (fig. 14).

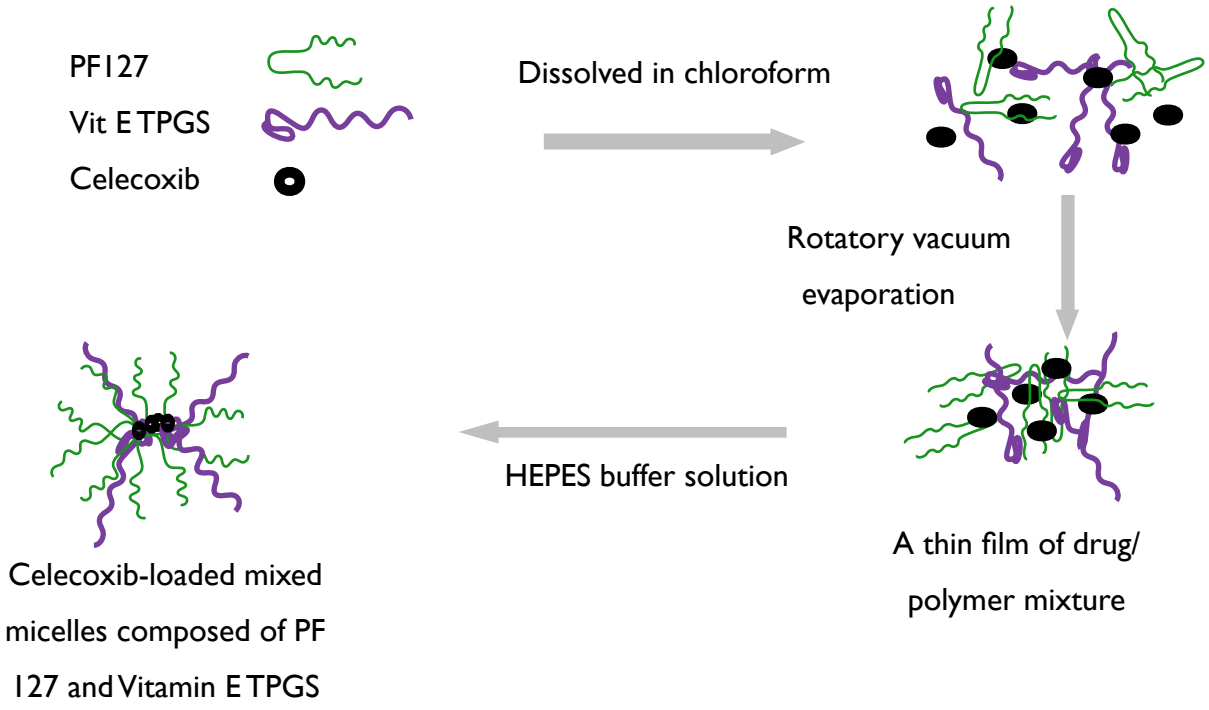


Figure 14 - Schematic illustration of celecoxib-loaded mixed micelles composed of PF127 and Vitamin E TPGS.

5.2. Characterization of NMFs

5.2.1. Size, PDI and Zeta Potential

Table 11 - Characteristics of NMFs prepared (size, PDI and zeta potential).

Formulations	Ratio of PF127:Vitamin E TPGS	Size (nm) ±SD	PDI	Zeta Potential
A	7:3	46.94±0.089	0.123	-7.89
B	5:5	44.01±0.094	0.102	-3.48
C	3:7	43.76±0.091	0.091	-7.86
D	7:3	48.04±0.151	0.15	-3.44
E	5:5	47.25±0.085	0.097	-0.26
F	3:7	44.17±0.12	0.115	-9.26

Nanomicellar size and PDI were determined for all the prepared formulations. Results are expressed in table 11.

Particle size is an important parameter as it can directly affects physical stability, the drug release, biodistribution and celular uptake [172].

It was observed that the celecoxib-loaded NMFs are slightly larger than blank NMFs. The micelle size obtained by DLS depends on both the block copolymer composition and drug loading. The micelle size after formation, was mainly influenced by the interaction of the hydrophobic fractions [110]. In blank NMFs the hydrophobic interactions, only occurs between poly-oxypropylene (PPO) and Vitamin E succinate chains of the PF 127 and Vitamin ETPGS, respectively. In celecoxib-loaded NMFs the hydrophobic interactions occurs between polymeric hydrophobic chains (PPO and Vitamin E succinate) and celecoxib, which results in an increase of the nanomicellar size. A small increase in nanomicellar size after drug entrapment has also been seen in other studies. For exemple, an increase in the average size was observed (9.45 nm to 18.43 nm) after Quercetin (hydrophobic drug) loading mixed micelles composed of P123/TPGS [110].

It was also observed a decrease in the average size as the amount of Vitamin E TPGS in

the formulation increase (A > B > C and D > E > F). This might be attributed to the influence of copolymer composition. Vitamin E TPGS has a smaller molecular weight and a smaller hydrophobic group than PF 127. Therefore as Vitamin E TPGS replace PF 127 the hydrophobic volume decrease, which might result in to a smaller size [173]. Previous studies about mixed nanomicelles for ocular drug delivery, obtained a nanomicellar size between 10 and 80 nm [13, 101, 118]. In this work, the smallest size was obtained in formulation F (44.17±0.12 nm). Therefore, we hypothesize that is sufficient to across thought aqueous scleral channels/pore that have a size range between 20 and 80 nm. Since these NMFs are in the same size range as membrane receptores, protein and other biomolecules, such carriers may have the ability to bind with cellular barriers [111]. Moreover, the celecoxib-loaded NMF present in sclera may act as a depot and slowly release the drug to deeper ocular tissues. On the other hand, if the mixed nanomicelles release celecoxib in scleral tissue, a constant celecoxib release to retinal tissues may be achieved which is an added advantage with NMFs. NMF can follow the same route of drug transport (transcleral pathway) that was previously observed with sub-conjuntival injection of celecoxib-poly (lactide-co-glycolide; PLGA) microparticles [8, 174].

Dispersity, also known as polidispersity index (PDI) indicate particle size heterogeneity [175]. PDI is an important factor that can influence the shape, stability and overall performance of the nanomicellar formulation. The kinetics of copolymers which exist between the micellar and non-micellar state are also influenced by the dispersity of the polymers. Therefore, a low dispersity (<1.2), are preferred for the development of controlled release systems [103]. In this work, the obtained polidispersity index is acceptable, range from 0.090-0.150.

The zeta potential is a key indicador of the stability of colloidal dispersions. The magnitude of the zeta potential indicates the degree of electrostatic repulsion between adjacent, similarly charged particles in a dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. when the potential is small, attractive forces may exceed this repulsion and the dispersion may break and flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate.

Surface charge could influence the cell-micelle interactions and thus play a critical role in determining ocular disposition of nanomicelles from precorneal space. The absolute values of the zeta potentials of all the preparations were negative, similar to verified in other studies [11, 13, 118]. The topical application of these NMFs may result in repulsion with cell surface and mucin layer, because both are negatively charged [11]. Since that Vitamin E TPGS and

PF127 are non-ionic stabilizing agents, the negative zeta potential values of this system can be attributed to celecoxib. The addition of a cationic polymer, like chitosan to the formulation may prolong the precorneal residence time, however may also enhance the transcorneal permeability by promoting favorable interactions between the nanomicelle and corneal surface, which is not the goal [101]. The suggestion would rather be to increase the viscosity of the formulation, which would result in increased retention time and consequently across of NMFs through the conjunctival/sclera pathway.

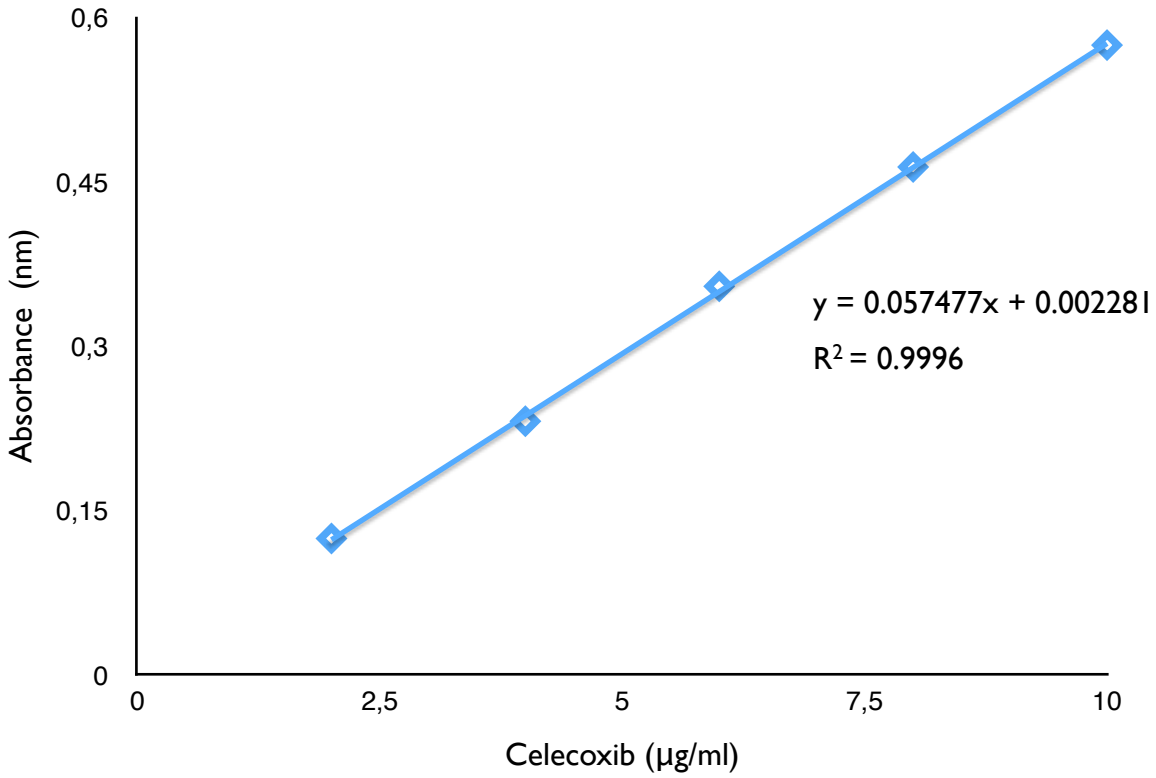
5.2.2. Drug-Loading and Entrapment Efficiency

Absorbance was determined at 257 nm, because this wavelength (λ) corresponds to the maximal absorption of celecoxib. The calibration curve was constructed with five celecoxib standards (concentrations between 2-10 µg/ml prepared from 10 µg/ml working standard solution). Table 12 shows the absorbance values recorded for each of the standards at 257 nm. The equations of the lines obtained as well as the value of the correlation coefficient (R^2) of each of the curves are shown in graphic 1. As evidenced by the value of R^2 (0.9996), the calibration curve shows good linearity between absorbance and concentration, so we can appropriate quantification of celecoxib when the values to be determined are within the concentration limits used.

Table 12 - The absorbance values of standards of 2, 4, 6, 8, and 10 ml of working standard solution at 257 nm.

Celecoxib (µg/ml)	2	4	6	8	10
Absorbance (nm)	0.1244	0.2211	0.3545	0.4634	0.5746

Grafic 1: Calibration curve of celecoxib at 257 nm



The major factors which influenced the EE% and DL% of mixed micelles are the nature and concentration of the solute, nature the core forming block, core block length and the nature and block length of the outer shell for the micelle [155]. Based on these reasons, mixed micelles carriers composed of PF 127 and Vitamin E TPGS with different proportions were studied. Celecoxib entrapment and loading into NMFs was determined with UV-Vis spectrophotometer method as described previously.

The formulations D, E and F showed excellent drug entrapment and loading efficiencies, which are summarized in table 13.

With the results obtained we can observe that as the amount of TPGS added increases (D to F), the EE% and DL% increase as well. The formulation F evidenced the highest EE% and DL%, 92.02% and 9.202% respectively. This could be explained by more cohesive core structure because of the stable reaction between the aromatic ring in Vitamin E TPGS, PO groups in PF 127 and incorporated celecoxib, that may cause stronger hydrophobic interaction between drug and polymers.

Table 13 - Characteristics of MNFs prepared (EE% and DL%).

Formulations	**Ratio of PF127:Vitamin E TPGS**	Absorbance at 257 nm	**EE%**	**DL%**
D	7:3	0.4131	71.83	7.183
E	5:5	0.509	88.16	8.816
F	3:7	0.5312	92.02	9.202

5.2.3. Optical Clarity

Due to extreme small nano size of the celecoxib-loaded NMF, light did not produce any scattering, which denotes a clear/transparent aqueous solution. Ophthalmic compositions of the present celecoxib NMFs are substantial clear with an absorption units below 0,05 measured at 400 nm (table 14). The low absorbance of the formulation indicates the clarity of the formulation which is devoid of any particulate matter. Optical clarity of the formulations was compared with distilled deionized water as blank. This study indicates that all the formulations are similar to water with no particulate matter present in the celecoxib-loaded NMFs.

Table 14 - Characteristics of MNFs prepared (Optical clarity).

Formulations	**Ratio of PF127:Vitamin E TPGS**	**Optical clarity (400nm)**
D	7:3	**0.041**
E	5:5	**0.027**
F	3:7	**0.024**
Blank	DD water	**0.022**

6. CONCLUSIONS

In summary, an aqueous, clear nanomicellar celecoxib-loaded formulation was successfully prepared with a blend of Vitamin E TPGS and PF 127 polymers. We prepared six MNFs with different ratios (3:7, 5:5 and 7:3) of Vitamin E TPGS / PF 127, with or without celecoxib. The formulations presented a size ranging from 44 to 49 nm, an acceptable polidispersity index (0.090-0.150) and a negative zeta potentials. The prepared celecoxib-loaded MNFs showed excellent EE% and DL% and demonstrated absence of any particulate matter.

According with the obtained results can conclude that when the percentage of Vitamin E TPGS in the formulation increases, it decreases the size and increase the EE% and DL% of the MNFs. The formulation F was the one that presented a smaller size and a larger entrapment efficiency of 44.17±0.12 nm and 92.02%, respectively. This formulation is more adequate for following studies.

7. FUTURE PERSPECTIVES

In the future, it is intended to overcome the shortcomings of this work regarding the synthesis, characterization and studies of cytotoxicity and ocular distribution of topically applied celecoxib-loaded NMFs.

To optimize the formulation it is necessary to increase the viscosity for improve the residential time and to control the osmolality and pH for maintain them in the physiological range.

Another important parameter is the stability of NMFs; it is should be analyzed by determining the CMC and the dilution studies.

In order to analyze ocular biocompatibility, cytotoxicity studies should be done with the various tissues in which the nanomicelles will be in contact.

Finally, it would be necessary to determine the amount of celecoxib in the various ocular tissues, ascertaining whether a high amount was achieved in the tissues of the posterior segment (retina/choroid) and was insignificant or undetected in the other tissues.

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