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Novas estratégias para optimização do efeito terapêutico de fármacos utilizados no tratamento do glaucoma

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Ao José Elias

¿Para qué sirve la utopía?

“Utopía está en el horizonte. Me acerco dos pasos, ella se aleja dos pasos. Camino diez pasos y el horizonte corre diez pasos más allá. Por mucho que yo camine, nunca la alcanzaré. ¿Para qué sirve la utopía? Para eso sirve: para caminar.”

Eduardo Galeano, en Las palabras andantes

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(autor desconhecido)

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RESUMO

Torre da Universidade de Coimbra (Portugal)

ABSTRACT

Resumo

O glaucoma é um grupo de doenças que tem em comum uma neuropatia ótica característica, com perda das células ganglionares e cujo principal fator de risco é o aumento da pressão intraocular. A administração de fármacos para o tratamento destas enfermidades oculares é extremamente necessária e, preferencialmente, por meio de vias que atinjam o tecido local, visando reduzir a ocorrência de efeitos indesejáveis e a absorção sistémica. As formas farmacêuticas oftálmicas convencionais (ex: soluções, suspensões, pomadas) apresentam baixa biodisponibilidade na córnea, devido aos mecanismos de defesa do olho e à drenagem nasolacrimal. Recentes esforços de pesquisa têm-se centrado no desenvolvimento de novos sistemas de cedência de fármacos oftálmicos. Neste trabalho procurou-se desenvolver sistemas que fossem capazes de aumentar a solubilidade de fármacos e cedê-los continuamente em níveis elevados e controlados. Entre as novas abordagens terapêuticas em oftalmologia, optou-se pelo uso das micelas poliméricas e ciclodextrinas. Estas representaram excelentes ferramentas no aumento da solubilidade dos inibidores da anidrase carbónica, nomeadamente a acetazolamida e a etoxzolamida, e também podem vir a facilitar o seu acesso através da córnea. Ao mesmo tempo prepararam-se hidrogeles (lentes de contacto) capazes de atuar como sistemas de entrega controlada de fármacos no fluido lacrimal pós-lente. Para o seu desenho utilizaram-se duas abordagens: (i) incorporação das ciclodextrinas (de forma direta ou na preparação de monómeros) à rede polimérica para fazer uso da sua capacidade de formar complexos de inclusão com os fármacos e, (ii) copolimerização e síntese de monómeros que apresentam grupos funcionais similares à dos aminoácidos, que constituem o local ativo da enzima anidrase carbónica. Esta abordagem biomimética combinada com a técnica de impressão molecular originou redes poliméricas que possuem

cavidades com alta afinidade pelos fármacos e proporcionou um carregamento mais elevado dos mesmos e uma melhor controlo do processo de libertação.

Abstract

Glaucoma is a group of diseases that have in common a characteristic optic neuropathy with loss of ganglion cells, whose main risk factor is increased intraocular pressure. The administration of drugs for the treatment of eye disorders becomes extremely necessary and, preferably, through routes that reach the local tissue to reduce the occurrence of side effects and systemic absorption. Conventional ophthalmic dosage forms (e.g. solutions, suspensions, ointments) lead to low bioavailability in the cornea, due to the protective mechanism of the eye and her nasolacrimal drainage. Recent research efforts have focused on developing new systems ophthalmic delivery. In this work we seek to develop systems that are able to increase the solubility of drugs and to continually deliver high and sustained levels of the same. Among the new therapeutic approaches in ophthalmology, we chose polymeric micelles and cyclodextrins as tools for increasing the solubility of carbonic anhydrase inhibitors, including acetazolamide and ethoxzolamide, and also for facilitating the cornea penetration. At the same time we prepared hydrogels useful as components of soft contact lenses able to sustain drug release in the post-lens lacrymal fluid. Two approaches were tested for their design: (i) incorporation of cyclodextrins (as such or prior preparation of monomers), the polymer network to make use of their ability to form complexes with drugs and (ii) copolymerization of monomers which have similar functional groups to the amino acids that constitute the active site of the enzyme carbonic anhydrase. This biomimetic approach combined with molecular imprinting technique originated polymer networks that have cavities with high affinity for drugs and provided a higher loading of drugs and an improved control of the release process.

PUBLICAÇÕES

Coimbra vista de Santa Clara (Portugal)

PUBLICATIONS

Publicações de artigos

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Receptor-based biomimetic NVP/DMA contact lenses for loading/eluting carbonic anhydrase inhibitors. **Journal of Membrane Science** 383 (1) 60-69. Andreza Ribeiro, Francisco Veiga, Delfim Santos, Juan J. Torres-Labandeira, Angel Concheiro, Carmen Alvarez-Lorenzo, (2011).

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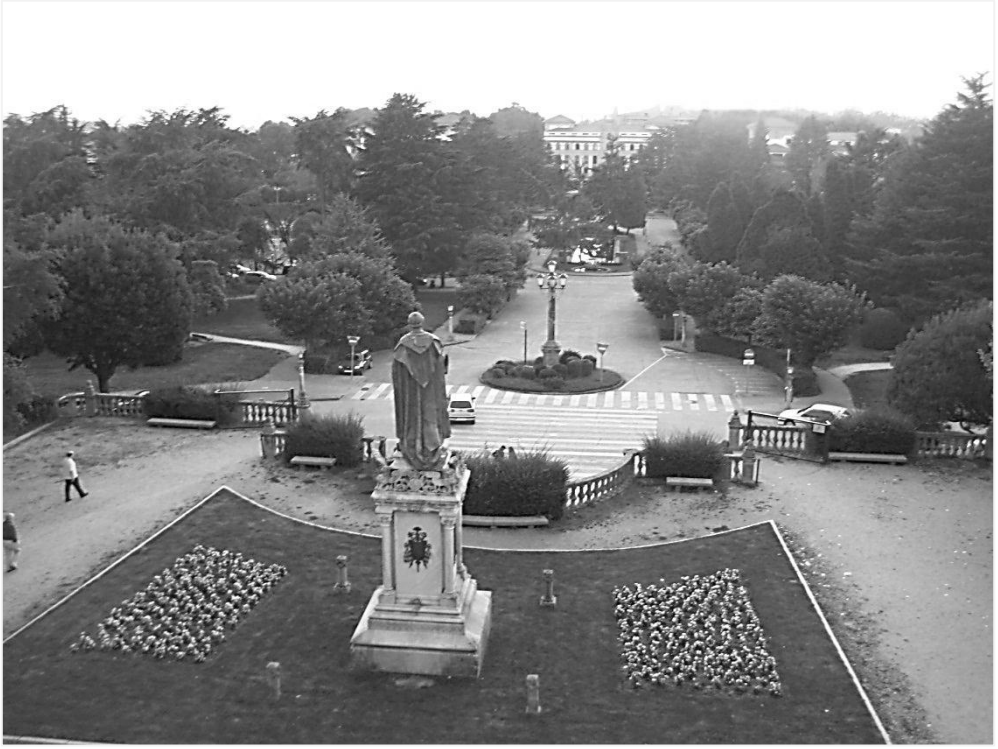

Universidade de Santiago de Compostela, Campus Sur (Espanha)

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INTRODUÇÃO GERAL

Universidade de Coimbra (Portugal)

CAPÍTULO 1

1. Introdução

1.1. Olho

O olho humano é o órgão responsável pela visão e tem uma grande importância vital [1, 2]. O olho pode ser dividido em duas partes, o segmento anterior que inclui a córnea, a junção córnea esclera (limbo), a rede trabecular, o canal de Schlemm, a íris e o cristalino, e o segmento posterior que inclui tudo o que se encontra após o cristalino, ou seja, o humor vítreo, a retina, o coróide, a esclerótica e o nervo ótico [2]. As doenças do segmento posterior do olho são as maiores causas de cegueira irreversível.

1.1.1. Anatomia do olho

O olho (Figura 1.1) tem a forma de uma esfera, encontra-se localizado na parte anterior da cavidade óssea (órbita) e é protegido pelas pálpebras. As estruturas que circundam o olho protegem-no e, ao mesmo tempo, permitem que ele se mova livremente em todas as direções. O aparelho visual é composto por um conjunto sensorial composto pelo olho, via ótica e centros visuais e um conjunto não sensorial representado pelos vasos e os nervos. A proteção do olho dá-se pela órbita, pálpebras, conjuntiva e aparelho lacrimal e a mobilidade é assegurada pelos músculos oculomotores [3].

O globo ocular é constituído por três camadas. A camada externa que é composta pela córnea, a esclera e o limbo. A intermédia ou úvea, que é composta pela íris, que contém a abertura central denominada pupila o corpo ciliar, responsável pela produção do humor aquoso e suporte do cristalino e pela coróide ou camada vascular. São três as cavidades oculares: a cavidade vítrea, a câmara posterior e a câmara anterior. A cavidade vítrea é a maior e está localizada posteriormente ao cristalino e adjacente à retina sensorial. A câmara posterior é a menor e

compreende o espaço entre a íris e o cristalino, enquanto que a câmara anterior localiza-se entre a íris e a face posterior da córnea.

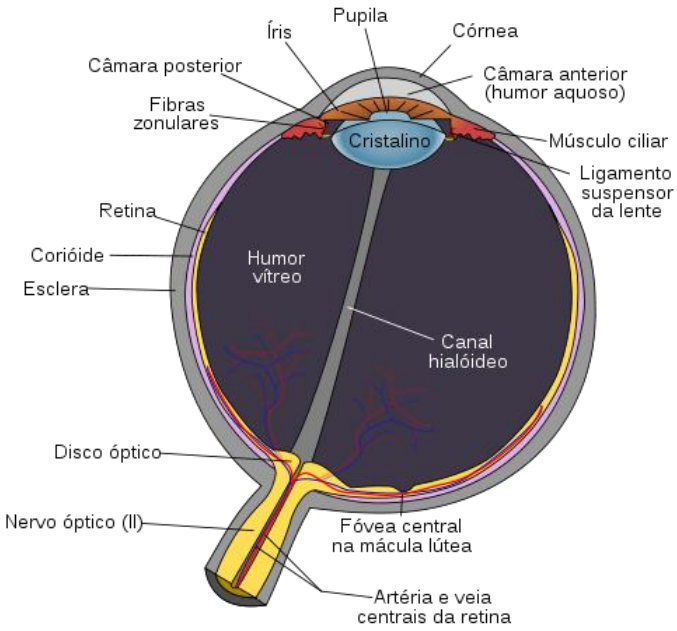

Figura 1.1. Estutura do olho humano.

1.1.2. Segmento anterior do olho

A córnea é um tecido avascular claro e transparente cujo oxigénio e os nutrientes são assegurados pelo fluido lacrimal e humor aquoso. É composta por 5 camadas: o epitélio, a membrana de Bowman, o estroma, a membrana de Descemet e o endotélio. O epitélio contém 5 camadas de células ligadas por estreitas junções o que o torna uma forte barreira para moléculas pequenas e compostos lipofílicos. O estroma é um tecido fibroso, espesso (450µm) em grande parte acelular e é composto principalmente de água. O endotélio é formado por uma camada de células com grandes junções intercelulares. A conjuntiva é uma fina membrana

transparente, que reveste a superfície interna da pálpebra e se reflete sobre o globo ocular. A membrana da conjuntiva é vascular e é humedecida pelo filme lacrimal pré-corneal. A parte exposta do olho é também coberta por uma fina camada deste fluido [2]. O humor aquoso é formado pelos processos ciliares e circula através da pupila e do sistema trabecular. Trata-se de uma substância viscosa, transparente e incolor que preenche a câmara anterior do olho. O humor aquoso é renovado de forma lenta e constante e o seu excesso é escoado pelo canal de Schlemn. Quando ocorre uma falha na drenagem do humor aquoso ocorre um aumento da pressão ocular, sendo uma das causas do glaucoma.

1.1.3. Segmento posterior do olho

A esclerótida também conhecida como esclera é uma camada que envolve externamente o globo ocular. A retina é o revestimento interior da parte posterior do olho é uma estrutura fina, transparente e bastante organizada. É composta por células sensíveis à luz, os cones e bastonetes, cuja função é transformar o estímulo luminoso em estímulo nervoso que é transmitido ao cérebro pelo nervo ótico. O vítreo ou corpo vítreo é essencialmente um material gelatinoso, composto por ácido hialurónico, colágeno e proteínas plasmáticas que preenche quase todo o espaço intraocular. O nervo ótico é um nervo mielinizado responsável pelo transporte da informação visual do olho para o cérebro. Este nervo é constituído por um feixe de fibras nervosas que se originam na retina, penetrando no crânio pelo canal ótico.

1.1.4. Aparelho lacrimal

O aparelho lacrimal é formado por uma parte secretora, que é constituída pelas glândulas lacrimais e acessórias e outra excretora, formada pelo sistema de drenagem lacrimal. A integridade da córnea, conjuntiva e pálpebras estão na

dependência da secreção contínua de lágrimas e também da sua correta drenagem. O sistema lacrimal é composto, basicamente, pelas glândulas lacrimais, pálpebras superiores e inferiores, saco conjuntival, ponto ou "puncta", e ductos nasolacrimais [4]. O papel funcional do sistema excretor lacrimal é o de drenagem do filme lacrimal da superfície ocular para as narinas [5]. As lágrimas são secretadas e distribuídas sobre a superfície ocular durante o ato de piscar das pálpebras. O filme lacrimal protege a superfície ocular da influência ambiental e minimiza danos decorrentes da exposição corneal.

O sistema de recolha consiste em recolher o excesso de lágrima pelo canalículo, o saco lacrimal, e o ducto nasolacrimal, e tem a sua abertura na passagem nasal inferior [3, 5]. A produção lacrimal é feita pela glândula lacrimal, pode ser dividida em básica ou lacrimejamento reflexo e emocional [6]. O fluxo da lágrima normal é cerca de 1,2 μL/min [7] e tem um volume residente de aproximadamente 7-9 μL. A máxima quantidade de fluido suportado sem ocorrer derramamento é de aproximadamente 30 μL. A produção de lágrimas por reflexo é induzido por estímulos periféricos, como por exemplo, a irritação química ou mecânica, a temperatura (como o frio) e a luz. Estes estímulos podem aumentar o lacrimejar em uma centena de vezes, mesmo até 300 μL/min, resultando na eliminação do corpo estranho e consequentemente de fármacos aplicados [7].

1.1.5. Visão geral da cedência de fármacos oculares

São várias as possíveis vias de administração de fármacos nos tecidos oculares. Tradicionalmente a administração pela via tópica ocular e a subconjuntival são usadas no segmento anterior, enquanto a administração intravítrea é a utilizada para o segmento posterior. O desenvolvimento das formas farmacêuticas pode ter grande influência sobre o resultado e a duração da ação dos fármacos.

1.1.6. Terapia tópica ocular

A instilação tópica de soluções oculares, como os colírios, no saco conjuntival inferior é o procedimento mais correntemente utilizado para a administração de fármacos oftálmicos. No entanto, um dos maiores problemas encontrados na administração de colírios é a sua farmacocinética, que descreve uma rápida e extensa perda do medicamento logo após a sua aplicação. Grande parte do medicamento sofre uma eliminação da área pré-corneal através dos eficientes mecanismos de proteção do olho, resultando numa reduzida biodisponibilidade. Estima-se assim, que menos de 5% da dose aplicada alcance o segmento posterior do olho. Além disso, vários fármacos potencialmente ativos em oftalmologia apresentam uma reduzida solubilidade em água, inviabilizando a sua incorporação em veículos convencionais, como as soluções aquosas. Algumas formulações administradas pela via tópica, como os géis e as pomadas, prolongam o tempo que o medicamento permanece na superfície ocular e podem promover uma maior absorção intraocular, contudo, causa desconforto, sensação pegajosa, visão turva, induzindo reflexos como o piscar.

Com o objetivo de contornar estes inconvenientes, prolongar a permanência de fármacos na área pré-corneal e, consequentemente, para melhorar a biodisponibilidade ocular, diferentes tipos de sistemas têm sido objeto de investigação, tais como o uso de soluções mucoadesivas [8], sistemas coloidais [9-12], formulações semi-sólidas e dispositivos de inserção ocular [13, 14]. Nas últimas décadas, dispositivos sólidos para libertação controlada de fármacos começaram a ser desenvolvidos [15-17]. Estes funcionam como reservatórios de fármacos, tendo como principal objetivo incrementar a permanência do fármaco na área pré-corneal. Tais sistemas, como o Ocusert® (Alza, EUA), melhoram a precisão da dose e a redução na absorção sistémica do fármaco, levando a uma

diminuição dos efeitos colaterais [18]. Apesar das notáveis vantagens terapêuticas deste tipo de sistemas, diversos fatores limitam a sua utilização, tais como, as dificuldades de manipular, a sensação de corpo estranho, o desconforto e o alto risco de expulsão acidental [19]. Entretanto, estas desvantagens podem ser superadas, mantendo o desempenho de libertação, fazendo uso de novos sistemas terapêuticos. O desenvolvimento de metodologias que viabilizem o acesso de fármacos ao tecido-alvo mantendo a sua concentração em níveis satisfatórios por tempo determinado, pode ser tão importante quanto o desenvolvimento de novos princípios ativos terapêuticos. O aumento da permeabilidade da córnea e/ou, o prolongamento do tempo de contacto da forma farmacêutica com a superfície ocular, representam fatores importantes para o aumento da biodisponibilidade. A complexação de fármacos de uso ocular em ciclodextrinas [20-22], o uso de nanocarreadores como as micelas [23], ou o uso de lentes de contacto (LCs) gelatinosas carregadas com fármacos [24-26] têm sido extensivamente propostos com a finalidade de aumentar a biodisponibilidade e estabilidade, e diminuir a irritabilidade de fármacos após a administração tópica ocular [27, 28].

1.1.7. Fatores que influenciam a cedência de fármacos tópicos oculares

1.1.7.1. Eliminação de fármacos na área pré-corneal

Após administração tópica (Figura 1.2), a solução aquosa mistura-se com o fluido lacrimal e passa a estar dispersa ao longo da superfície ocular. No entanto, como já referido, vários fatores pré-corneanos, como a drenagem da solução gotejada, a não absorção pela córnea e a indução de lacrimejamento limitam a absorção ocular devido à redução do tempo de contacto entre o medicamento aplicado e a córnea [2]. Associam-se também a estes fatores, a ligação do fármaco às proteínas do filme lacrimal, a sua metabolização e a sua difusão, através da córnea e

conjuntiva, para a circulação sistémica [29]. Depois da instilação de uma gota ocular (convencionalmente entre 30-50 µL), o tempo que esta permanecer no fundo do saco da conjuntiva e no filme lacrimal, mantendo-se o contacto com a córnea, contribui de maneira importante para sua absorção intraocular. O gradiente de concentração do medicamento, entre a lágrima e a córnea, também influencia a difusão passiva através deste tecido, já que a sua penetração possui uma relação linear com a concentração no filme lacrimal. Para se atingir o nível terapêutico adequado, são necessárias elevadas concentrações e/ou frequentes administrações, o que pode aumentar o risco de efeitos adversos sistémicos e interações medicamentosas. Na sequência da aplicação de uma gota sobre a zona pré-córneal do olho, uma grande parte da solução medicamentosa perde-se rapidamente da superfície ocular através do sistema de drenagem lacrimal, mucosa nasal e faringe. Pouco tempo depois, o volume lacrimal residente de 7-9 µL volta ao normal [7, 30]. Os principais locais para que ocorra a absorção sistémica são a mucosa nasal e a mucosa da conjuntiva ocular [31]. A absorção de fármacos lipofílicos, que pode ocorrer através da mucosa nasal durante a drenagem, pode causar efeitos colaterais adversos, como a hipertensão, taquicardia e asma brônquica, e até mesmo reações tóxicas.

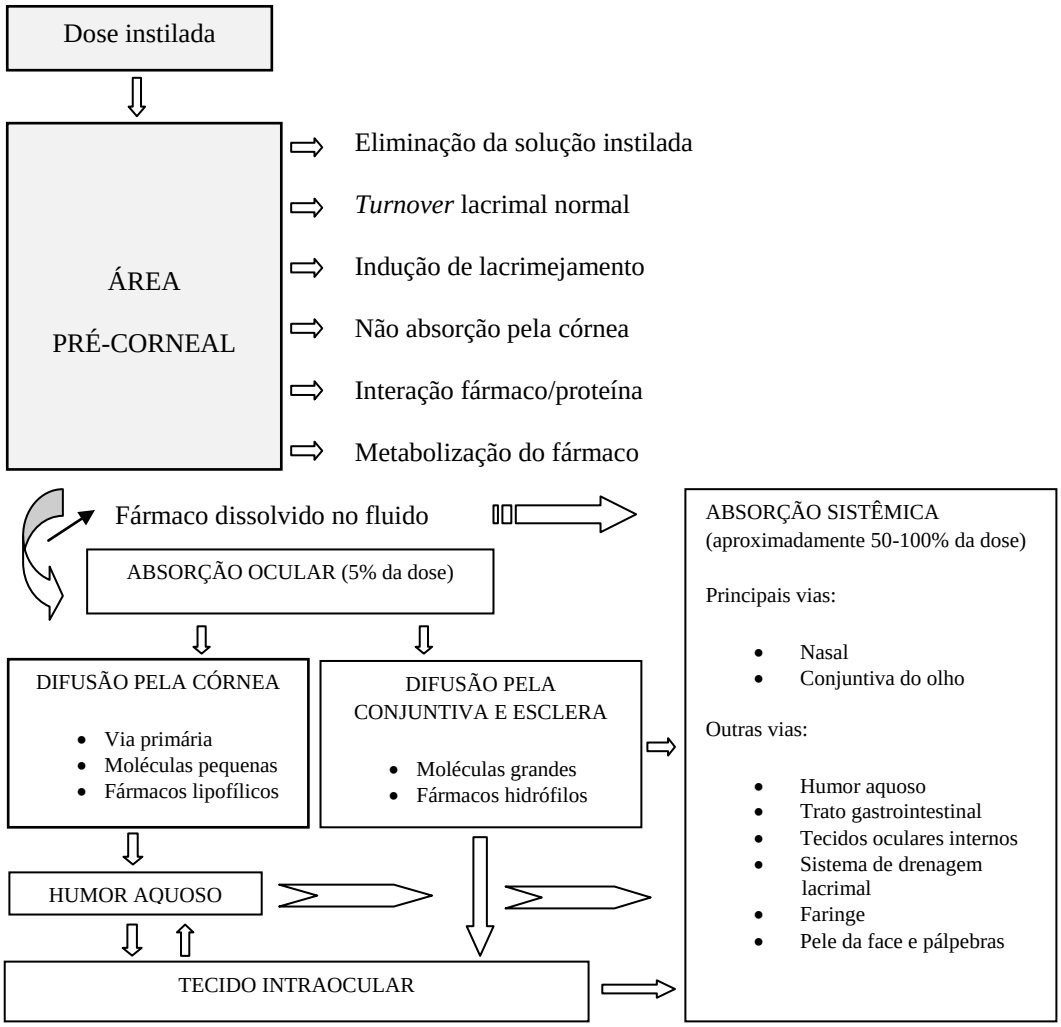

Figura 1.2. Fatores pré-corneais que influenciam a biodisponibilidade de soluções oftálmicas de aplicação tópica e as vias de absorção após aplicação dos fármacos de uso ocular (Adaptado de [32]).

1.1.7.2. Permeabilidade da córnea

Entre os fatores que influenciam a cedência de fármacos na região ocular, a córnea propriamente dita, é um fator barreira, limitando a penetração tópica de

fármacos administrados no olho. A permeabilidade corneal é um fator de grande importância e determinante da concentração de fármacos no humor aquoso [19]. A córnea é geralmente considerada como sendo uma importante, mas não exclusiva, via ocular para a permeação dos fármacos aplicados topicamente [33]. A conjuntiva e a esclera são mais permeáveis do que a córnea [34], no entanto, o fármaco é removido pela circulação sanguínea antes de atingir os tecidos do interior do olho. Comparado com muitos outros tecidos epiteliais, nomeadamente o tecido brônquico, o intestinal, o nasal e o traqueal, o epitélio da córnea é relativamente impermeável, mas mais permeável do que o estrato córneo da pele [35]. Pelo facto do tecido epitelial ser lipofílico, torna-se a principal barreira para a permeação de fármacos na córnea. No entanto, o passo limitante de permeação para os fármacos lipofílicos é o coeficiente de partilha entre o epitélio e o estroma [36]. A conjuntiva é altamente vascularizada, possui uma área de superfície (16-18 cm^2) maior que a da córnea (1 cm^2) [37] e dependendo do fármaco administrado, pode ser 2 a 30 vezes mais permeável [34, 38].

Outro fator que pode influenciar a permeabilidade aos fármacos à córnea é a presença de proteínas transportadoras do tipo bomba de efluxo (glicoproteína-p, proteínas de resistências a multifármacos (MRP) e proteína de resistência ao cancro da mama (BCRP)) que são expressas no epitélio córneal [39]. Vellonen e colaboladores [40] encontraram proteínas MRP1, MRP5 e BCRP expressas em tecidos epiteliais de córnea humana [40].

O coeficiente aparente de permeabilidade da córnea é normalmente determinado por meio da córnea isolada, disposta num sistema de difusão celular. Os estudos de permeabilidade *in vitro* da córnea, fornecem informação sobre os efeitos do fármaco na sua estrutura e da permeabilidade da formulação. No entanto, os estudos *in vitro* de permeabilidade através da córnea não prevêem perdas durante

o processo pré-corneano, portanto, não predizem a biodisponibilidade *in vivo* de fármacos administrados topicamente. Avtar e colaboradores [41] desenvolveram um modelo matemático simples para explicar o perfil de difusão de fármacos administrados topicamente na região anterior do olho através da córnea, bem como o efeito de diversos parâmetros sobre a concentração no humor aquoso de fármacos lipofílicos e hidrófilos. Os resultados indicaram que um aumento na taxa de consumo metabólico do fármaco no epitélio da córnea, reduz a concentração na câmara anterior, tanto para as moléculas lipofilicas, como para as hidrófilas. O modelo também permitiu confirmar, que uma diminuição na taxa de eliminação e no volume de distribuição do fármaco na câmara anterior aumenta significativamente a concentração no humor aquoso. Foi ainda observado que a taxa de decréscimo da concentração dos fármacos na câmara anterior é maior para moléculas lipofílicas. Finalmente, concluíram que estes resultados podiam contribuir para optimizar estratégias e melhorar a biodisponibilidade de fármacos no humor aquoso.

1.1.7.3. Propriedades físico-químicas

As propriedades físico-químicas das substâncias também são fatores que estão envolvidos na capacidade de difusão de fármacos através da córnea. A córnea pode ser imaginada com uma estrutura tri-laminar: "lípido-água-lípid1o", que corresponde ao epitélio, estroma e endotélio corneal [42]. O epitélio e endotélio funcionam como barreira para substâncias hidrófilas e o estroma como barreira para componentes hidrofóbicos.

Os fármacos lipofílicos penetram no epitélio da córnea, através da via transcelular e as moléculas hidrófilas utilizam a via paracelular [43]. A lipofilicidade dos fármacos é uma importante propriedade de penetração na córnea. No entanto, a solubilidade aquosa do fármaco é uma propriedade essencial para uma libertação

eficaz. A superfície do olho está constantemente a ser limpa e humedecida pelo fluido lacrimal. Sendo assim, é difícil para as moléculas dos fármacos serem absorvidas pelo epitélio da córnea, a menos que estas sejam solúveis no filme lacrimal. O dilema é que, um potencial fármaco ideal para uso oftálmico deva ter simultaneamente características hidrófilas e hidrofóbicas para obter boa permeação pela córnea, contudo apenas algumas moléculas conseguem cumprir este critério. Devido a este facto, alguns recursos, como o uso de ciclodextrinas (CDs) [32, 44-46] e síntese de pró-fármacos [47-51] têm sido utilizados no sentido de melhorar as propriedades físico-quimicas dos fármacos oftálmicos e consequentemente melhorar a sua libertação.

Além da lipofilia e da solubilidade aquosa de um fármaco, o tamanho da molecula, a carga e o grau de ionização, também afetam a absorção pela córnea [52]. A forma não ionizada do fármaco usualmente penetra na córnea mais facilmente do que a forma ionizada, deste modo o pH e a capacidade tampão de uma solução administrada topicamente no olho podem ter um efeito significativo sobre a absorção do fármaco [53].

1.2. Glaucoma

Hipocrates descreveu o termo glaucosis fazendo referência aos olhos que apresentavam “cegueira com a pupila cor do mar” [54]. Inicialmente o glaucoma não era diferenciado da catarata (hypochyma), contudo, entre os anos de 98-117 DC Rufus de Ephesus descreveu o olho, posicionou corretamente o cristalino e diferenciou a hypochyma do glaucoma (glaucosis). Foi somente em 1622 que Richard Banister, descreveu o que hoje conhecemos como glaucoma absoluto, que é a fase mais avançada da doença, onde o indivíduo perde a visão. No entanto, apenas em 1830, a importância da pressão intraocular foi reconhecida por William Mackenzie também se constatou que a sua redução era fundamental na evolução

da doença, de tal modo que ele propôs a realização da paracentese (drenagem do fluido) da esclerótica como procedimento para o tratamento. O exame do fundo do olho só foi possível com a descoberta do oftalmoscópio, feita por Hermann Helmholtz, em 1851 [55]. Após a criação e evolução dos tonómetros conseguiu-se chegar a medidas mais precisas da pressão intraocular (PIO) o que, associado aos estudos morfológicos e funcionais do olho levou a ampliar o conceito referente ao que é hoje conhecido por glaucoma [56].

O glaucoma é a designação de um grupo de doenças caracterizado por distintas manifestações clínicas e histopatológicas que possuem como denominador comum à neuropatia ótica. O glaucoma é a segunda maior causa de cegueira no mundo e estima-se que 80 milhões de pessoas serão afetadas em 2020 [57]. Estima-se que aproximadamente cem mil pessoas sofrem de glaucoma em Portugal e que 33000 apresentam cegueira irreversível [58]. Um dado epideomológico relevante do glaucoma é que indivíduos negros apresentam maior incidência e, muito mais agressivo do que em indivíduos brancos [59, 60].

O principal fator de risco no glaucoma é caracterizado por um aumento na pressão intraocular (PIO). Contudo sabe-se que existem casos em que pessoas com PIO elevada não desenvolvem a doença e que pessoas com PIO dentro da normalidade podem vir a ser afetadas pelo glaucoma [61]. A elevação da pressão ocular pode afetar o nervo ótico devido a uma compressão da cabeça do nervo (prolongada e/ou repetidamente), o que leva com que as fibras que o formam acabem por morrer. Uma situação diferente é quando a pressão do olho é aumentada e dificulta a chegada de suprimento sanguíneo na cabeça do nervo levando à morte destas fibras. O mais provável na prática é que uma combinação destas duas situações ocorra. Se não for tratado, o glaucoma pode causar um dano permanente no disco ótico da retina, que pode progredir para cegueira.

Indiscutívelmente, o único fator de risco para o glaucoma que pode ser identificado e tratado é a PIO. Entre a córnea e o cristalino (Figura 1.1) existe uma cavidade que é preenchida pelo humor aquoso. Como explicado anteriormente, o humor aquoso é produzido no corpo ciliar do olho, fluindo através da pupila para a câmara anterior. A malha trabecular drena o líquido para o canal de Schlemm e finalmente para o sistema venoso (Figura 1.3). O humor aquoso é constantemente produzido e drenado, de modo que o seu volume e pressão mantém-se constantes. Quando ocorre algum desequilíbrio neste ciclo, seja pelo aumento da produção do humor aquoso, ou por uma diminuição da sua drenagem, há um aumento do líquido nesta cavidade, causando o aumento da pressão dentro do olho. A PIO média numa população normal varia entre 8 – 21 milímetros de mercúrio (mmHg). A PIO acima de 21 mmHg pode ser considerada suspeita e possivelmente anormal [62]. Contudo, sugere-se que a neuropatia ótica glaucomatosa seja multifatorial, ou seja, outros fatores não dependentes da pressão têm sido considerados tais como os fatores imunológicos, vasculares, genéticos, miopia, diabetes mellitus entre outros [63, 64].

O glaucoma pode ser classificado, segundo os mecanismos de obstrução da drenagem do humor aquoso, em primário de ângulo aberto, primário de ângulo fechado e secundário. O glaucoma de ângulo fechado ocorre quando há uma obstrução física da malha trabecular e, consequentemente, a um problema na drenagem deste líquido. No glaucoma de ângulo aberto a malha trabecular está livre de obstruções, porém a sua capacidade de drenagem está reduzida. A forma mais comum de glaucoma, e que afeta aproximadamente 90% dos pacientes, é o glaucoma de ângulo aberto ou também chamado glaucoma crónico simples. Este é assintomático e diferencia-se por uma perda da visão periférica que ocorre lentamente e que só é percebida em estágios bastante avançados. O tratamento precisa de ser iniciado precocemente para evitar a perda total da visão. O

glaucoma de ângulo fechado ou estreito é caracterizado por aumentos súbitos de pressão intraocular. O glaucoma de ângulo fechado pode causar dor e reduzir a acuidade visual, e pode levar dentro de um curto periodo de tempo a uma perda visual irreversível. O glaucoma secundário ocorre por várias complicações clínicas ou cirúrgicas, como: inflamação, tumor, trauma, hemorragia, cataratas, lesões oculares ou uso de outras medicações como os corticosteróides.

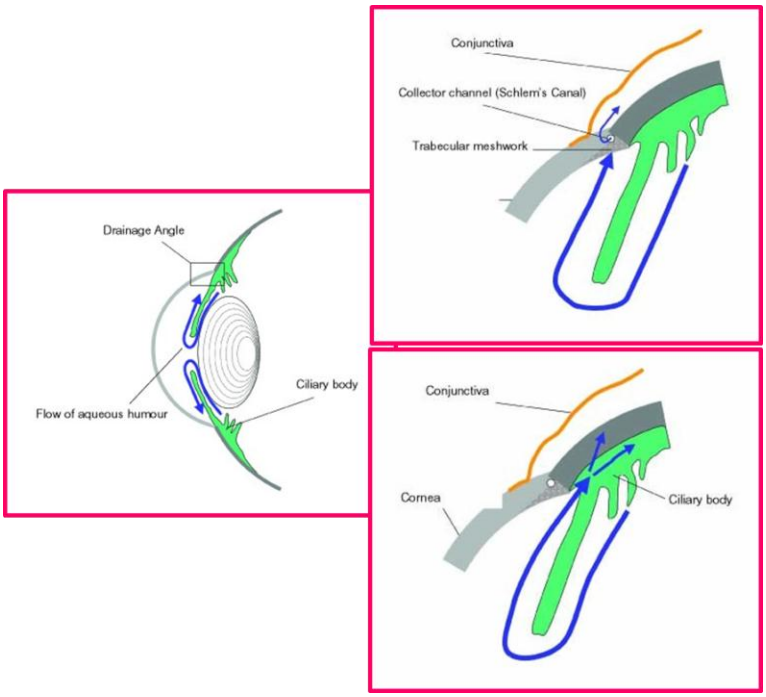

Figura.1.3. Diagrama de uma secção transversal da parte frontal do olho mostrando o ângulo de drenagem. A seta mostra o fluxo do líquido do humor aquoso.

No glaucoma congénito o bebé já nasce com a doença, observa-se a ausência do desenvolvimento dos canais oculares e uma redução da permeabilidade trabeocular. O termo glaucoma infantil é utilizado para o glaucoma congénito que

surge durante os primeiros anos de vida, e o termo glaucoma juvenil para designar qualquer glaucoma que pode aparecer em crianças a partir dos 10 anos de idade.

1.2.1. Tratamento do glaucoma

O tratamento do glaucoma mais comum faz-se utilizando a terapia tópica com o uso de colírios. Outras formas de tratamento podem incluir os medicamentos orais, os implantes, a terapia a laser, a cirurgia e, uma combinação desses métodos [56, 65]. Entretanto, o tratamento do glaucoma não é feito para devolver a visão perdida, mas sim baixar a pressão intraocular e evitar o progressivo dano ao nervo ótico. Devido a isso é muito importante o diagnóstico precoce e o tratamento contínuo. A adesão ao tratamento com medicamentos é um dos principais problemas para os pacientes afetados pelo glaucoma [66]. Por se tratar de uma patologia assintomática, os portadores da doença deixam de usar a medicação por fatores económicos ou por esquecimento devido ao facto do tratamento com os colírios tradicionais serem muitas vezes de aplicações diárias e frequentes.

A trabeculectomia é a cirurgia convencional mais comum realizada para tratamento do glaucoma e é considerada a cirurgia de eleição para os casos de glaucoma congénito e para os casos em que não há resposta ao tratamento clínico e que continuam progredindo [67, 68].

1.2.2. Fármacos usados no tratamento do glaucoma

Há diversas classes com diferentes medicamentos para o tratamento do glaucoma. A pilocarpina foi o primeiro fármaco introduzido para a terapia da hipertensão ocular. A classe mais empregada é a dos beta-bloqueadores, como o timolol. Outras opções são alfa-agonistas como brimonidina, inibidores da anidrase carbónica (CAIs), como dorzolamida e acetazolamida, ou ainda, as

prostaglandinas como o latanoproste. A terapia combinada utilizando dois fármacos hipotensores complementares vem sendo muitas vezes utilizada na terapia clínica [69]. A administração de fármacos hiperosmóticos como o manitol é reservada para tratamentos que requerem certa emergência, como um ataque agudo [56]. Os fármacos frequentemente usados para o tratamento da hipertensão ocular e para o glaucoma estão listados na Tabela 1.1. Nos últimos anos, o uso de estratégias neuroprotetoras para o tratamento de glaucoma tem vindo a ser enfatizado. A prevenção da morte de células ganglionares da retina com terapias neuroprotetoras, que se focam em outros fatores que não a PIO, é discutida como um futuro tratamento para o glaucoma [69]. Com o conhecimento cada vez maior dos mecanismos que envolvem a produção do humor aquoso e os obstáculos que envolvem a sua drenagem, novos agentes terapêuticos (inibidores de proteínas rho-quinase, serotonérgicos, canabinóides, melatonina, nucleotídeos, corticosteróides agonistas) têm demonstrado ser bastante relevantes no tratamento do glaucoma [70].

O sucesso no tratamento tópico ocular visa, fundamentalmente, o transporte de doses efetivas de agentes farmacológicos diretamente para os locais a serem tratados. A baixa penetração dos fármacos nos tecidos oculares limita o número de fármacos indicados para uso em oftalmologia e exige cuidados com os que estão disponíveis no mercado devido a possíveis ocorrências de efeitos adversos. O grande desafio consiste em desenvolver e optimizar estratégias que consigam reduzir estes efeitos e melhorar o tratamento.

Tabela 1.1. Fármacos utilizados no tratamento da hipertensão ocular e glaucoma (Adaptado de [71]).

Classes	Fármaco	Vias de administração	Mecanismo de ação	Efeitos colaterais
Parassimpatico-miméticos	Pilocarpina Epinefrina Demecarium Carbachol	Tópica	Aumenta a drenagem do humor aquoso	Miose, espasmos ciliares,dores de cabeça, visão turva, irritação local.
CAIs	Acetazolamide Metazolamide Etoxzolamida Dorzolamida Brinzolamida	Orais e tópica	Reduzir a produção do humor aquoso	Diminuição do apetite, letargia, cálculo renal, reações cutâneas.
Antagonistas β-adrenérgico	Propranolol Atenolol Betaxolol Timolol Levobunolol Metipranolol Carteolol	Tópica	Diminui a produção do humor aquoso e facilita a saída através da via uveoescleral	Irritação ocular, broncoespasmo, bradicardia.
Agonistas α-adrenérgicos	Apraclonidina Brimonidina Clonidina	Tópica	Reduzir a produção do humor aquoso	Alergia local, boca seca, cefaleia leve, redução na pressão arterial sistémica.
Prostaglandinas	Latanoproste Bimatoproste Cloprostenolol Travoproste Tafluproste Unoprostrone	Tópica	Aumenta o fluxo uveoescleral e o escoamento do humor aquoso	Irritação leve, desconforto, queimação e ardor ocular, aumento no crescimento dos cílios, escurecimento irreversível da íris.

1.2.3. Inibidores da anidrase carbónica

Os inibidores da anidrase carbónica são uma classe de medicamentos utilizados para o tratamento do glaucoma que atuam reduzindo a secreção do humor aquoso. As anidrases carbónicas formam uma família de enzimas que catalisam a conversão de dióxido de carbono e água em ácido carbónico, prótões e iões bicarbonato (ou vice-versa). O sítio ativo da maioria das anidrases carbónicas contém um ião zinco, e por isso são classificadas como metaloenzimas [72].

A reação catalisada pela anidrase carbónica é:

$$\mathbf{H_2O + CO_2 \leftrightarrow H^+ + HCO_3^-}$$

Há três famílias de anidrase carbónica que ocorrem na natureza, nomeadamente alfa, beta e gama. A forma mais estudada é a alfa anidrase carbónica e está presente nos animais. Existem pelo menos 16 isoformas diferentes em mamíferos com diferentes atividades catalíticas, localização subcelular (ex: citosol) e distribuição tecidual. O sítio ativo de muitas das CAs, como já referido, contém um ião zinco (Zn^{2+}) que é essencial para sua catálise. Estudos de cristalografia de raios-X forneceram informações mais detalhadas sobre a presença do ião zinco no sitio ativo da anidrase carbónica II (Figura 1.4). Embora existam diferentes isoformas, o sítio ativo da maioria delas é composta por uma cavidade cónica que contém um ião Zn^{+2} coordenado por três resíduos de histidina (His), His94, His96 e His119 e uma molécula de solvente (ou ião hidróxido, dependendo do pH) como quarto ligante formando uma geometria tetraédrica.

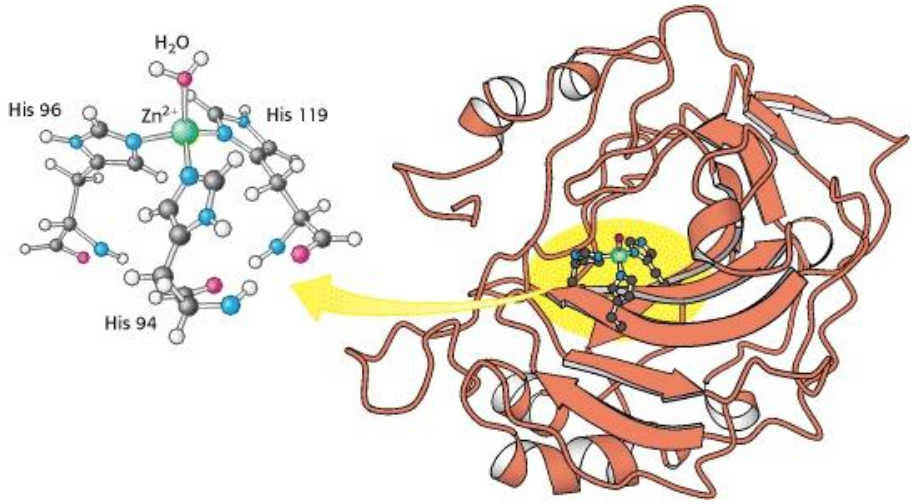

Figura 1.4. Estrutura da anidrase carbónica II. O zinco, esta ligado aos anéis imidazólico de três resíduos de histidina, bem como a uma molécula de água. Na direita aparece a localização do zinco na enzima [73].

1.3. Novas estratégias em formulações para o tratamento do glaucoma

O desenvolvimento de formulações que permitam a entrega e a permeação de fármacos oculares é um desafio constante na tecnologia farmacêutica. Apesar dos muitos resultados positivos com vários agentes terapêuticos é importante assinalar que o tratamento do glaucoma possui uma grande barreira no que diz respeito à adesão ao tratamento, o que muitas vezes está relacionado com a forma farmacêutica utilizada. Por esse motivo, torna-se fundamental o desenvolvimento de formulações farmacêuticas que possibilitem um aumento da adesão por parte dos paciêntes e uma entrega e biodisponibilidade de fármacos oftálmicos nos tecidos oculares. Muitas estratégias têm vindo a ser pesquisadas e mostram-se com grande potencialidade na utilização no tratamento das doenças oculares como o glaucoma. A partir das estratégias mais estudadas destacam-se o uso de dispositivos oculares [74], lipossomas, hidrogeles, dendrímeros [57], micelas,

micro e nanopartículas [75]. Contudo, como descrito anteriormente a natureza de alguns fármacos podem influenciar o desenvolvimento das formulações farmacêuticas de cedência de fármacos antiglaucomatosos. Neste sentido de entre todas as estratégias referenciadas aqui será dado um maior enfoque ao uso de ciclodextrinas e micelas no âmbito do aumento da solubilidade aparente de fármacos e o uso de dispositivos do tipo lentes de contacto medicamentosas para a cedência de fármacos.

1.3.1. Ciclodextrinas

As ciclodextrinas (CDs) são oligossacarídeos de forma cilíndrica, composto por seis (α-ciclodextrina (α-CD)), sete (β-ciclodextrina (β-CD)), oito (γ-ciclodextrina (γ-CD)) (Figura 1.5) ou mais unidades glucopiranose ligadas por ligações glicosídicas do tipo α-1,4 e são conhecidas por CDs naturais. Possuem uma estrutura tronco-cónica e, devido à orientação dos grupos hidroxilos resultam numa estrutura com cavidade central lipofílica, e uma superfície externa hidrófila com capacidade de formar complexos de inclusão com várias moléculas liposolúveis [32]. Durante a formação dos complexos de inclusão não são formados ligações covalentes e quando os complexos estão em solução aquosa são facilmente dissociados [76]. Para além das CDs naturais diferentes derivados de CDs foram sintetizados. Esses derivados são geralmente produzidos por reações de aminações, esterificações ou eterificações dos grupos hidroxila primários e secundários da estrutura da CD. Praticamente todos os derivados têm uma alteração do volume da cavidade hidrofóbica, uma melhora da sua solubilidade e estabilidade, e são capazes de ajudar a controlar a atividade química de moléculas hóspedes e reduzir a sua toxicidade [77]. A aplicação de CDs na solubilização de fármacos pouco solúveis em meio aquoso, aumentando a sua biodisponibilidade e estabilidade traduz-se num maior emprego terapêutico [78].

A eficácia dos fármacos aumenta potencialmente devido ao aumento da sua solubilidade, ou seja, ocorre uma diminuição da dose necessária para obter uma atividade terapêutica ótima, reduzindo a sua toxicidade. [79, 80]. Portanto, CDs são descritas como novos adjuvantes quimicamente estáveis que aumentam a biodisponibilidade ocular de fármacos [81].

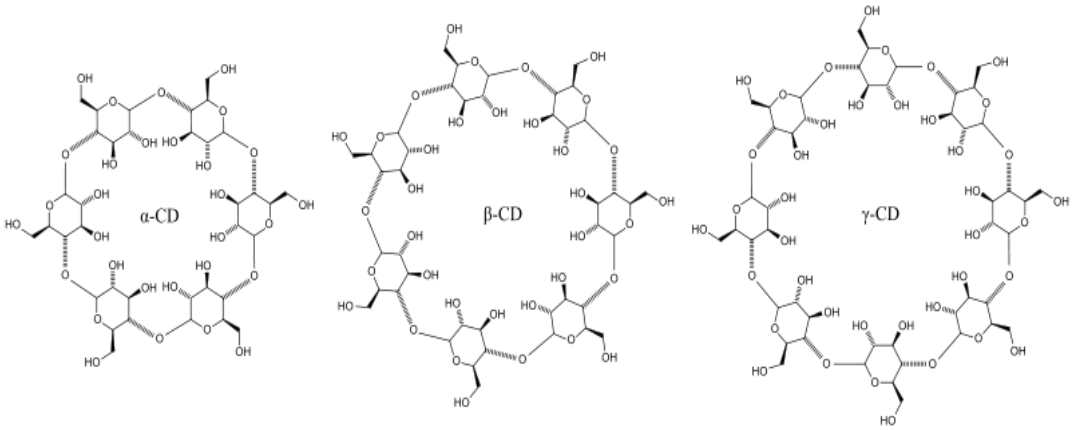

Figura.1.5. Estrutura química das três principais ciclodextrinas naturais.

1.3.1.1 Toxicologia

Alguns derivados de CDs têm sido aplicados em formulações oculares como a hidroxipropil β-CD, γ-CD, maltosil β-CD e a sulfobutilether β-CD ((SBE)β-CD) [82]. Uma preocupação evidente com o uso de CDs é que estas não venham a causar qualquer dano irreversível à córnea [83]. Para ser realmente considerada segura, recomenda-se que a permeabilidade intrínseca da córnea não seja modificada pelas CDs. Embora já utilizadas em formulações comerciais, as CDs metiladas [77, 84] não são consideradas para preparações oftálmicas devido à sua toxicidade e irritação semelhante ao que é esperado para α e β-CDs [85]. No entanto, as CDs metiladas (Mβ-CD) [86] e α-CDs [87] foram consideradas seguras na administração tópica ocular quando usadas em baixas concentrações.

As CDs metiladas podem induzir alterações na permeabilidade intríseca da córnea, e isto deve se à capacidade, sob certas condições, de extrair componentes das membranas biológicas, como o colesterol e fosfolípidos, fazendo o mesmo papel de um agente surfactante [88]. Também podem ter uma atividade hemolítica [85, 89]. Ambos (SBE)β-CD e hidroxipropil β-CD (HPβ-CD) mostraram não alterar a permeabilidade ou causar irritação à córnea [90]. Jarvinen e colaboladores demonstraram que o uso de (SBE)β-CDs diminuiu a irritação oftálmica de um pró-fármaco da pilocarpina [91]. Eles observaram uma redução seletiva na irritação sem uma diminuição na eficácia miótica, quando as CDs estavam presentes. A capacidade das CDs complexadas de interagirem com membranas biológicas é muito reduzida e os efeitos prejudiciais à membrana só são geralmente observados *in vivo* na presença de concentrações relativamente elevadas [32]. As CDs hidrofílas não atravessam a barreira ocular assim como os promotores de absorção convencionais, por exemplo, o cloreto de benzalcónio [92]. Investigadores [93, 94] demonstraram que o cloreto de benzalcónio, frequentemente usado em colírios comerciais, atua desorganizando as barreiras biológicas, sendo mais citotóxico do que as CDs estudadas [95].

1.3.1.2. Ciclodextrinas na cedência de fármacos oculares

O uso de CDs em preparações oftálmicas tem recebido considerável atenção nas últimas décadas [21, 22, 96]. Os parâmetros cinéticos são fundamentais nas preparações oftálmicas contendo CDs. Para a absorção de fármacos através das membranas oculares, as moléculas têm que estar inicialmente dissolvidas no fluido lacrimal. No entanto, muitos fármacos utilizados em oftalmologia são pouco solúveis em meios aquosos, resultando em baixa absorção e baixa biodisponibilidade. As CDs podem agir como transportadoras de fármacos que disponibilizam a molécula de fármaco até à mucosa do exterior do olho, ou seja, a

camada de mucina, e em seguida libertá-lo para a membrana lipofílica, como a córnea [76]. Além disso, as CDs podem aumentar a concentração de fármacos e a biodisponibilidade contribuindo para obtenção de formulações mais eficazes e tratamentos com esquemas terapêuticos menos frequentes para pacientes com doenças oculares [76].

Um estudo de Zhang e colaboladores [20] em coelhos, utilizando complexos de inclusão, cetoconazol e HPβ-CD, mostrou um aumento de 12 vezes da biodisponibilidade do fármaco quando foi comparado com uma solução aquosa do mesmo sem CD. A junção de uma ou várias estratégias permite aumentar a eficiência de complexação das CDs. Destacando-se o ajuste do pH do meio de complexação, a formação de complexos multicomponentes com ácidos orgânicos e/ou bases orgânicas e/ou aminoácidos, e ainda a formação de complexos multicomponentes com polímeros hidrossolúveis. Granero e colaboladores combinaram o efeito da HPβ-CD e de um composto básico (trietanolamina) na preparação de complexos ternários com a acetazolamida, e conseguiram um aumento na solubilidade do fármaco, confirmando uma possível utilização em formulações oftálmicas [97]. Outra estratégia foi aplicada a antibióticos do tipo quinolona, que consistiu na utilização simultânea de iões metálicos (Al^{3+} e Mg^{2+}), CDs (β-CD e HPβ-CD), com controle de pH e a adição de polivinilpirrolidona (PVP) para promover uma maior solubilidade do antibiótico. Os autores verificaram um maior aumento da solubilidade quando houve a utilização do Mg^{2+} e HPβ-CD. O PVP garantiu a estabilidade da formulação impedindo a precipitação do fármaco, quer pelo aumento da sua viscosidade, quer pelo estabelecimento de interações com o fármaco. Concluiu-se que este sistema poderia ser uma potencial formulação para uso oftálmico [98]. Valls e colaboradores [99] avaliaram um modelo de aparelho concebido para o estudo, *ex vivo*, de permeação de fármacos aplicados topicamente, através dos tecidos da

córnea de coelhos. Uma formulação contendo um complexo de diclofenac e β-CD foi utilizada para avaliar o modelo. Os resultados foram satisfatórios na validação do sistema, e a difusão de diferentes formulações com o mesmo fármaco mostrou ser dependente dos aditivos utilizados.

1.3.1.3. As ciclodextrinas como agentes funcionais nas lentes de contacto

Fazendo uso da sua capacidade de formar complexos de inclusão as ciclodextrinas podem ser exploradas de um modo racional na funcionalização de hidrogeles [100-104]. Xu e colaboladores [105] prepararam hidrogeles de poli(metacrilato-PVA-co-mono-metacrilato-β-ciclodextrin) incorporando um monómero (mono-metacrilato-β-CD) (pPVA-β-CD) previamente sintetisado. Os resultados demonstraram que os hidrogeles de pPVA-β-CD possuem boa transmitância, e que a incorporação de β-CD nos hidrogeles fez com que a deposição de proteínas fosse diminuida. Os resultados indicaram que a quantidade de fármaco contido nos hidrogeles aumentou progressivamente, enquanto a taxa de libertação diminui com o aumento da concentração da β-CD. E que a incorporação da β-CD auxiliou na diminuição da velocidade de libertação inicial da acetazolamida e manteve-se sustentada por15 dias. Os autores concluíram que os hidrogeles de pPVA-β-CD têm potencial aplicação como dispositivos biomédicos para libertação sustentada de fármacos oculares. Rosa dos Santos e colaboradores [106] sintetizaram hidrogeles acrílico de hidroxi etil metacrilato copolimerizados com metacrilato de glicidila (GMA) e ligaram a β-CD na rede através de uma reação com os grupos glicidila. Fazendo com que fosse estabelecida uma ligação éter através dos grupos hidroxila. As propriedades mecânicas e de biocompatibilidade dos hidrogeles foi mantida, melhoraram significativamente a capacidade de carregar do fármaco diclofenac em 1300% e conseguiram controlar a taxa de libertação. Os hidrogeles foram capazes de sustentar a entrega do fármaco no fluido lacrimal por duas

semanas. Estes sistemas foram considerados particularmente úteis e citocompatíveis para o desenvolvimento de implantes medicamentados ou dispositivos biomédicos.

1.3.2. Micelas

Micelas são partículas coloidais de compostos anfifílicos (surfactantes ou copolímeros do tipo bloco) formados espontaneamente em solução. São chamadas de sistemas auto-estrurados ou do termo anglo-saxão "self-assemblies". São moléculas anfifílicas com regiões hidrofóbicas e hidrófilas que podem associar-se, formando uma variedade de agregados como: as micelas esféricas, cilíndricas e discoidais, as vesículas, os lipossomas, os microtúbulos, as bicamadas, as micelas reversas e as microemulsões.

A formação das micelas não ocorre a qualquer concentração, apenas a partir de uma concentração mínima, na qual ocorre à formação do agregado micelar, chamada de concentração micelar crítica (CMC) e esta é geralmente determinada a partir de uma variação brusca do sistema em função da concentração [107]. Com a formação de micelas, várias propriedades físicas da solução micelar são afetadas tais como, a viscosidade, a condutividade elétrica, a tensão superficial e a pressão osmótica.

As micelas poliméricas possuem um núcleo hidrofóbico e o exterior hidrófilo, apresentam a vantagem de, em pequenas concentrações, formarem sistemas micelares, o que não é comum quando se utiliza tensoativos de baixo peso molecular. Devido à presença de um núcleo hidrofóbico, as micelas são úteis para a solubilização e estabilização de fármacos liposolúveis. O fármaco pode ser solubilizado no interior hidrofóbico das micelas ou conjugado com o próprio polímero. Estas micelas são consideradas importantes sistemas nanocarreadores,

devido à sua estabilidade cinética, boa termodinâmica e capacidade de libertar lentamente os fármacos [108]. Os copolímeros em bloco formadores das micelas são macromoléculas compostas por duas ou mais unidades estruturais diferentes. São formados por uma sequência linear de um tipo de unidade estrutural (mero) do tipo *A*, quimicamente ligada à outra sequência linear de um mero do tipo *B*. Essas sequências lineares são chamadas de blocos. De acordo com a organização dos blocos na cadeia, os copolímeros em bloco são classificados como sendo do tipo ramificado ou linear. Ainda podem ser classificados como dibloco ou tribloco, dependendo da distribuição dessas unidades repetidas ao longo da cadeia. A formação de micelas de copolímeros compostos por blocos anfifílicos, contendo unidades de poli(óxido de etileno) (PEO) e poli(óxido de propileno) (PPO) como os poloxameros (ou Pluronic®) e poloxaminas (ou Tetronic®), tem despertado grande interesse [109-111] na área da tecnologia farmacêutica.

As poloxaminas (Figura 1.6) são novos copolímeros do tipo em bloco que vêm sendo explorados para uso em diversas áreas incluindo a ocular [108, 112-115]. Estas possuem uma estrutura em forma de X, ou seja, é formada por quatro braços ou blocos de PEO-PPO ligadas por um grupo etilenodiamina central [116]. As poloxaminas estão disponíveis comercialmente (Tabela 1.2) sob uma ampla variedade de composições (diferentes proporções de blocos EO/PO) e pesos moleculares [108].

Tabela 1.2. Propriedades das poloxaminas atualmente comercializadas pela BASF sob o nome de Teronic ®. (Adaptado de [108])

Tetronic	M (Da)	Unidades de EO por bloco (a)	Unidades de PO por bloco (b)	HLB	Solubilidade em água a 25ºC (p/p %)	Ponto de névoa à 1 % (ºC)	pKa
304	1650	3.7	4.3	12-18	>10	75	4.3; 8.1
701	3600	2.1	14.0	1-7	Insolúvel	18	4.0; 7.9
901	4700	2.7	18.2	1-7	Insolúvel	20	5.1; 7.6
904	6700	15	17	12-18	>10	74	4.0; 7.8
908	25000	114	21	>24	>10	>100	5.2; 7.9
1107	15000	60	20	18-23	>10	>100	5.6; 7.9
1301	6800	4	26	1-7	Insolúvel	16	4.1; 6.2
1304	10500	21.4	27.1	12-18	>10	---	---
1307	18000	72	23	>24	>10	>100	4.6; 7.8
90R4	6900	16	18	1-7	>10	43	---
150R1	8000	5	30	1-7	Insolúvel	20	4.8; 7.5

A importância destes sistemas em diversas aplicações terapêuticas deve-se à sua capacidade de solubilizar e transportar fármacos liposolúveis. Além disso, alguns destes polímeros são adequados para o uso em vetorização passiva em células tumorais (permeabilidade e retenção) por bloquear e modular a atividade das bombas de efluxo resistentes a multifármacos [111, 117, 118].

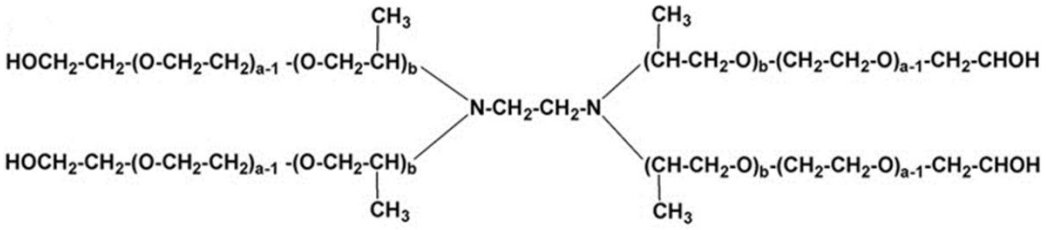

Figura 1.6. Estrutura de uma poloxamina.

Uma propriedade interessante das poloxaminas é a sua sensibilidade ao pH e à temperatura, que se dá devido aos dois grupos de aminas terciárias [119]. Chiappetta e colaboradores [120] avaliaram a influência do pH, sobre a capacidade de solubilização do agente antibactericida triclosan, em micelas

poliméricas de poloxamina T1107. A solubilização do triclosan foi possível a diferentes concentrações micelares, contudo, o poder de agregação diminuía com o aumento do pH e consequentemente a sua solubilização. As micelas contendo fármaco foram utilizadas em estudos *in vitro* de atividade bactericida e demonstraram serem ativas contra uma ampla gama de patógenos, como *Sthaphylococcus aureus* meticilina resistente (MRSA) e *Enterococcus faecalis* vancomicina resistente (VRE) [120]. As poloxaminas também foram utilizadas com sucesso para a solubilização do fármaco efavirenz, um antiviral utilizado na terapia da síndrome da imunodeficiência humana (SIDA). Os estudos de libertação do efavirenz demonstraram uma cinética de ordem zero que foi sutentada por 24 horas, sendo de grande interesse para o uso oral ou parenteral [121]. Micelas preparadas com Tetronic 904 foram capazes de solubilizar o anti-inflamatório não esteróide, nimesulida [122]. A solubilização da sinvastatina e a prevenção da hidrólise do grupo lactona, que é essencial para a sua absorção intestinal, foi obtida com o uso de sistemas micelares utilizando poloxaminas [123, 124].

Além da propriedade de atuar como solubilizante de fármacos liposolúveis e possibilitar uma maior penetração dos fármacos, as poloxaminas podem ser usadas para melhorar a atividade antimicrobiana de composições oftálmicas, facilitar a remoção de proteínas e ou lípidos das superfícies de lentes de contacto e evitar a formação de depósitos de proteínas e lípidos [125, 126]. Estes copolímeros são eficazes em baixas concentrações, pelo que, podem ser muitas vezes introduzidos diretamente no olho. São compatíveis com os agentes antimicrobianos utilizados para preservar composições farmacêuticas aquosas contra a contaminação microbiana e/ou para desinfetar outros dispositivos médicos. Algumas variedades têm a aprovação pela ("*US Food and Drug*

Administration") FDA para ser utilizados como componentes em medicamentos e para dispositivos biomédicos para uso humano [112].

1.3.3. Lentes de contacto

As lentes de contacto (LCs) são um dispositivo ótico, que é usado sobre a córnea do olho de modo a que a lente permanece na superfície do olho durante o movimento do abrir e fechar das pálpebras. São amplamente utilizados para a correção nas deficiências visuais, e também como dispositivos terapêuticos no tratamento de doenças oculares [127]. As LCs são classificadas de acordo com o seu módulo de elasticidade podendo ser LCs duras ou rígidas e moles ou gelatinosas.

A tecnologia das LCs cobre atualmente várias aplicações terapêuticas, incluindo os dispositivos de diagnóstico como LCs inteligentes SENSIMED triggerfish® [128, 129] que permitem que os médicos monitorizem a PIO de seus pacientes por um período de 24 horas, assim como para cedência de fármacos para o tratamento de doenças oculares [130, 131].

São muitos os parâmetros importantes para a formulação de uma adequada LC, como o tipo de polímero, a espessura, a curvatura, o diâmetro da lente e o teor de água. Considerando-se o tipo do polímero, a permeabilidade ao oxigénio (Dk) é um factor muito importante, e pode ser determinado em condições laboratóriais [132]. Quanto maior o valor de Dk, maior será a permeabilidade ao oxigénio [133]. Polse e colaboradores [134] sugeriram valores de Dk superiores a 20 para o uso de lentes com o olho aberto ou, superior a 75 para os períodos prolongados quando o olho está fechado, sendo estes valores suficientes para evitar a hipóxia ou edema da córnea.

Juntamente com as características intrínsecas dos polímeros, outro fator importante é o teor de água da lente. As moléculas de água são o meio de transporte de oxigénio numa lente gelatinosa, assim, quanto maior o teor de água, maior será a permeabilidade ao oxigénio. O teor de água de uma lente gelatinosa depende tanto das subunidades dos monómeros, quanto do número de ligações cruzadas. Com o aumento do número destas ligações, a água é excluída da matriz do hidrogel e diminui o fluxo de oxigénio. Outro fator importante no desenvolvimento de uma LC é o seu movimento no globo ocular que depende da sua curva base e do diâmetro da lente. É importante que a lente flutue sobre o filme lacrimal pré-corneal permitindo assim a troca de oxigenação da córnea durante o piscar e o movimento da lente. Uma curva base mais íngreme ou um diâmetro maior, reduz o movimento da lente, reduzindo assim a entrega do oxigénio à córnea.

1.3.3.1. Lentes de contacto gelatinosas

A diferença básica entre as LCs rígidas das lentes gelatinosas é que estas possuem a capacidade de absorver quantidades consideráveis de água na sua estrutura, são maleáveis e elásticas. Esta propriedade é devida à utilização de monómeros e comonómeros hidrófilos durante a polimerização. Um número variável destes materiais com diferentes características é utilizado na produção de LCs hidrofílas.

Quanto maior a quantidade de água, maior será a tendência de formação de depósitos de substâncias provenientes dos componentes da lágrima, sendo o tipo e a quantidade deste depósito dependentes das propriedades de cada material. Logo, importantes considerações devem ser tomadas quando no desenvolvimento de LCs. A primeira LC gelatinosa e considerada como um protótipo foi desenvolvida por Otto Wichterle e Lim, [135] que utilizou o polímero 2-hidroxi etil metacrilato

(pHEMA). Estas lentes contêm aproximadamente 38-40% de água quando completamente hidratadas, excelente molhabilidade e oferecem mais benefícios que as LCs rígidas, pelo seu conforto e reduzido tempo de adaptação para os pacientes [1]. Estes hidrogeles são produzidos pela polimerização de monómeros individuais com o agente reticulante, etileno glicol dimetacrilato (EDGMA) [136]. Após este primeiro momento, outros monómeros que podem consistir de uma variedade de subunidades hidrófilas ou hidrofóbicas foram introduzidos para a fabricação de LCs gelatinosas. A n-vinilpirrolidona (NVP), é um exemplo, de monómero hidrófilo, que tem um grupo amida, é polar e oferece excelente biocompatibilidade com tecidos vivos [137, 138]. Outro monómero utilizado para produzir LCs para aplicações diárias é o gliceril metacrilato (GMA), que é mais hidrófilo do que o HEMA, devido aos dois grupos hidroxilo existentes na sua estrutura [136, 139, 140]. O ácido metacrílico (MAA), também muitas vezes é empregado como monómero hidrófilo e quando utilizado resulta em uma LC com grupos ionizados (carregados negativamente) dentro da matriz do polímero, permitindo assim que a lente absorva mais água. Infelizmente, este polímero também tem desvantagens, devido às suas sensíveis alterações na tonicidade e pH [136].

O silicone é um material hidrofóbico que é geralmente combinado com monómeros de hidrogel convencional para produzir LCs como, por exemplo, o lotrafilcon A. Hidrogeles com a adição de silicone foram desenvolvidos e melhoraram drasticamente a oferta de oxigénio para a córnea (seis vezes maior) em relação a outros materiais de hidrogel [141]. Devido às propriedades intrínsecas das moléculas de silicone que permitem com que mais oxigénio possa permear através da lente, a sua utilização resulta em uma menor hipóxia em comparação com as LCs de hidrogel convencional. Entretanto, a componente hidrófila dos hidrogeles facilita o transporte de líquidos e, portanto, o movimento

da lente. Kim e Chauhan [142] desenvolveram um hidrogel com adição de silicone para uso como LCs gelatinosas que podem libertar fármacos oftálmicos por um longo período de tempo, mantendo todas as propriedades importantes de uma LC.

O conteúdo de água em uma LC pode chegar a 79%, dependendo das proporções dos monómeros hidrofóbicos ou hidrófilos utilizados. A espessura da LC também afeta a transmissibilidade ao oxigénio. Enquanto o Dk representa a permeabilidade do material, utilizado nas LCs e serve para comparar os materiais, cada LC caracteriza-se por um coeficiente de transmissibilidade, Dk/L, em que o L é a sua espessura. Recentemente foram desenvolvidos diferentes materiais para a fabricação de LCs. Estes podem ser vistos na Tabela 1.3 e divididos em quatros grupos em função da quantidade de água (alta ou baixa) e características iónicas (iónica ou não-iónica).

Tabela 1.3. Classificação dos materiais de LC hidrófilas quanto à hidratação e à ionicidade (FDA-USA)[143]

GRUPO 1	**GRUPO 2**	**GRUPO 3**	**GRUPO 4**
Baixa hidratação (< 50% H_2O) Polímeros não-iónicos	Alta hidratação (> 50% H_2O) Polímeros não-iónicos	Baixa hidratação (<50% H_2O) Polímeros iónicos	Alta hidratação (> 50% H_2O) Polímeros iónicos
Balafilcon A (36%)	Alphafilcon A (66%)	Bufilcon A (45%)	Bufilcon A (55%)
Crofilcon A (39%)	Atlafilcon A (64%)	Deltafilcon A (43%)	Etafilcon A (58%)
Dimefilcon A (36%)	Hefilcon C (57%)	Droxifilcon A (47%)	Methafilcon (55%)
Genfilcon A (47,5%)	Hioxifilcon A (55%)	Etafilcon A (43%)	Oculfilcon B (53%)
Hefilcon A&B (43%)	Lidofilcon A (70%)	Ocufilcon A (44%)	Oculficon C (55%)
Hioxifilcon B (48%)	Lidofilcon B (79%)	Phemfilcon A (38%)	Oculficon D (55%)
Isofilcon (36%)	Melfilcon A (69%)		Oculficon E (65%)
Lotrafilcon A (24%)	Netrafilcon A (65%)		Perfilcon A (71%)
Mafilcon A (33%)	Ofilcon A (74%)		Phemfilcon A (55%)
Phemfilcon A (30%)	Omafilcon A (60%)		Tetrafilcon B (58%)
Polymacon (38%)	Scafilcon A (71%)		Vifilcon A (55%)
Tefilcon (38%)	Surfilcon A (74%)		
Tetrafilcon A (43%)	Xylofilcon A (67%)		

1.3.3.2. Lentes de contacto na cedência ocular de fármacos

As LCs gelatinosas têm sido investigadas em termos de dispositivos de libertação controlada de fármacos de uso oftálmico devido ao facto de serem dispositivos confortáveis, biocompatíveis e apresentarem um significativo aumento de residência de fármacos na mucosa ocular. O emprego destes dispositivos faz com que um determinado fármaco possa estar mais tempo em contacto e difundir-se no tecido alvo. O desenvolvimento de LCs medicamentosas tem um papel importante numa variedade de doenças da superfície ocular (inflamações e infecções) [144] e no glaucoma [145, 146].

A primeira vez que se utilizaram as LCs como um sistema de cedência de fármaco foi carregando-as pelo método de imersão em soluções aquosas de fármaco [147-151]. Outra forma que foi estudada consistia em colocar uma solução de fármaco na concavidade da lente antes de ser colocada no olho, ou a instilação do colírio na sua superfície após a inserção [151-153]. Estudos recentes demonstram que o fármaco é libertado a partir da LC para o filme lacrimal, entre a córnea e a lente, onde pode permanecer ali por muito tempo. Durante o intervalo de tempo entre o abrir e fechar da pálpebra, a superfície externa da lente torna-se seca, fazendo com que a quantidade de fármaco que difunde para epitélio corneano seja de aproximadamente cinco vezes maior do que o montante libertado para o fluido lacrimal que banha a sua superfície externa [154]. Isso explica por que o fármaco gotejado ou pré-embebido na LC pode melhorar tanto a biodisponibilidade ocular, como a resposta farmacológica em relação à administração de colírio convencional.

Xinming e colaboladores [131] descreveram critérios desejáveis para que uma LC gelatinosa seja considerada ideal como um sistema de cedência de fármaco oftálmico, entre elas, que a lente seja capaz de veicular uma concentração máxima

para o tratamento e que seja capaz de ceder de forma sustentada e que seja estável durante a preservação e durante o seu transporte. Rosa dos Santos e colaboradores [106] desenvolveram um sistema que foi capaz de evitar que o fármaco fosse libertado no líquido de conservação comum às LCs gelatinosas. A lente deve idealmente apresentar perfis de libertação de ordem zero, sem a libertação do fármaco rapidamente, e a concentração do fármaco tem que ser sustentada numa concentração máxima segura e com uma concentração mínima eficaz no líquido lacrimal. O material escolhido para formulação da LC também é um fator importante, considerando que a LC deve manter a transparência durante a libertação do fármaco e uma aceitável permeabilidade ao oxigénio [131]. Kim e colaboradores [142] sintetisaram hidrogeles contendo silicone, 1-vinil-2-pirrolidona (NVP) e n, n-dimetilacrilamida (DMA) e demonstraram que a composição pode ser ajustada para se obter uma libertação sustentada de timolol, por um período que varia de 10 dias a alguns meses. Kapoor e Chauhan mostraram que hidrogeles de pHEMA contendo um surfactante, Brij 97, e carregados com um fármaco imunossupressor ciclosporina (CIA) que é utilizado no tratamento de várias doenças oculares, apresentaram uma libertação prolongada, justificando o uso das LCs para cedência ocular [25, 155]. Schultz e colaboladores investigaram a capacidade de carga e de libertação, em lentes oculares comerciais Vasurfilcon® (Ciba Vision), dos seguintes farmácos: timolol e brimonidina e, incluíram para os testes clínicos realizados em pacientes portadores de glaucoma, duas outras lentes Etafilcon A (Vistakon) e Vifilcon (Ciba Vision). Os autores concluíram que a rede dos hidrogeles era capaz de captar passivamente os fármacos e libertá-los em meio salino, sugerindo assim que as LCs são sistemas que podem ser utilizados como ferramentas para o controle da PIO [74].

1.3.4. Polímeros biomiméticos e tecnologia de impressão molecular

A biomemitização é uma técnica que se inspira nas estruturas naturais com o propósito de imitar e desenhar estruturas ou processos que tenham aplicação na vida humana [156]. Tem vindo a ser utilizada em várias áreas e tornou-se uma interessante e desafiadora técnica nas áreas dos biomateriais [157-160]. A partir do conhecimento biomolecular de muitos processos biológicos podem-se desenvolver materiais sintéticos com alta seletividade e grande potencial de utilização [161]. Os polímeros molecularmente impressos ("*Molecularly Imprinted Polymers*"-MIPs) são matrizes artificiais e destacam-se pela sua capacidade de desenvolver sistemas de reconhecimento biomimético semelhante aos sistemas específicos antigeno-anticorpo e ou enzima-substrato [162]. Para a sintese de um MIP é usada uma molécula como molde (ex: fármaco) que interage com grupos funcionais dos monómeros durante a formação do polímero. A técnica é capaz de produzir polímeros porosos, dotados de sítios específicos que são estereoquimicamente moldados com uma alta capacidade de reconhecimento [163-165]. A organização tridimensional dos grupos funcionais dos MIPs é obtida através do estabelecimento de uma ligação covalente ou não-covalente entre monómeros funcionais e a molécula-molde durante o processo de polimerização [166, 167]. No geral, o processo de impressão molecular inclui algumas etapas, conforme mostrado na Figura 1.7. A primeira etapa consiste em misturar o monómero estrutural juntamente com os monómeros funcionais e a molécula molde. Ocorre uma autoestruturação e um esqueleto polimérico é formado ao redor da molécula-molde. Posteriormente adiciona-se ao meio o agente reticulante e iniciador da reação de polimerização. Finalmente, a polimerização é induzida por meio de calor e ou luz UV, na ausência de oxigénio. A molécula molde original é removida da rede polímerica formada através de processos de lavagem ou hidrólises. A escolha do método de remoção da molécula-molde vai depender

do tipo da ligação formada [167]. A remoção da molécula-molde, após a formação do polímero, origina uma estrutura complementar (forma e tamanho) à sua. Com esta estratégia, o resultado é uma "memória" molecular no polímero, ou seja, criam-se microcavidades ou sítios que podem ser ocupados novamente pela molécula-molde ou por outra estrutura análoga, por meio do restabelecimento das interacções de ligação que haviam ocorrido durante o processo de síntese do polímero ou através do estabelecimento de interações mais favoráveis [166, 168]. O facto de estes polímeros poderem mimetizar receptores biológicos aumenta significativamente o interesse pela técnica de MIPs [157, 169]. A busca por reagentes que possuam maior compatibilidade com o sistema biológico é uma alternativa para a obtenção de melhores resultados. Materiais biomiméticos podem interagir, seletivamente com o microambiente biológico mimetizando-o [157], são sistemas sintéticos simples de preparar, com baixo custo e estáveis [170]. Embora estes materiais ainda não sejam utilizados clinicamente na libertação de fármacos, muitos são os pesquisadores [171-174] que têm demonstrado o potencial desta tecnologia no desenvolvimento de formas farmacêuticas com potencial clínico.

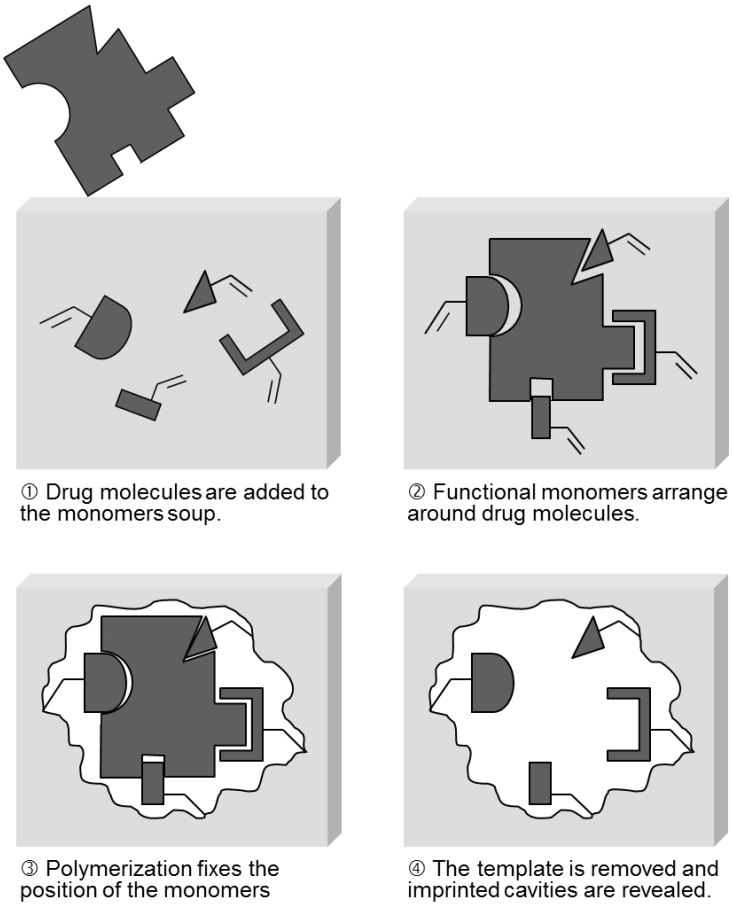

Figura 1.7. Representação esquemática do processo de impressão molecular (Concedido por [150]).

1.3.4.1. Tecnologia de impressão molecular e as lentes de contacto

Nos últimos anos, investigadores [146, 169, 172, 175, 176] têm usado a técnica de MIP em preparações de hidrogeles empregados como LCs para cedência de fármacos. Com isso, pretendem aumentar a possibilidade de carregamento de fármacos oftálmicos e prolongar o tempo de libertação sustentada em LCs gelatinosas. Um critério importante é a escolha de monómeros funcionais. Estes devem possuir grupos funcionais capazes de interagir com o fármaco (molécula-

molde) e formar uma espécie de complexo estável. A interação deve ser capaz de gerar um aumento no coeficiente de partição entre a rede polimérica e a solução de carga de fármaco. A libertação de fármaco pode ser modulada em resposta à competição por sítios específicos ou pela presença de iões. Polímeros biomiméticos podem potencializar a interação e a resposta aos estímulos. Venkatesh e colaboradores valeram-se dos princípios biomiméticos para sintetizar LCs gelatinosas capazes de carregar farmácos anti-histamínicos e sustentar a sua libertação por 5 dias [169]. Alvarez-Lorenzo e colaboladores utilizaram a técnica de calorimetria de titulação isotérmica como ferramenta para estudar as melhores interações entre a molécula de norfloxacina (NRF) e o monómero ácido acrílico (AA) e obter cavidades impressas com maior afinidade pelo fármaco. Os hidrogeles sintetizados usando a relação molar NRF:AA (1:3) e NRF:AA (1:4) mostraram ter maior habilidade no controlo da libertação do fármaco [172]. Ali e colaboradores, demonstraram experimentalmente que LCs sintetizadas utilizando a técnica de *molecular imprinting* foram capazes de libertar o farmaco fumarato de cetotifeno numa cinética de ordem zero [176]. Na última década, moléculas terapêuticas de pequeno peso molecular (anti-histamínicos, antibióticos, antiflamatórios e antiglaucoma) foram utilizadas na produção de LCs recorrendo à técnica de impressão molecular [24, 146, 172, 173, 177]. Em outro estudo, Ali e colaboradores planearam e sintetizaram LCs gelatinosas capazes de carregar ácido hialurónico, que é uma molécula com grande peso molecular, utilizada para o tratamento do olho seco e variando os monómeros funcionais foram capazes de libertar a molécula de ácido hialurónico de maneira sustentada por 24 horas [175]. Demonstrou-se uma possível oportunidade de desenvolver dispositivos capazes de utilizar moléculas de tamanho grandes em sistemas molecularmente impressos para o uso ocular.

1.4. Referências

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OBJETIVOS

Rio Mondego, Coimbra (Portugal)

CAPÍTULO 2

Objetivos

Este trabalho de tese de doutoramento tem como objetivo principal o desenvolvimento de novos sistemas de administração tópica ocular, como os inibidores de anidrase carbónica, visando o tratamento do glaucoma. Avaliando as novas formulações e estratégias terapêuticas e incluindo a perspectiva de uma futura ampliação destes métodos, em benefício dos doentes. Sabendo que a anidrase carbónica é uma enzima que se encontra omnipresentemente distribuída em tecidos e órgãos díspares do sistema de administração oftálmica, admitindo uma provável melhora terapêutica tanto no que diz respeito à eficácia como na segurança.

Para se conseguir chegar ao objetivo geral foram utilizadas duas estratégias:

A) Preparação de dissoluções aquosas fazendo uso de micelas poliméricas e ciclodextrinas com a finalidade de aumentar a solubilidade aparente de fármacos pouco solúveis;
B) Desenvolvimento de lentes de contacto medicamentosas que permitam ceder concentrações terapêuticas de fármacos no filme lacrimal pós-lente por períodos prolongados.

O estudo foi delineado seguindo e adotando objetivos concretos que constituem diferentes etapas do trabalho:

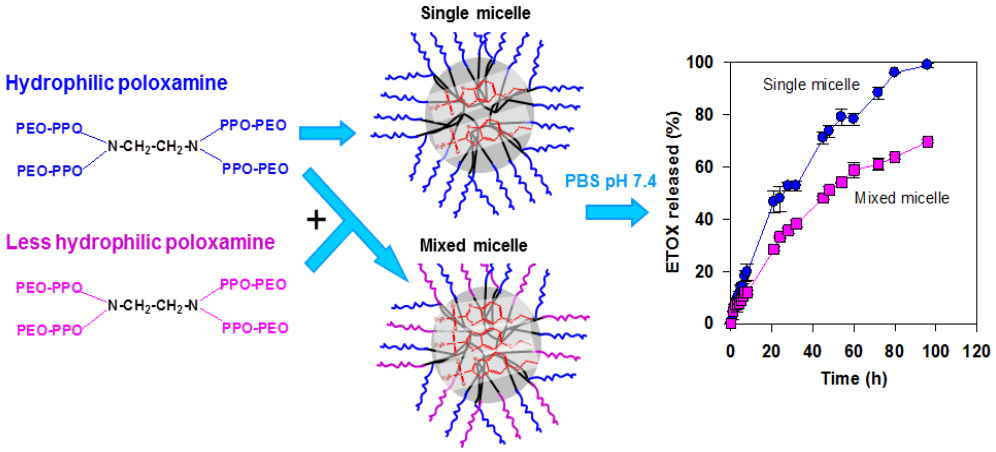
1. Explorar a utilidade dos copolímeros em bloco, de poli (óxido de etileno) - poli (óxido de propileno), da família das poloxaminas, como um novo material para a solubilização de fármacos, fazendo uso da sua capacidade formadora de micelas. Determinar a concentração micelar crítica, o tamanho e a estabilidade das micelas em meio isotónico empregando fluido lacrimal, e avaliar a sua

biocompatibilidade, capacidade de carregamento e libertação da etoxzolamida utilizando ensaios *in vitro*.

2. Avaliar a capacidade de duas ciclodextrinas naturais, β-CD e γ-CD, para formar complexos de inclusão com inibidores da anidrase carbónica, nomeadamente, acetazolamida e etoxzolamida. As ciclodextrinas foram utilizadas para a preparação de hidrogeles incorporando-as como: (i) monómeros funcionais aptos para copolimerização com outros monómeros; (ii) entidades pendentes que se ligam a redes poliméricas já polimerizada; com a finalidade de melhorar a carga e conseguir uma libertação sustentada dos fármacos a partir dos hidrogeles.

3. Desenvolver redes poliméricas biocompátiveis que possam captar quantidades consideráveis de inibidores da anidrase carbónica (acetazolamida e ethoxzolamida), utilizando técnicas biomiméticas. Pretende-se criar domínios no hidrogel, com alta afinidade pelo fármaco, que imitam o sítio ativo da enzima a que se unem *in vivo*. Para isto selecionaram-se os monómeros que melhor mimetizavam os grupos funcionais dos aminoácidos do sítio ativo da enzima e avaliaram-se as possibilidades que a técnica de impressão molecular oferece para conseguir uma ocupação ótima pelos monómeros.
Caracterizar os hidrogeles quanto às suas propriedades físico-químicas e mecânicas, transparência ótica, biocompatibilidade, capacidade de carregamento e cedência de fármacos utilizando ensaios *in vitro*.

SINGLE AND MIXED POLOXAMINE MICELLES AS NANOCARRIERS FOR SOLUBILIZATION AND SUSTAINED RELEASE OF ETHOXZOLAMIDE FOR TOPICAL GLAUCOMA THERAPY



CHAPTER 3

Abstract

Polymeric micelles of single and mixed poloxamines (Tetronic ®) were evaluated regarding their ability to host the antiglaucoma agent ethoxzolamide for topical ocular application. Three highly hydrophilic varieties of poloxamine (T908, T1107 and T1307) and a medium hydrophilic variety (T904), possessing a similar number of propylene oxide units but different contents in ethylene oxide, were chosen for the study. The CMC and the cloud point of mixed micelles in 0.9% NaCl were slightly greater than the values predicted from the additive rule, suggesting that the co-micellization is somehow hindered. Micellar size ranged between 17 and 120 nm and was not altered after the loading of ETOX (2.7-11.5 mg drug/g poloxamine). Drug solubilization ability ranked in the order: T904 (50-fold increase in the apparent solubility) >T1107≅T1307>T908. The mixed micelles showed an intermediate capability to host ethoxzolamide but a greater physical stability, maintaining almost 100% drug solubilized after 28 days. Furthermore, the different structural features of poloxamines and their combination in mixed micelles enabled to tune drug release profiles, sustaining the release in the one to five days range. These findings together with promising HET-CAM biocompatibility tests make poloxamine micelles as promising nanocarriers for carbonic anhydrase inhibitors in the treatment of glaucoma.

Keywords

Polymeric micelles, ethoxzolamide, poloxamine, ocular delivery, CAI solubilization, controlled release, PEO-PPO block copolymer.

3.1. Introduction

Carbonic anhydrase inhibitors (CAIs) drugs represent an important option for the treatment of glaucoma due to their efficacy in decreasing the rate of aqueous humor secretion. Carbonic anhydrases are metalloenzymes present in the anterior uvea of the eye that catalyze the conversion of CO_2 to bicarbonate and proton [1]. The use of CAIs, namely sulfonamides such as acetazolamide, as a way to lower the intraocular pressure (IOP) in the treatment of glaucoma is mostly centered in the oral route. Unfortunately, the oral administration of CAIs is associated with relevant systemic adverse effects like depression, fatigue, gastrointestinal irritation, metabolic acidosis, metallic taste, loss of libido and paresthesias [2] due to the ubiquitous distribution of carbonic anhydrases in all the tissues of the body. Thus, the topical administration of CAIs to the eye may notably decrease these side effects and be more patient compliant. However, the development of suitable ophthalmic formulations of CAIs has to face up their limited aqueous solubility, particularly in the case of those that have adequate log *P* value to pass through the cornea [3, 4].

Ethoxzolamide (ETOX) is a hydrophobic CAI (log *P* = 2.08) with a high activity against carbonic anhydrases and a favorable corneal permeability (100 times greater than the most popular one, acetazolamide) [5-7]. ETOX structure has being used as the starting point to design new molecules, such as dorzolamide and brinzolamide [8, 9], which were approved as the firsts topical CAIs ([3, 10]. Another hydrophilic derivative, 6-hydroxyethoxzolamide, has shown efficient drug corneal penetration and ocular hypotensive effect in albino rabbit eyes [11]. The 6-amino-2-benzothiazolesulfonamide formulated as topical gel reduced the pressure in human eyes, but not when prepared as suspension [12]. On the other hand, it was shown that ETOX can be solubilized by forming inclusion complexes with cyclodextrin derivatives and that topically active formulations that combine

ETOX and timolol can be thus prepared [13, 14]. More recently, biomimetic contact lenses capable to load ETOX and to control the drug release were prepared with the intention of prolonging the contact time between the drug and the eye and enhancing the ocular bioavailability of the drug [15].

Polymeric micelles of block copolymers are a potentially valuable tool to overcome some limitations involved in CAIs formulation for ophthalmic application. The core-shell structure may notably enhance the apparent aqueous solubility, leading to a greater concentration gradient favorable for diffusion, and also provide sustained release since the dilution factor in the lachrymal fluid is less than in other administration routes [16]. Polymeric micelles have been shown suitable ocular carriers for anti-inflammatory drugs [17] or even plasmids and genes [18, 19]. Transcorneal permeation studies through excised rabbit cornea indicated that non-steroidal anti-inflammatory drugs (NSAID) formulations in polymeric micelles can enhance about 2-fold drug permeation compared to that of an aqueous suspension of the same concentration because the dissolution step is overcome [17]. Recent studies have demonstrated that poloxamine micelles are able to host relatively hydrophobic drugs and to increase their apparent solubility and stability [20, 21]. As opposed to the linear counterparts (poloxamers) that are only thermoresponsive, poloxamines (four arms of poly(ethylene oxide)-poly(propylene oxide) connected through an ethylenediamine group) are appealing amphiphiles owing to the greater chemical versatility and dually pH- and temperature-responsive behavior [22]. Furthermore, some block copolymers and particularly certain varieties of poloxamers and poloxamines are able to block the P-glycoprotein efflux pumps and to enhance drug penetration in different tumor cells [23-25]. Such a feature may also contribute to increase the corneal penetration by inhibition of the P-glycoprotein present in the corneal tissue [26].

The aim of this work was to elucidate the potential of single and mixed polymeric micelles of branched poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO)

block copolymers of the poloxamine family (Tetronic®) as nanocarriers suitable for the ocular delivery of ETOX. The self-associative behavior of mixtures of these X-shaped copolymers in 0.9% NaCl has been explored in detail to gain an insight into the potential performance of these systems for localized ocular delivery. ETOX solubilization, micelle stability, tissue irritability and *in vitro* ETOX release were also evaluated. To the best of our knowledge, this is the first study evaluating the performance of polymeric micelles for the encapsulation of CAIs in the treatment of glaucoma.

3.2. Materials and Methods

3.2.1 Materials

Ethoxzolamide (ETOX) was from Sigma-Aldrich Chemicals (Madrid, Spain). Tetronic 904 (T904, M_w 6700, 40% PEO, 15 EO and 17 PO units per arm), 908 (T908, M_w 25,000, 80% PEO, 114 EO and 21 PO units per arm), 1107 (T1107, M_w 15,000, 70% PEO, 60 EO and 20 PO units per arm), and 1307 (T1307, M_w 18,000, 70% PEO, 72 EO and 23 PO units per arm) were donated by BASF (New Milford, CT, USA). Purified water was obtained by reverse osmosis (MilliQ®, Millipore, Spain). Others reagents were of analytical grade.

3.2.2. Preparation of single and mixed polymeric micelles

Solutions of T904, T908, T1107 and T1307 at 10 % (w/v) were prepared by adding the copolymer to cold 0.9% NaCl aqueous medium. Solutions were stored for 24 hours at 25ºC before the assays. Mixed micelles of T904:T1107 and T904:T1307 were prepared by mixing the above prepared solutions at 75:25, 50:50 and 25:75 weight ratios. Mixed micelles of T1107:T1307 were prepared at

a 50:50 weight ratio. In the case of mixed micelles, the overall copolymer concentration was also 10%.

3.2.3. Critical micellar concentration

Critical micellar concentration (CMC) of single and mixed poloxamine solutions was estimated by means of dynamic laser scattering (DLS) employing a Zetasizer Nano-Zs (Malvern Instruments, Worcestershire, UK) fitted with a He-Ne (633 nm) laser and a digital correlator at a scattering angle of $\theta = 173^{\circ}$ to the incident beam. Copolymer solutions (0.001-10%) were filtered through cellulosic membranes of 0.22 μm (Westboro, MA, USA) and equilibrated at 25 ˚C prior to the analysis. Measurements were made at 25^{o}C in triplicate.

3.2.4. Cloud point (CP)

The measurements were performed by submerging glass tubes that contained 2.0 mL of the micellar system (10%) without drug in an oil bath at room temperature. Then, the temperature was increased at a rate of 1ºC/min until an abrupt change in the visual appearance of the system from clear to turbid was observed [27]. Assays were carried out in duplicate.

3.2.5. Micellar solubilization of ETOX

Solutions of single and mixed polymeric micelles (5 mL) were transferred into vials containing ETOX in excess (8 mg) and kept under magnetic stirring at 25 ºC ±1 for 72 h. Then, the solutions were filtered through PTFE membranes of 0.45 μm pore size (Sartorius, Goettingen, Germany) to remove the insoluble drug. The concentration of the dissolved drug was quantified by UV spectrophotometry at

304 nm (CARY 1E UV-Visible Spectrophotometer Varian, Palo Alto, CA, USA) using a calibration curve of ETOX ethanolic solutions (5-20 mg/L). Solubility factors (f_S) were calculated according to the equation:

$$f_S = \frac{S_a}{S_{S.Aq.}} \qquad \text{Eq.(3.1)}$$

where S_a and $S_{SAq.}$ represent the ETOX apparent solubility in micelles and the experimental intrinsic solubility in 0.9% NaCl (22.84 mg/L).

3.2.6. Kinetic stability of drug-containing micelles

ETOX-loaded micellar systems were stored at 25 ˚C and monitored over 28 days. The absorbance of aliquots (50 µL) diluted in ethanol (2950 µL) was recorded at 304 nm in order to quantify the amount of ETOX remaining in solution. At the same time points, the hydrodynamic diameter (D_h) and the polydispersion index (PDI) of the micelles were recorded by DLS using the same operational conditions described above. All measurements were carried out in triplicate.

3.2.7. Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) assay

Fertilized broiler chicken eggs (not older than 3 days; Avirojo, Pontevedra, Spain) were incubated with the large end upwards in an Ineltec CCSP0150 climatic chamber (Tona, Barcelona, Spain) at 37±0.3 °C and 60±2% relative humidity. Eggs were rotated (five times per day) for 8 days to prevent the attachment of the embryo to one side of the egg. Then, the ICCVAM-recommended test method protocol was followed [28]. The upper part of the eggshell (air cell) was removed using a Dremel 300 equipped with a rotary saw (Breda, Netherlands). The intact

inner membrane was moistened with 0.9% NaCl solution and the eggs were placed in the climatic chamber for a maximum of 30 min. The 0.9% NaCl solution was sucked out and the inner membrane was removed with a forceps. A micellar solution (300 µL at 25 ºC) was placed on the chorioallantoic membrane and the irritation potential (hemorrhage, vascular lysis and coagulation) was monitored for 300 seconds. The experiments were carried out in triplicate. Negative (0.9% NaCl solution) and positive (0.1 N NaOH) controls were tested under the same conditions. Irritation scores (IS) were calculated from the time (in seconds) at which hemorrhage (H), lysis (L) or coagulation (C) started, as follows [28]:

$$IS = \left[\left(\frac{301 - H_{time}}{300}\right)\cdot 5\right] + \left[\left(\frac{301 - L_{time}}{300}\right)\cdot 7\right] + \left[\left(\frac{301 - C_{time}}{300}\right)\cdot 9\right] \qquad \text{Eq. (3.2)}$$

According to the IS values, the materials can be classified as non-irritating (0-0.9), weakly irritating (1-4.9), moderately irritating (5-8.9) or severely irritating (9-21) [28].

3.2.8. *In vitro* release studies

ETOX release from the loaded micellar systems was studied using Franz diffusion cells with diffusion area of 0.785 cm^2 and fitted with cellulose dialysis membrane (MWCO 3500, Spectrum Lab., Rancho Dominguez, CA, USA), previously immersed for 30 min in distilled water and washed with buffer. Isotonic phosphate saline buffer (7 mL, pH 7.4) containing 0.3% SDS was used as receptor medium. The donor compartment was filled with 500 µl of the drug-loaded micellar systems and covered to prevent evaporation. The receptor solution was stirred with a magnetic bar and maintained at 32 ± 0.5°C throughout the experiment. This temperature mimics the ocular environment. The ETOX

concentration in the receptor solution was monitored over time by UV spectrophotometry at 304 nm (UV-vis spectrophotometer, Agilent 8453, Waldbronn, Germany) by taking 700 µL samples at pre-established time points. The same volume was replaced with fresh buffer medium (700 µL). Assays were carried out in triplicate.

3.3. Results and Discussion

3.3.1. Self-aggregation of poloxamines

The present work explored the capacity of three highly hydrophilic (HLB >18) varieties of poloxamine (T908, T1107 and T1307) and a medium hydrophilic (HLB 12-18) variety (T904) and their combinations to form mixed polymeric micelles as a nanotechnology platform to encapsulate the CAI-drug ETOX towards the topical treatment of glaucoma. Previous studies showed that to produce mixed poloxamer/poloxamer polymeric micelles both copolymers need to present two main features: (i) hydrophobic blocks of similar molecular weight and (ii) different hydrophilic/hydrophobic balance [29, 30]. These two premises were taken into account for choosing the poloxamines for the study. Our hypothesis was that co-micellization of highly hydrophilic poloxamines (T1107 and T1307) with a more hydrophobic derivative (T904) would lead to encapsulation extents of ETOX characteristic of T904 and at the same time would improve the physical stability of the drug-loaded micelles; drug-loaded T904 micelles are shown to be physically instable [31, 32]. Poloxamer/poloxamine mixed micelles were previously capitalized to improve the physical stability of efavirenz-loaded micelles [33]. As opposed to poloxamers that are linear molecules with a single central PPO block, poloxamines display a molecular architecture where the PPO content is the sum of four segments connected through a central ethylenediamine

unit. In this regard, we recently hypothesized that poloxamines could behave as two linked PEO-PPO-PEO triblocks [24]. Thus, some steric hindrance in the co-micellization process could be anticipated.

The micellization process of single poloxamines has been previously evaluated in detail in water, HCl medium and other aqueous salt solutions (e.g., NaCl and Na_2SO_4) [21, 34, 35]. It should be noticed that these block copolymers are quite sensitive to the physicochemical conditions of the dispersant medium, particularly the pH and the ionic strength, which lead to changes in the protonation extent of the ethylenediamine central group and consequently alter the hydrophobic interactions that govern the self-assembly phenomena [23, 36].

The CMC of single and mixed systems was determined by DLS (Table 1) and compared with the CMC value predicted according to the following expression [37]:

$$\frac{1}{CMC} = \frac{X_1}{CMC_1} + \frac{X_2}{CMC_2} \qquad \text{Eq.(3.3)}$$

where X_1 and X_2 represent the molar fractions of the components 1 and 2, and CMC_1 and CMC_2 the CMC values of components 1 and 2, respectively.

The CMC values of single poloxamine systems (Table 3.1) were in the same order of magnitude of those previously obtained in HCl 10 mM using the pyrene fluorescence technique [21]. The greater the molecular weight of the copolymer, the lower the CMC. This behavior was less dependent on the HLB. The slight decrease of CMC in NaCl with respect to HCl would stem from the salting out induced by Na^+ ions. Then, the analysis focused on the mixed micelles. In general, CMC data were similar to those theoretically predicted, though slight differences were observed. Positive and negative deviations from ideality point out an unfavorable and a favorable mixing process, respectively. When favorable

interactions are strong, co-micellization is improved and the experimental CMC value is smaller than the theoretical one. The greater the difference between the theoretical and the experimental value, the more favored or disfavored the co-micellization. T1107:T1307 (50:50) micelles showed a CMC value that was greater than the one shown by both copolymers separately, suggesting that the co-micellization was hindered. It is worth stressing that T1107 and T1307 do not comply with the condition for the generation of mixed micelles that both components need to display different HLB values [29, 30].

The addition of growing T1107 or T1307 amounts to T904 led to a gradual decrease of the CMC of pure T904 from 0.75 mM to 0.47 and 0.52 mM, respectively, for T904:T1307 (25:75) and T904:T1107 (25:75). These findings would suggest that the aggregation is primarily driven by the micellization of T1107 and T1307 (two copolymers that show relatively low CMC), followed by the later incorporation of T904 into the core of the initially formed micelles. This hypothesis was previously formulated for poloxamer/poloxamine mixed micelles studied by ESR [33]. On the other hand, all CMC values remained greater than those shown by single T1107 and T1307, suggesting that the addition of T904 to the T1107 and T1307 micelles had a slight to moderate detrimental effect on the self-aggregation of the highly hydrophilic counterparts. Interestingly, T904:T1107 (75:25) micelles showed a CMC value of 0.77 mM, this value being the greatest of all the systems under evaluation and greater than the one shown by pure T904. These results indicated that the formation of these micelles is strongly hindered. Overall, these results are in agreement with previous reports where F127:P85 and F127:P123 mixed micelles showed a positive deviation and F88:P123 showed a negative one [37].

The turbidity of 10% micellar solutions was monitored as a function of temperature with the aim to establish the cloud point (CP) and the effect of NaCl on the aggregation process [27]. As expected from its smaller HLB, T904

evidenced phase separation at a much lower temperature (65ºC) than that of the more hydrophilic poloxamines (97-105ºC; Table 1). Remarkably, the CP of 10% T904 in 0.9% NaCl was 10ºC lower than the value measured in phosphate-citrate buffer solution of pH 5 [33]. All systems showed one single CP, revealing of the formation of mixed micelles [38]. In addition, values of mixed micelles were always smaller than that of the pure hydrophilic poloxamine, probably due to the generation of a more hydrophobic system upon the addition of T904. These findings were in full agreement with data reported elsewhere [33]. On the other hand, with the exception of T1107:T1307 (50:50), experimental CP values of mixed micelles were higher than the theoretical ones (estimated as for the CMC). The CP is associated with the inter-micellar interactions in the binary system and it is expected to differ from that of the single micelles. These results constitute further evidence that even if taking place, the co-micellization of poloxamine mixtures is a quite disfavored process.

Table 3.1. Experimental and predicted CMC and cloud point values for single and mixed systems in 0.9% NaCl at 25 ºC.

Copolymers	Experimental CMC (mM)	Theoretical CMC (mM)	Experimental cloud point (˚C)	Theoretical cloud point (ºC)
T904	0.75	-	65	-
T908	0.24	-	97	-
T1107	0.40	-	105	-
T1307	0.33	-	100	-
T1107:T1307 (50:50)	0.49	0.37	99	102
T904:T1107 (25:75)	0.52	0.50	97	84
T904:T1307 (25:75)	0.47	0.45	93	80
T904:T1107 (50:50)	0.65	0.59	86	74
T904:T1307 (50:50)	0.61	0.56	87	72
T904:T1107 (75:25)	0.77	0.67	76	68
T904:T1307 (75:25)	0.63	0.66	71	68

3.3.2. Micellar size

Size of single and mixed micelles was recorded before and after the loading with ETOX (Table 3.2). Multimodal distributions were observed in all the cases except for T904 and its combinations with T1107 and T1307 at a 75:25 weight ratio. In the case of ETOX-free micelles, main size fractions of sizes between 17 and 120 nm (peak 1) correspond to polymeric micelles, while the other ones belong to the smaller unimers or dimers (4-7 nm, peak 2) or insoluble matter (>200 nm, peak 3) [21]. It is worth stressing that the percentage of unimers/dimers in samples of pure T1107, T1307 and T908 ranged between 25 and 48% and it was substantially greater than that in T904 (4.5%). This phenomenon relied on the incomplete micellization of hydrophilic PEO-PPO block copolymers at 25^{o}C [20].

T904 micelles were smaller (approximately 20 nm) than those of the other poloxamines (45-70 nm) owing to the lower molecular weight. When mixed micelles were analyzed, the size depended on the relative composition. In general, the greater the T904 content, the smaller the size. For example, T904:T1107 and T904:T1307 (75:25) micelles displayed sizes similar to those of pure T904. Conversely, the size of 25:75 mixed systems was approximately 40 nm, while 50:50 showed an intermediate value. Incorporation of ETOX into the micelles, in the amounts discussed below, did not cause relevant changes in the micellar size and size distribution. These findings indicate that ETOX does not induce micellization as previously shown for efavirenz [36] and only a clear size increase from 66.8 and 52.4 nm to 119.8 and 69.4 nm was observed for single T908 and T1107 micelles, respectively, at day 0 (Table 3.2).

Table 3.2. Micellar size (Dh), size distribution and polydispersity index (PDI) of (A) ETOX-free polymeric micelles, (B) ETOX-loaded poloxamine micelles at day 0 and (C) ETOX-loaded poloxamine micelles stored at 25 ºC over 28 days. The final copolymer concentration was 10%.

Copolymers		**Peak 1**		**Peak 2**		**Peak 3**		**PDI**
		D_h (nm)	**%**	**D_h (nm)**	**%**	**D_h (nm)**	**%**	
(A)	T904	-	-	22.7 (0.3)	100.0	-	-	0.39 (0.00)
(B)	T904	-	-	17.3 (0.1)	100.0	-	-	0.27 (0.02)
(C)	T904	3.8 (0.6)	7.0	16.9 (1.3)	93.0	-	-	0.30 (0.13)
(A)	T908	4.8 (0.1)	45.3	65.4 (3.5)	18.7	545.5 (76.7)	36.0	0.40 (0.01)
(B)	T908	5.1 (0.0)	38.3	107.7 (13.1)	32.6	349.5 (38.5)	29.1	0.24 (0.03)
(C)	T908	4.6 (0.0)	53.5	56.1 (6.7)	29.0	418.4 (31.2)	17.5	0.46 (0.01)
(A)	T1107	5.5 (0.1)	25.0	52.4 (0.5)	75.0			0.73 (0.04)
(B)	T1107	5.8 (0.2)	22.1	69.8 (3.4)	77.9			0.81 (0.04)
(C)	T1107	4.5 (0.0)	37.2	28.7 (10.9)	8.6	264.0 (43.1)	54.2	0.63 (0.03)
(A)	T1307	5.7 (0.1)	27.9	54.9 (2.9)	72.1	-	-	0.68 (0.06)
(B)	T1307	5.6 (0.1)	19.4	47.7 (1.5)	80.6	-	-	0.84 (0.01)
(C)	T1307	5.3 (0.1)	23.3	50.4 (0.7)	76.7	-	-	0.38 (0.07)
(A)	T1107:T1307 (50:50)	5.3 (0.1)	27.3	43.6 (0.8)	72.7			0.66 (0.04)
(B)	T1107:T1307 (50:50)	5.4 (0.1)	30.5	47.8 (0.2)	69.5	-	-	0.55 (0.03)
(C)	T1107:T1307 (50:50)	5.4 (0.0)	25.5	46.8 (0.3)	74.5	-	-	0.42 (0.05)

Tabela.3.2. Continuation.

(A)	T904:T1107 (50:50)	6.4 (0.6)	29.7	30.7 (4.4)	70.3	-	-	0.52 (0.01)
(B)	T904:T1107 (50:50)	4.8 (0.3)	18.5	22.8 (1.2)	81.5	-	-	0.27 (0.15)
(C)	T904:T1107 (50:50)	3.7 (0.2)	11.9	21.0 (1.7)	52.5	326.6 (42.6)	35.6	0.59 (0.02)
(A)	T904:T1307 (50:50)	5.6 (0.2)	22.3	28.7 (0.3)	77.7	-	-	0.49 (0.06)
(B)	T904:T1307 (50:50)	5.5 (0.3)	27.4	20.2 (0.5)	72.6	-	-	0.26 (0.04)
(C)	T904:T1307 (50:50)	4.7 (0.1)	21.0	21.7 (2.1)	59.6	222.6 (74.7)	19.4	0.45 (0.21)
(A)	T904:T1107 (25:75)	5.7 (0.2)	27.7	42.5 (1.3)	72.3	-	-	0.52 (0.02)
(B)	T904:T1107 (25:75)	5.9 (0.1)	27.8	38.4 (0.8)	72.2	-	-	0.47 (0.01)
(C)	T904:T1107 (25:75)	5.3 (0.2)	22.3	36.6 (0.9)	77.7	-	-	0.43 (0.17)
(A)	T904:T1307 (25:75)	5.9 (0.2)	30.3	39.0 (1.7)	69.7	-	-	0.49 (0.02)
(B)	T904:T1307 (25:75)	6.2 (0.3)	32.3	39.2 (1.4)	67.7	-	-	0.49 (0.02)
(C)	T904:T1307 (25:75)	5.5 (0.1)	26.6	36.1 (1.9)	73.4	-	-	0.31 (0.09)
(A)	T904:T1107 (75:25)			20.1 (0.1)	100.0	-	-	0.29 (0.01)
(B)	T904:T1107 (75:25)			20.0 (0.1)	100.0	-	-	0.28 (0.01)
(C)	T904:T1107 (75:25)	5.3 (0.4)	16.1	23.0 (0.7)	83.9			0.43 (0.02)
(A)	T904:T1307 (75:25)	-	-	19.1 (0.1)	100.0	-	-	0.29 (0.01)
(B)	T904:T1307 (75:25)	-	-	18.3 (0.5)	100.0	-	-	0.27 (0.01)
(C)	T904:T1307 (75:25)	5.2 (0.3)	16.7	21.4 (1.4)	83.3			0.30 (0.11)

3.3.3. ETOX solubilization

ETOX displays a relatively high melting point of 189^{o} C, revealing the presence of strong solute-solute interactions. To efficiently encapsulate the drug within polymeric micelles, the drug-core interaction needs to be stronger than the solute-solute ones. All poloxamine micelles led to sharp increases in drug solubility (Table 3.3), at least one order of magnitude compared to ETOX aqueous solubility (22.8 mg/L). T904 solely micelles increased up to 50 times the apparent solubility of the drug. The amount of drug hosted by the micelles referred to gram of polymer ranged between 2.7 mg for T908 to 11.5 mg for T904 (Table 3). Solubilization ability ranked in the order: T904>T1107≅T1307>T908.

Mixed micelles did not show a synergistic solubilization as previously shown with efavirenz [33], but improved the physical stability of the system with respect to ETOX-loaded T904 single micelles. Both DLS measurements of micellar size and size distribution (Table 3.2) and spectrophotometric determination of ETOX solubilized (Table 3.3) indicated that drug-loaded micelles are quite stable over 28 days in 0.9% NaCl medium, remaining encapsulated 82-94% ETOX in single micelles and nearly 100% ETOX in the mixed micelles. Therefore, ETOX formulations in poloxamine micelles could be envisioned as an aqueous solution with a shelf life longer than one month. It has been previously shown that poloxamine micellar solutions of 10% T904, T908, T1107 and T1307 enhance simvastatin solubility by factors of 8.5, 2.4, 4.7 and 21, respectively, and protect the labile lactone group from hydrolysis [21]. T904 micelles increased the solubility of the anti-HIV drug efavirenz by 7930.5-fold [33]. The solubility factors achieved for ETOX are intermediate, though closer to those of simvastatin. Even though these solubility extents were smaller than those previously reported by Loftsson et al. with 12.5% hydroxypropyl-β-cyclodextrin/0.1% hydroxypropyl methylcellulose [13], they would be appropriate for the topical treatment of IOP.

Moreover, since PEO-PPO block copolymers at greater concentration form thermoresponsive gel-like to gel matrices [39], these nanocarriers could be employed as a technology platform for the production of ETOX-loaded viscous systems where the drug is completely soluble within the micelles and the contact with the ocular mucosa is extended.

Table 3.3. Apparent solubility (Sa) and solubility factor (f_S) of ETOX in micellar systems and Sa (%) after 28 days for drug-saturated 10 % poloxamine solutions in 0.9% NaCl, at 25 ºC.

Copolymers	**S_a (mg/mL)**	**% (28 days)**	**ETOX/ polymer (mg/g)**	***f_S***	**ETOX/ hydrophobic block (mg/g)**
T904	1.16 (0.11)	94.9 (4.1)	11.5 (1.07)	50.68 (4.68)	19.26 (1.78)
T908	0.28 (0.02)	82.6 (12.1)	2.73 (0.21)	11.97 (0.90)	13.65 (1.03)
T1107	0.61 (0.02)	94.6 (3.1)	6.14 (0.27)	26.93 (1.17)	20.46 (0.89)
T1307	0.63 (0.07)	90.1 (7.5)	6.32 (0.70)	27.70 (2.98)	22.36 (0.03)
T1107:1307 (50:50)	0.44 (0.01)	100.0 (5.4)	4.37 (0.12)	19.16 (0.50)	14.56 (0.38)
T904:T1107 (25:75)	0.65 (0.01)	100.0 (2.4)	6.46 (0.12)	28.36 (0.54)	17.24 (0.33)
T904:T1307 (25:75)	0.71 (0.01)	100.0 (3.9)	7.10 (0.01)	30.97 (0.04)	18.83 (0.02)
T904:T1107 (50:50)	0.85 (0.01)	95.4 (4.0)	8.45 (0.11)	37.07(0.48)	18.78 (0.24)
T904:T1307 (50:50)	0.86 (0.02)	94.5 (6.1)	8.57 (0.19)	37.57 (0.82)	19.04 (0.41)
T904:T1107 (75:25)	0.80 (0.01)	100.0 (0.3)	7.96 (0.05)	34.89 (0.21)	15.15 (0.09)
T904:T1307 (75:25)	0.82 (0.02)	100.0 (5.7)	8.16 (0.18)	35.80 (0.80)	15.54 (0.34)

3.3.4. HET CAM assay

T1107 is an FDA-approved component of multipurpose solutions for cleaning/storage of contact lenses usually at concentrations of 1% (Tonge et al., 2001). Since poloxamine micellar solutions contain 10% copolymer concentration, the micellar compatibility with eye tissues was characterized.

Namely, the potential ocular irritancy was evaluated according to the HET-CAM test following the NICEATM-ICCVAM protocol [28]. The hen's embryo, more precisely the chorioallantoic membrane, is as an alternative to the Draize eye rabbits test for the evaluation of ocular formulations. The HET-CAM test is a simple, fast and cheap test that provides measurable indices of the biocompatibility of a material [40, 41]. The micellar systems tested did not induce haemorrhage, lysis or coagulation both before and after being loaded with ETOX. Thus the IS of all micelles was 0.0, as occurred for the negative control (0.9% NaCl). By contrast, the positive control caused an IS of 19.7 ± 0.1, fulfilling the criteria for an acceptable test. Thus, this biocompatibility screening test indicates that poloxamine formulations may have good tolerance when used as ocular formulations.

3.3.5. *In vitro* release studies

A diffusion test was carried out in order to gain an insight into the ETOX release profile from the poloxamine micelles (Figure 3.1). Although all micellar systems provided sustained release, remarkable differences were observed depending on the variety of the poloxamine. Figures 3.2 and 3.3 show detailed plots of ETOX release profiles in percentage. In the case of the single micelles, T908 provided the fastest release (77.1 % in 24 h), followed by T1107 (47.8 % in 24 h), T1307 (39.6 % in 24 h) and T904 (32.1 % in 24 h). This behavior indicates that the greater the hydrophilicity of the micelles, the smaller the capacity to retain the drug within the core. Interestingly, mixed micelles showed intermediate release rates, introducing an additional feature that enables to fine tune the release rate. Since each formulation has a different load of ETOX, the diffusion coefficients were estimated for a more precise comparison. Thus, Higuchi equation was

applied to the first 60% drug released and plotted versus the square root of time [42]:

$$\frac{Q}{A} = 2.C_0.\left(\frac{D_t}{\pi}\right)^{1/2} \quad \text{Eq.(3.4)}$$

where *Q/A* is the amount of ETOX released per unit area, C_0 is the initial ETOX concentration in the micellar system, and *D* is the drug diffusion coefficient through the micelle. The diffusion coefficients are reported on Table 3.4. It is interesting to note that these coefficients were greater for single T1107 and T1307 micelles than for T904 (those with the highest loading) and T908 (those with the lowest loading). These results suggest that the release profile is governed by a combination of parameters that include the micellar HLB and the ETOX concentration gradient between the micelles and the release medium. Mixed micelles of T1107 and T1307 notably decreased the *D* values from $2.3 \cdot 10^{-9}$ to $1.6 \cdot 10^{-9}$ cm^2/s. In the case of mixed micelles of T904 with T1107 or T1307 the decrease in *D* values was even more remarkable, particularly when the content in T904 was below 50%. ETOX diffusion coefficient progressively increased as the proportion of T904 in the mixed micelles raised. These results follow an unexpected trend and would rely on a more substantial hindrance of the co-micellization process of T1107 and T1307 when greater T904 contents are used. *D* values of ETOX from polymeric micelles were smaller than those previously established in similar assays for free acetazolamide and acetazolamide released from a cyclodextrin complex [13]. These findings would stem from the fact that due to their remarkably greater hydrodynamic diameter, micelles do not surpass the membrane and serve as drug reservoirs, making the drug release process more sustained. This is an interesting advantage because, as opposed to cyclodextrins complexes that can cross the mucosa, copolymer micelles will not be absorbed. In

addition, a more sustained release would enable a much better fine tuning of the release profile. On the other hand, it should be stressed that the solubility values attained remain smaller than those achieved with cyclodextrins and, in this context, *in vivo* assays would be required to confirm that a similar and more prolonged IOP decrease can be attained with this new approach.

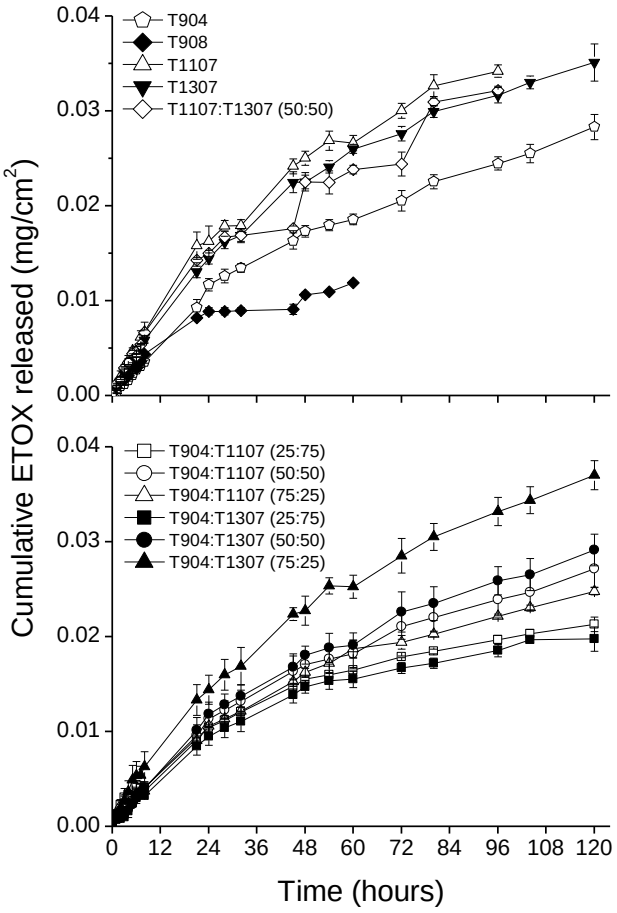


Figure 3.1. ETOX release profiles in isotonic phosphate saline buffer medium (pH 7.4) from single (upper plot) and mixed (lower plot) poloxamine micellar systems, at 32oC. Mean values and standard deviations (n=3).

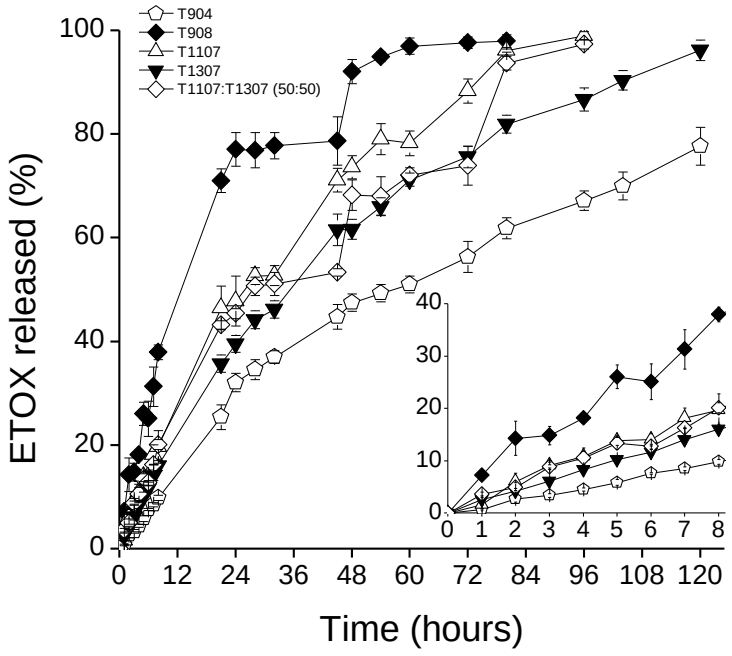


Figure 3.2. ETOX release (%) profiles in isotonic phosphate saline buffer medium (pH 7.4) from single poloxamine micelles and T1107:T1037 mixed micelles, at 32oC. The insert shows the first 8 hours release pattern.

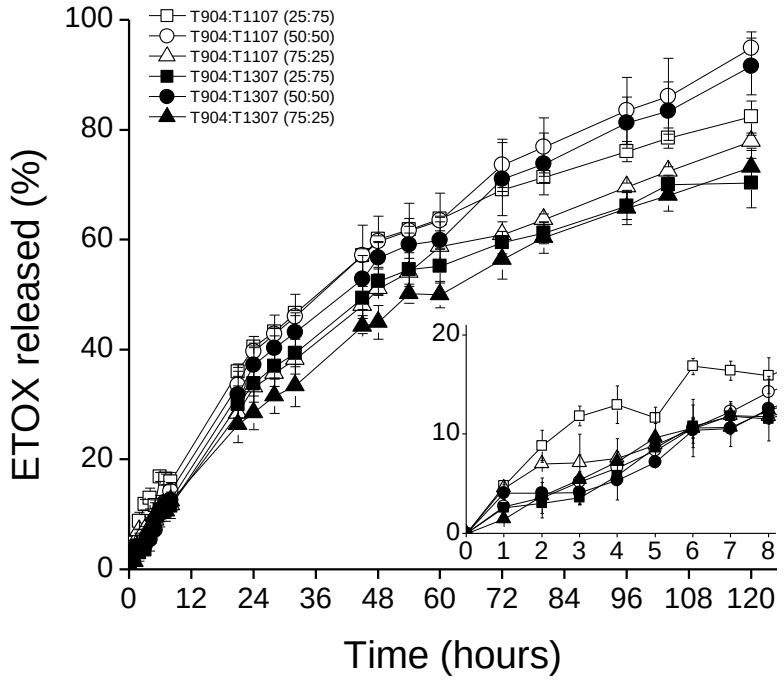


Figure 3.3. ETOX release (%) profiles in isotonic phosphate saline buffer medium (pH 7.4) from mixed T904:T1107 and T904:T1307 micellar formulations, at 32oC. The insert shows the first 8 hours release pattern.

Table 3.4. Results of diffusion coefficients (D) obtained from Higuchi equation. Means values, and in parentheses standards deviations (n=3).

Copolymers	**D cm^2/s (x 10^{-9})**	**R^2**
T904	1.500 (0.106)	0.9811
T908	0.061 (0.006)	0.9391
T1107	2.330 (0.314)	0.9533
T1307	2.410 (0.165)	0.9693
T1107:T1307 (50:50)	1.630 (0.098)	0.9590
T904:T1107 (25:75)	0.510 (0.006)	0.9839
T904:T1307 (25:75)	0.592 (0.059)	0.9807
T904:T1107 (50:50)	0.889 (0.160)	0.9758
T904:T1307 (50:50)	1.290 (0.205)	0.9311
T904:T1107 (75:25)	0.926 (0.016)	0.9802
T904:T1307 (75:25)	4.870 (0.426)	0.9874

3.4. Conclusion

The encapsulation and release of ETOX from poloxamine micelles was investigated for the first time. T904 solely micelles increased drug solubility up to 50 times and the combination of T904 and T1107 or T1307 provided mixed micelles with higher solubilization capability than those of T1107 or T1307 alone. Incorporation of ETOX did not modify the micellar size and size distribution and the resultant systems passed the HET-CAM ocular irritancy test. Furthermore, co-micellization of poloxamines of different hydrophilicity led to more physically stable systems that sustained ETOX release more efficiently than micelles of each single component. In sum, although an unfavorable mixing process was observed, the co-micellization of poloxamines bearing similar number of PO but different of EO units at various weight ratios improves the stability of drug-loaded micelles and enables the tuning of drug loading and release, being an useful tool to adapt the release profile to specific requirements.

3.5. References

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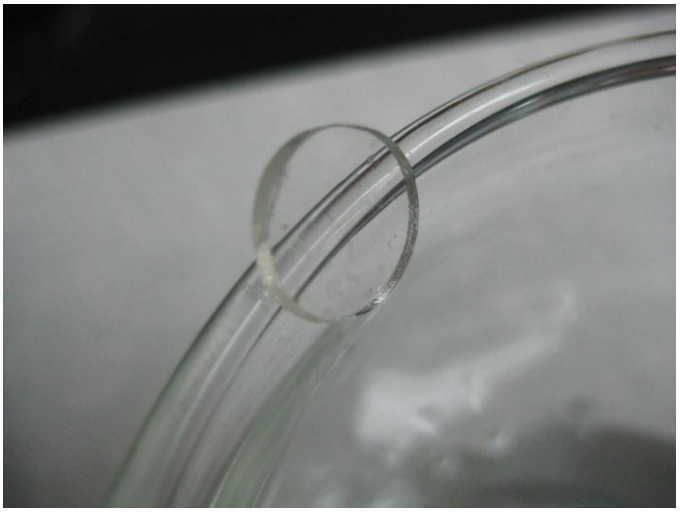
HYDROGELS WITH BUILT-IN OR PENDANT CYCLODEXTRINS AS ANTI-GLAUCOMA DRUG DELIVERY SYSTEMS



CHAPTER 4

Abstract

Carbonic anhydrase inhibitors (CAIs), such as acetazolamide (ACT) and ethoxzolamide (ETOX) are gaining interest for the localized treatment of glaucoma and other ocular disorders. However, the poor solubility of these drugs and the short precorneal residence time limit their use. The aim of this work was to explore the possibilities of using cyclodextrins (CDs) for modulating the loading and the release rate of ACT and ETOX from N, N-dimethylacrylamide-co-N-vinylpyrrolidone (DMA-co-NVP) hydrogels. DMA and NVP are common components of high water-content soft contact lenses. Two different approaches were evaluated to insert β-CD and γ-CD in the hydrogel structure: i) synthesis of CD monomers and copolymerization with DMA and NVP; and ii) grafting of natural CDs to preformed hydrogels. The effects of the preparation method, CD nature and CD-drug stability constant on relevant functional features of the hydrogels as well as on cytocompatibility and drug delivery performance were studied in detail. Functionalization with cyclodextrin provides highly biocompatible and optically clear hydrogels and with capability to modulate the release performance of the hydrogel network.

Keywords

Cyclodextrin, glaucoma, contact lenses, N,N-dimethylacrylamide, N-vinylpyrrolidone.

4.1. Introduction

Glaucoma is the generic name of a group of progressive optical neuropathies characterized by degeneration of retinal ganglion cells and their axons, with resultant visual field defects and loss of vision [1]. Recent data indicate that the ratio of people with open angle and angle closure glaucoma will raise from 60.5 million to 79.6 million in 2020; glaucoma being the second leading cause of blindness worldwide [2]. Carbonic anhydrase inhibitors (CAIs), such as acetazolamide (ACT) and ethoxzolamide (ETOX), are particularly useful systemic (oral) antiglaucoma drugs for reducing the elevated intraocular pressure (IOP) characteristic of this disease [3]. Their action mechanism consists in the inhibition of carbonic anhydrases at the eye and, thus the reversible conversion of carbon dioxide to bicarbonate and the secretion of aqueous humor. However, carbonic anhydrases are ubiquitously distributed in the body and systemic CAIs administration may lead to relevant collateral effects [4]. Topical formulations of the first generations of CAIs were initially unsuccessful due to their poor ocular bioavailability, related to a poor penetration coefficient and poor aqueous solubility. These limitations could be at least partially overcome by preparing inclusion complexes with cyclodextrins (CDs) [5-7]. Nevertheless, the search for topical formulations able to sustain the release and to provide better patient compliance is still on going.

The development of strategies to overcome the barriers for topical ocular delivery of drugs is a major challenge for pharmaceutical scientists [8, 9]. In this sense, drug-eluting contact lenses can offer novel chances for the management of eye pathologies [10, 11]. Soft contact lenses (SCLs) can be loaded with drugs by soaking in drug solutions and, once applied onto the eye, they may sustain the release in the postlens lachrymal fluid [12]. SCLs increase significantly the residence time of the drug in the precorneal area, compared to the short time (2-5

min) achieved with common as eye drops. The longer drug residence time on the cornea surface promoted by the SCL may result in higher drug flux through the anterior segment structures and, consequently, greater ocular bioavailability and lower side effects [13]. Nevertheless, as drug delivery devices there are still a number of limitations associated with the use of SCLs. Usually the amount of drug incorporated in the lens matrix by presoaking is low due to solubility a poor drug in the aqueous phase of the SCL and/or to a low affinity of the drug for the polymeric network [14]. Several methods have been assayed to improve drug loading and controlled release, such as the use of functional monomers and molecular imprinting [15-17], the drug impregnation applying supercritical fluid impregnation [18], or the incorporation of the drug into colloidal structures, nanoparticles or microparticles to be dispersed in the polymeric network [13, 19, 20]. We have previously observed that biomimetic SCLs, with domains that resemble the composition and conformation of the active site of carbonic anhydrase, exhibit a remarkably longer affinity for ACT and ETOX than common SCLs [10, 11]. Recently, grafting of CDs to the SCL structure has been shown to endow the networks with the ability to host drugs by forming dynamic inclusion complexes, which can regulate drug uptake and release through an affinity-driven mechanism, as previously reported for other CD hydrogels [21].

The aim of this work was to explore the possibilities of using CDs for modulating the loading and the release rate of ACT and ETOX from N, N-dimethylacrylamide-co-N-vinylpyrrolidone (DMA-co-NVP) hydrogels. DMA and NVP are common components of high water-content SCLs. Two different approaches were evaluated to insert the CDs in the SCL structure: i) synthesis of CD monomers and copolymerization with DMA and NVP; and ii) grafting of natural CDs to preformed hydrogels. Two natural CDs (β-CD and γ-CD) were tested in each approach. The effects of the preparation method, CD nature and

CD-drug stability constant on relevant functional features of the hydrogels as well as on cytocompatibility and drug delivery performance were studied in detail.

4.2. Experimental section

4.2.1. Materials

N,N-dimethylacrylamide (DMA), N-vinylpyrrolidone (NVP), ethylene glycol dimethacrylate (EGDMA), glycidyl methacrylate (GMA), N-(hydroxymethyl) acrylamide (NMA), acetazolamide (ACT) and ethoxzolamide (ETOX) were from Sigma-Aldrich Chemicals (St. Louis MO, USA). Azobisisobutyronitrile (AIBN) was from Acros Organic Co. (Geel, Belgium), γ-cyclodextrin (γ-CD) from Wacker Chemie AG (Munchen, Germany) and β-cyclodextrin (β-CD) from Roquette (Lestrem, France). Purified water was obtained by reverse osmosis (MilliQ®, Millipore Spain). All other reagents were analytical grade.

4.2.2. Phase solubility diagrams

Solutions of β-CD (0-0.0132 mol/L) or γ-CD (0-0.154 mol/L) were prepared in NaCl 0.9% and then 5-mL aliquots were added to glass vials containing ACT or ETOX in excess. Each system was prepared in sextuplicate; three replicates being immediately autoclaved (121ºC for 20 min). Then, the six replicates were kept at 37 ºC under shaking (50 osc/min) for 96 h. The resultant suspensions were filtered through a 0.45 μm membrane (Sartorius®, Spain). The filtrate was suitably diluted with ethanol and the absorbance measured at 264 nm (ACT) or 303 nm (ETOX) using a UV-visible spectrophotometer (Agilent 8453, Germany). The stability constants of the complexes were estimated as follows [22]:

$$K_{1:1} = \frac{Slope}{S_0.(1-Slope)} \quad \text{Eq. (4.1)}$$

For this equation, the slope was obtained from the plot of the drug solubilized vs. CD concentration, and S_0 from the equilibrium solubility of the drug in NaCl 0.9%. The complexation efficiency (CE) was calculated according as follows [23]:

$$CE = \frac{[D.CD]}{[CD]} = K_{1:1}.S_0 = \frac{Slope}{1-Slope} \quad \text{Eq. (4.2)}$$

4.2.3. Synthesis of acrylamidomethyl-CD monomers

β-CD (15.0 g) or γ-CD (17.12 g) and NMA (13.36 g) were added to 1% HCl aqueous solution (50 mL) in a reactor and kept under stirring at 80°C. After 30 min, acetone (300 mL) was added to stop the reaction and to precipitate β-CD-NMA and γ-CD-NMA monomers. The reactor was kept at 4 ºC for 12 h. Then, the precipitate was separated by filtration (Sartorius®, Madrid, Spain) and repeatedly washed with acetone (200 mL) and filtered (four cycles). The monomers were finally dried under vacuum for 2 days at room temperature and stored at 4 ºC [24].

4.2.4. Synthesis of CDs built-in hydrogels

The monomeric composition of the hydrogels is summarized in Table 4.1. NVP/DMA 20/80 molar ratio mixture was prepared just by mixing the adequate volumes of the monomers. β-CD-NMA or γ-CD-NMA were added to 8-mL aliquots of NVP/DMA solution and kept under stirring until complete dissolution. Then, EGDMA (80 mM) and AIBN (10 mM) were added to each solution. The

preparation of networks with hight contents γCD-NMA (300 to 800 mg; i.e., Cγ300, to Cγ800 in Table 1) required the previous dissolution of this monomer in 1 mL of DMSO. The monomer solutions were injected into moulds constituted by two glass plates pretreated with dimethyldichlorosilane and separated by a silicone frame of 0.9 mm thickness [25]. The moulds were heated at 50°C for 12 h and then at 70°C for 24 h more. The hydrogels were removed from the moulds and immersed in boiling water for 15 min to remove any residual non-reacted components. Discs (10 mm in diameter) were cut from the wet films and immersed in water for 24 h, in a NaCl 0.9% solution for 24 h, and then in water again replacing the medium every 12 h for some days until no absorbance of the medium in the UV-vis range was observed. The hydrogel discs were stored at the dried state.

4.2.5. Hydrogels with pendant CDs

Different amounts of GMA were added to NVP/DMA mixtures (Table 4.1). After addition of EGDMA (80 mM) and AIBN (10 mM), the monomer solutions were injected into moulds, polymerized and then washed as described above. The wet discs were immersed in 150 mL of dimethylformamide: 0.5M NaCl aqueous solution 50:50 v/v mixture containing 80 mM β-CD (Group β) or 80 mM γ-CD (Group γ) and 4.5 g NaOH, and kept at 80 °C for 24 h. Then the hydrogels were washed by immersion in water at 80 °C for 5 min (five cycles), in water at 70 °C for 24 h (three times), in ethanol (96%) for 24 h (three cycles) and in water at room temperature 24h (three cycles). Then, the discs were dried at room temperature for 48 h.

Table 4.1. Monomeric composition of the hydrogels.

Formulation	NVP (mL)	DMA (mL)	EGDMA (mL)	AIBN (g)	GMA (mL)	DMSO (mL)	β-CD-NMA (mg)	γ-CD-NMA (mg)
C0	1.65	6.35	0.12	0.0135	-	-	-	-
Cβ100	1.65	6.35	0.12	0.0135	-	-	100	-
Cγ50	1.65	6.35	0.12	0.0135	-	-	-	50
Cγ100	1.65	6.35	0.12	0.0135	-	-	-	100
Cγ150	1.65	6.35	0.12	0.0135	-	-	-	150
Cγ200	1.65	6.35	0.12	0.0135	-	-	-	200
Cγ300	1.65	6.35	0.12	0.0135	-	1.0	-	300
Cγ400	1.65	6.35	0.12	0.0135	-	1.0	-	400
Cγ500	1.65	6.35	0.12	0.0135	-	1.0	-	500
Cγ600	1.65	6.35	0.12	0.0135	-	1.0	-	600
Cγ700	1.65	6.35	0.12	0.0135	-	1.0	-	700
Cγ800	1.65	6.35	0.12	0.0135	-	1.0	-	800
G1A	0.00	8.00	0.12	0.0135	0.22	-	-	-
G2A	1.65	6.35	0.12	0.0135	0.22	-	-	-
G3A	3.27	4.73	0.12	0.0135	0.22	-	-	-
G1B	0.00	8.00	0.12	0.0135	0.44	-	-	-
G2B	1.65	6.35	0.12	0.0135	0.44	-	-	-
G3B	3.27	4.73	0.12	0.0135	0.44	-	-	-

4.2.6. Fourier transform infrared spectroscopy (FTIR)

FTIR-ATR (attenuated total reflection) spectra of raw cyclodextrins, β-CD-NMA and γ-CD-NMA monomers, and dried hydrogels were recorded over the range 400–4000 cm^{-1} in a Varian-670 FTIR spectrometer equipped with a GladiATR^{TM} (Madison Instruments, Madison WI, USA) fitted with diamond crystal.

4.2.7. Degree of swelling

Dried hydrogel discs were weighed (W_0) and immersed in water at room temperature. At pre-established time intervals, the discs were removed from the aqueous medium, their surfaces were carefully wiped and the weight recorded

(W_t). The experiments were carried out in duplicate. The swelling ratio was estimated as follows:

$$Q(\%) = \left(\frac{W_t - W_o}{W_0}\right).100 \qquad \text{Eq. (4.3)}$$

4.2.8. Optical transparency

Fully swollen hydrogels were mounted on the side of the inside surface of a quartz cuvette and the light transmittance was recorded, in duplicate at 600 nm (UV-vis spectrophotometer, Agilent 8453, Germany).

4.2.9. Content in functional CDs

Dried hydrogel discs were immersed in 10 mL of 3-methylbenzoic acid (3-MBA) aqueous solution (0.12 mg mL^{-1}) and kept for 48 h in the dark. The concentration of 3-MBA was spectrophotometrically monitored at 281 nm (Agilent 8453, Germany). The total amount of 3-MBA taken up by discs was calculated as the difference between the initial and the final amounts in the solution. The experiments were carried out in triplicate.

4.2.10. Cytocompatibility

Dried hydrogel discs were immersed in phosphate buffer pH 7.4 and autoclaved (121°C, 20 min). Then, the pieces were added to wells (24-wells plate) containing Balb/3T3 clone A31 cells (200,000 cells per well) in Dulbecco's Modified Eagle Medium DMEM F12 HAM 2 mL, (Sigma-Aldrich Chemicals, Madrid, Spain).The systems were kept in a humidified incubator at 5% CO_2 and 37 ºC for

24 h. Then aliquots (100µl) of medium were taken and transferred to 96-wells microplates, and mixed with the reaction mixture solution (100µl) contained in the Cytoxicity Detection Kit^{PLUS} LDH, (Roche, Barcelona, Spain). Blank (100 µl of culture medium), negative (50 µl of cells and 50 µl of medium) and positive (50 µl of cells and 50 µl of medium with 5 µl of lysis factor) controls were also prepared. The plates were incubated 30 min at 15-25 ºC protected from light. A stop solution (50µl) was added to the wells and the absorbance immediately measured at 490 nm (BIORAD Model 680 Microplate reader, USA). The experiments were carried out in triplicate. The cytocompatibility was estimated as follows:

$$Cytocompatibility(\%) = \frac{Abs_{\exp} - Abs_{negative\ control}}{Abs_{positive\ control} - Abs_{negative\ control}} . 100 \qquad \text{Eq. (4.4)}$$

4.2.11. ACT loading and release

Dried hydrogels discs (six replicates) were placed in 5 mL of ACT aqueous solution (0.20 mg/mL) and kept for two days at room temperature protected from the light. The amount of ACT loaded by each hydrogel was calculated as the difference between the initial amount of drug in the solution and the amount remaining after loading determined by UV spectrophotometry at 264 nm (Agilent 8453, Germany). Drug-loaded discs were rinsed with water, their surface was carefully wiped and the discs were immediately immersed in 7.5 mL of NaCl 0.9% solution at room temperature. The amount of ACT released was measured spectrophotometrically at 264 nm, in samples periodically taken and again placed in the same vessel, so that the liquid volume was kept constant.

4.2.12. ETOX loading and release

Dried hydrogels discs (six replicates) were placed in 5 mL of ETOX suspension (0.23 mg/mL) and kept for two days at room temperature. The ETOX-loaded discs were rinsed with water; their surfaces were carefully wiped and immediately immersed in 5 mL of NaCl 0.9% at room temperature. The amount of drug released was measured spectrophotometrically at 303 nm in samples periodically taken up and placed again into the same vessel. After 360 h in the release medium, the discs were rinsed with water and placed in vials with 5 mL of ethanol:water (70:30) mixture. The amount of drug extracted to the hydroalcoholic medium after 24 hours was quantified from the absorbance measurements at 303 nm.

4.3. Results and discussion

4.3.1. Phase solubility diagrams

The stoichiometry and stability constant of the inclusion complexes of ACT and ETOX were estimated from the phase solubility diagrams (Figures 1 and 2). Since it is well-known that the nature of the solvent significantly determines the affinity of the drugs for the cyclodextrins, the experiments were carried out in 0.9% NaCl solution in order to mimic the physico-chemical conditions of the lachrymal fluid. ACT and ETOX solubility in water at 25 °C is 0.70 mg/mL [3, 26] and 0.04 mg/mL respectively [27]. The apparent solubility of ETOX linearly increased with the concentration of β-CD and γ-CD due to the formation of inclusion complexes. Solubility of ETOX increased 10-fold at 0.013 mol/L β-CD and 21-fold at 0.154 mol/L γ-CD. The effect of the CDs on the solubility of ACT was smaller although still relevant; the increments in solubility being 3.8-fold in 0.013

mol/L β-CD and 1.5-fold at 0.154 mol/L γ-CD. It should be noticed that for both drugs, autoclaving helps the inclusion complex to be formed probably due to a temporal increase in drug solubility at high temperatures, which makes more drug molecules to be available to be hosted in the CD cavities [5, 28].

The phase solubility plots were A_L-type, which indicates that the complex is first order with respect to the complexing agent and first order with respect to the drug [22]. The stability constants of 1:1 complexes for the ETOX and ACT with β-CD were greater than those found for γ-CD (Table 4.2). The stability constant (Ks) calculated for the complexation of ETOX with β-CD and γ-CD were larger in non-autoclaved systems than in the autoclaved ones the effect of thermal treatment on ACT complexes was less relevant.

Table 4.2. Complexation efficiency (CE) and stability constants ($Ks_{(1:1)}$) of ACT and ETOX with β-CD and γ-CD in NaCl 0.9% solution at 37ºC, with and without pre-treatment.

Inclusion complexes	**Pre-treatment**	**CE**	**$Ks_{(1:1)}(M^{-1})$**	**R^2**
ACT:β-CD	None	0.152	39.1	0.957
ACT:γ-CD	None	0.033	11.9	0.999
ETOX:β-CD	None	0.060	644.9	0.997
ETOX:γ-CD	None	0.011	129.2	0.995
ACT:β-CD	Autoclaved	0.165	38.4	0.960
ACT:γ-CD	Autoclaved	0.066	19.1	0.828
ETOX:β-CD	Autoclaved	0.062	354.3	0.961
ETOX:γ-CD	Autoclaved	0.012	72.7	0.997

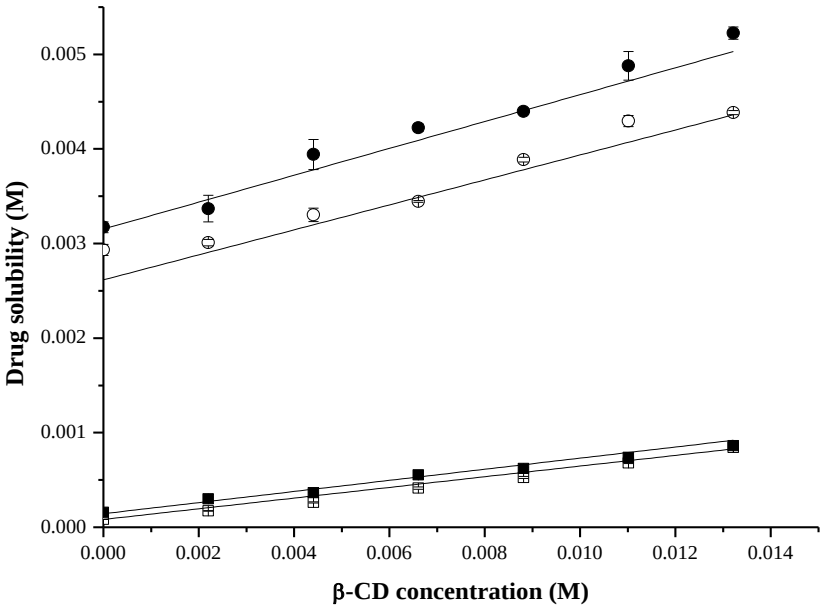


Fig.4.1. Phase solubility diagrams for ACT and ETOX with β-CD at 37°C in NaCl 0.9%: (○) ACT no autoclaved, (●) ACT autoclaved, (□) ETOX no autoclaved, (■) ETOX autoclaved. The error bars represent the standard deviations (n=3).

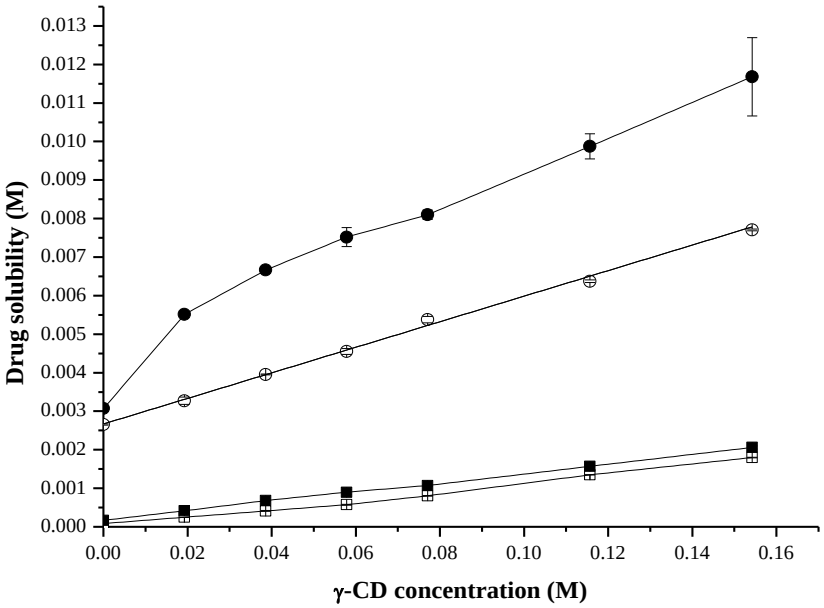


Fig.4.2. Phase solubility diagrams for ACT and ETOX with γ-CD at 37°C in NaCl 0.9%: (○) ACT no autoclaved, (●) ACT autoclaved, (□) ETOX no autoclaved, (■) ETOX autoclaved. The error bars represent the standard deviations (n=3).

4.3.2. Synthesis of CD built-in hydrogels

The synthesis of β-CD-NMA and γ-CD-NMA was confirmed by FTIR (Figure 3). Compared to the FTIR spectra of β-CD and γ-CD, the spectra of β-CD-NMA and γ-CD-NMA showed two additional bands at 1708 and 1544 cm^{-1}, which correspond to the amide I and II (C=O and NH stretching peak). Vinyl (C=C) stretching peak was observed at 1628 cm^{-1}.

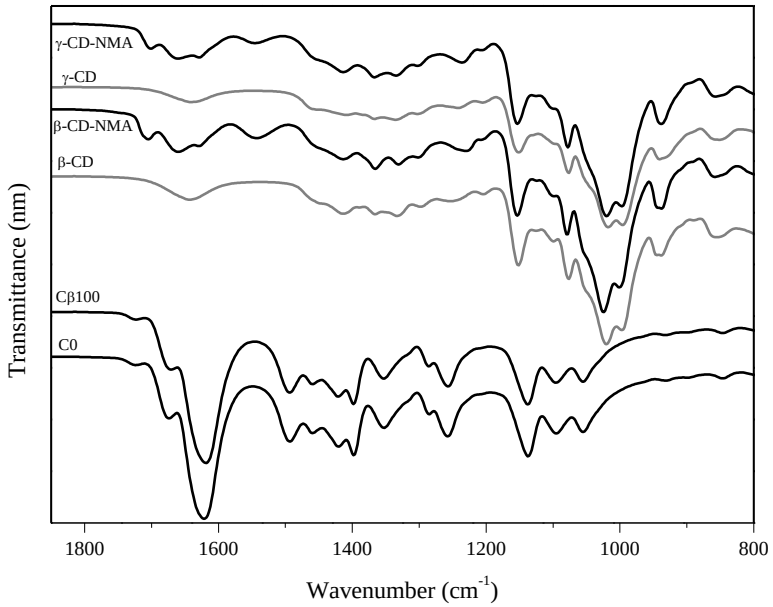


Fig.4.3. FTIR spectra of hydrogels C0 and Cβ100; β-CD and γ-CD nature, β-CD-NMA and γ-CD-NMA monomer.

The synthesis of the hydrogels was carried out by free radical polymerization of the NVP/DMA mixture with various proportions of β-CD-NMA (100 mg) and γ-CD-NMA (50-800 mg). FTIR spectra of NVP/DMA hydrogels without CD-NMA monomers showed peaks at 1720 cm^{-1} and 1394 cm^{-1} due to the carbonyl groups and the C–N stretching vibration of tertiary amide. The lactam group of the NVP and amide group of the DMA appeared at 1670 cm^{-1}(Figure 4.3).

All hydrogels swelled rapidly when immersed in water and reached the equilibrium in less than 2 h (Figure 4.4). The degree of swelling was about 80%, confirming the high affinity of the hydrogels for water. Minor dependence of water uptake on the proportion of γ-CD-NMA monomer was observed in the first hours.

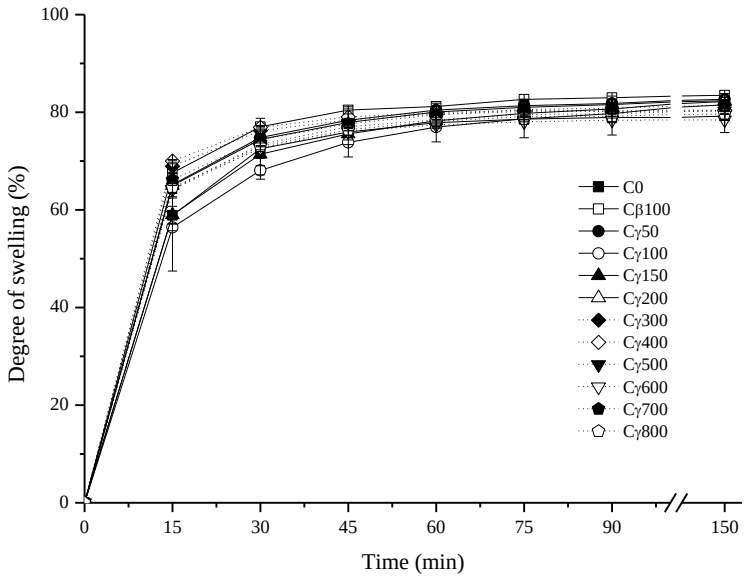


Fig.4.4. Swelling profiles of the CD built-in hydrogels. (n=2)

4.3.3. Synthesis of hydrogels with pendant CDs

Grafting of raw CDs to preformed networks was carried out by means of reaction with glycidyl methacrylate (GMA) as spacer agent. GMA is a bi-functional monomer, with both acrylic and epoxy groups. The attractiveness of GMA is related to the versatility of its epoxy group, which can react with amino groups [29] or with the hydroxyl groups, such as those of CDs [8, 30]. Copolymerization with DMA/NVP/GMA (Table 4.1) occurs via carbonic double bond cleavage and results in hydrogels with the original reactivity of the epoxy ring.

FTIR spectra of the hydrogels were similar before and after treatment with both cyclodextrin. The lactam group of the NVP and amide group of the DMA appeared at 1670 cm^{-1}. The grafting of CDs did not alter the swelling degree of

the hydrogels (Figure 4.5), which was similar to that recorded for DMA/NVP hydrogels without GMA (Figure 4)

As show in Figure 4.5, the swelling the SCLs with and without pendant CD is similar for all hydrogels and not less 80%. The high affinity of the dried hydrogels for water is attributed to the presence of hydrophilic monomers NVP and DMA. The initial swelling degree is relatively fast and occurs in fewer of 1 hour.

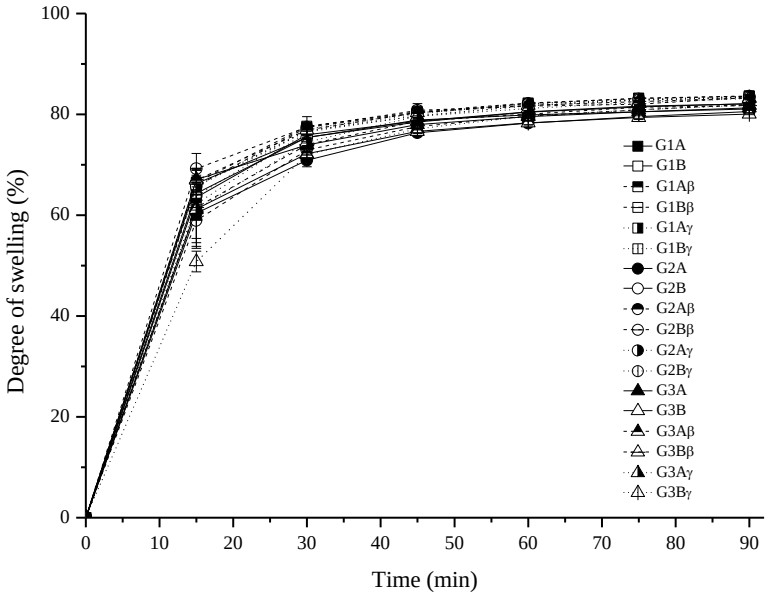


Fig.4.5. Swelling profiles of the hydrogels formulations with grafting CDs. (n=2)

4.3.4. Light transmission and oxygen permeability

Light transmission (600 nm) was above 80% for all swollen hydrogel. Oxygen permeability was neither modified by the copolymerization with the CD monomers nor the grafting of raw CDs, and resulted to be in the 65-87 barrers range, which is adequate for contact lenses.

4.3.5. Cytocompatibility

In general NVP and DMA based polymers and networks show excellent biocompatibility with living tissues [31]. Balb/3T3 cell line was used for testing the cytocompatibility of all hydrogels. Both groups of hydrogels with β-CD-NMA and γ-CD-NMA monomers or with grafted β-CD and γ-CD showed cell viability close to 100 % (Figure 4.6).

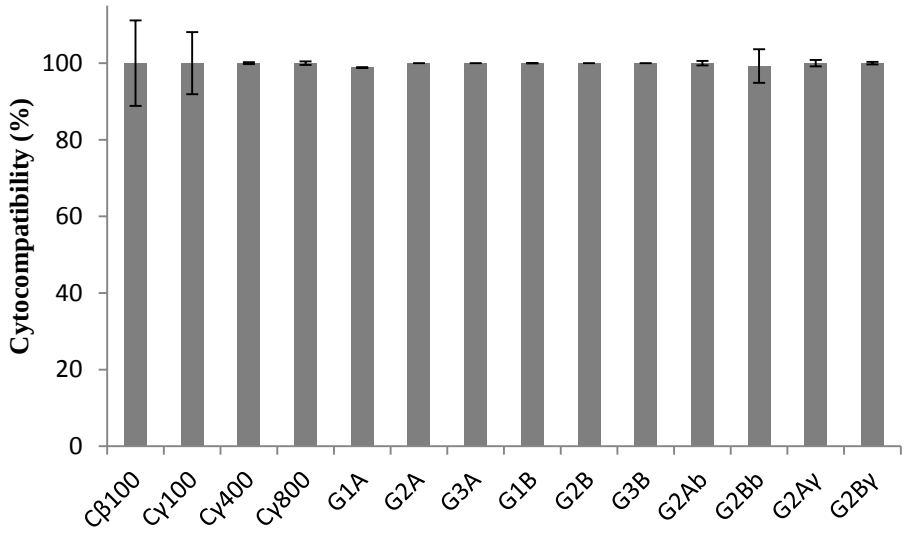


Fig.4.6. Viability of Balb/3T3 cells after 24 h in contact with the hydrogels.

4.3.6. CDs available for complex formation

The content in CDs was determined applying the typical organic compound (TOC) approach [32] using 3-MBA as a probe with high affinity for β-CD (1.3 $x10^{-7}$ M^{-1}) [32-34]. All hydrogels with built-in CDs and pendant CDs were loaded with 3-MBA. Hydrogels with built-in CDs did no show substantial 3-MBA loading in contrasts to hydrogels with pendant CDs. In the case of hydrogels made of CD monomers, that prepared with β-CD-NMA exhibited an affinity for 3-MBA

larger than that hydrogels prepared with γ-CD-NMA. The ability of NVP/DMA/GMA hydrogels to load 3-MBA was remarkably increased after the grafting of β or γ-CDS (Figure 4.7). Furthermore, an increase in the proportion of GMA used during polymerization led to hydrogels that grafted more β or γ-CDS and, consequently, with greater affinity for 3-MBA (Figure 4.7).

These findings indicate that copolymerization with GMA followed by grafting of CDs results in hydrogels that possess more functional CDs available for interacting through inclusion complex formation.

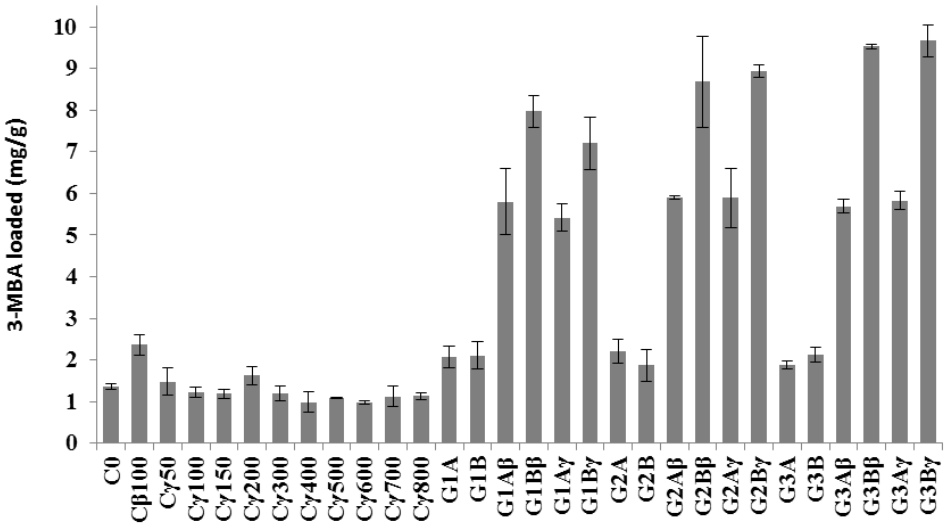


Fig.4.7. 3-MBA loading by the hydrogels with built-in or pendant CDs.

4.3.7. Acetazolamide loading and release

When a hydrogel is immersed in an aqueous drug solution, the amount of drug that can be loaded mainly depends on both the drug concentration in the soaking solution and the affinity of the drug to the network. Table 4.3 shows the amounts of ACT loaded by each SCL and the partition coefficient ($K_{N/W}$) values. The $K_{N/W}$ values were estimated from the following equation [35]:

$$Loading\ (total) = \left[\frac{(V_s + K_{N/W} V_p)}{W_p}\right] . C_0 \qquad \text{Eq. (4.5)}$$

where Vs is the volume of water sorbed by the hydrogel, Wp is the dried hydrogel weight, C_0 is the concentration of the drug in the loading solution and Vp is the volume of dried polymer. The $K_{N/W}$ values which are an index of the affinity of the drug for the network [21] were clearly larger for hydrogels G3Bβ and G3Bγ. Namely, those prepared with NVP/DMA 40/60 ratio, the highest proportion of GMA and, consequently, the greatest content in grafted β-CD or γ-CD. The amount of ACT loaded was not affected by addition of the β-CD-NMA and γ-CD-NMA monomers in hydrogel network. Once loaded with ACT, hydrogels sustained the release for 3-6 hours (Figure 4.9); the release rate being slightly lower for hydrogels made with the highest proportions of γ-CD-NMA monomers.

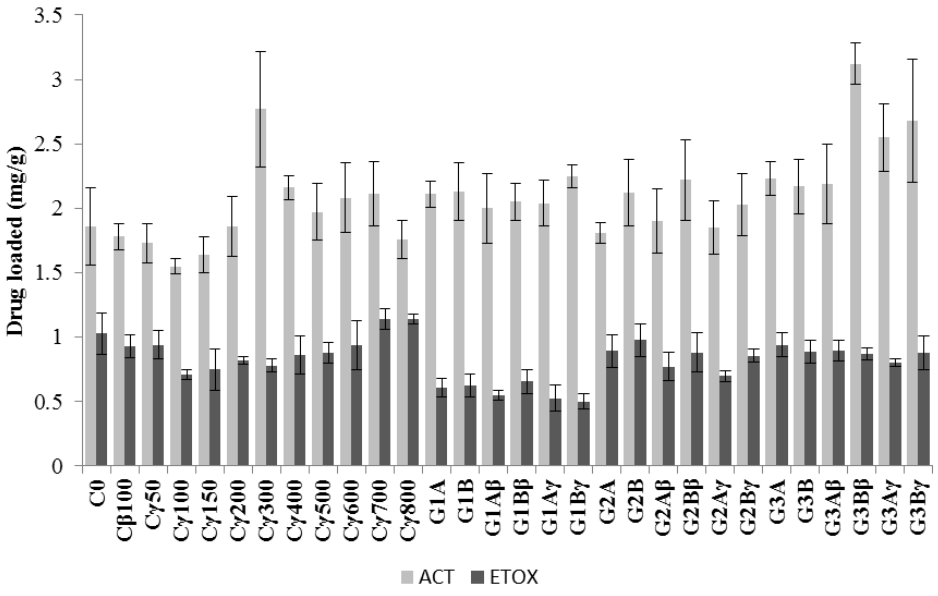


Fig.4.8. Drugs loading of the hydrogels with built-in and pendant CDs.

Table 4.3. Amounts of acetalozamide (ACT) and ethoxzolamide (ETOX) loaded and the network/water partition coefficients in hydrogels prepared with CD monomers or with grafted raw CDs.

Formulations	ACT (mg/g)	$K_{N/W}$	ETOX (mg/g)	$K_{N/W}$
C0	1.86 (0.30)	4.2 (1.46)	1.03 (0.16)	46 (6.07)
Cβ100	1.78 (0.1)	3.8 (0.43)	0.93 (0.09)	38 (4.19)
Cγ50	1.73 (0.15)	3.2 (0.67)	0.94 (0.11)	39 (5.36)
Cγ100	1.55 (0.06)	2.4 (0.24)	0.71(0.04)	28 (1.61)
Cγ150	1.64 (0.14)	2.9 (0.57)	0.75 (0.16)	28 (1.25)
Cγ200	1.86 (0.23)	4.0 (1.10)	0.82 (0.03)	33 (1.62)
Cγ300	1.90 (0.1)	4.6 (2.20)	0.78 (0.05)	33 (2.38)
Cγ400	2.16 (0.09)	5.8 (0.40)	0.86 (0.15)	36 (6.94)
Cγ500	1.97 (0.22)	4.7 (1.08)	0.88 (0.08)	37 (3.51)
Cγ600	2.08 (0.27)	5.7 (1.27)	0.94 (0.19)	40 (8.81)
Cγ700	2.11 (0.25)	5.7 (0.90)	1.14 (0.08)	49 (4.08)
Cγ800	1.76 (0.15)	4.2 (0.73)	1.14 (0.04)	50 (1.79)
G1A	2.11 (0.10)	4.8 (0.45)	0.608 (0.07)	25 (1.47)
G1B	2.13 (0.22)	5.1 (1.04)	0.623 (0.09)	23 (1.86)
G1Aβ	2.00 (0.27)	3.8 (1.25)	0.550 (0.04)	20 (1.76)
G1Bβ	2.05 (0.14)	4.1 (0.64)	0.655 (0.09)	26 (3.54)
G1Aγ	2.04 (0.18)	3.9 (0.79)	0.526 (0.10)	18 (3.35)
G1Bγ	2.25 (0.09)	5.7 (0.42)	0.500 (0.06)	18 (2.93)
G2A	1.81 (0.08)	3.7 (0.38)	0.892 (0.13)	35 (5.19)
G2B	2.12 (0.26)	4.7 (0.38)	0.976 (0.13)	37 (1.30)
G2Aβ	1.90 (0.25)	4.3 (1.20)	0.773 (0.11)	29 (1.86)
G2Bβ	2.22 (0.31)	4.3 (1.40)	0.882 (0.15)	34 (4.02)
G2Aγ	1.85 (0.21)	3.2 (0.87)	0.697 (0.04)	27 (1.71)
G2Bγ	2.03 (0.24)	4.3 (1.08)	0.857 (0.05)	35 (2.27)
G3A	2.23 (0.13)	5.5 (0.70)	0.941 (0.09)	39 (4.14)
G3B	2.17 (0.21)	5.7 (1.00)	0.887 (0.09)	37 (4.17)
G3Aβ	2.19 (0.31)	4.9 (1.43)	0.899 (0.08)	37 (3.92)
G3Bβ	3.12 (0.16)	9.7 (0.73)	0.870 (0.05)	36 (2.31)
G3Aγ	2.55 (0.26)	6.6 (1.22)	0.804 (0.03)	32 (1.24)
G3Bγ	2.68 (0.48)	7.7 (2.24)	0.876 (0.13)	34 (3.07)

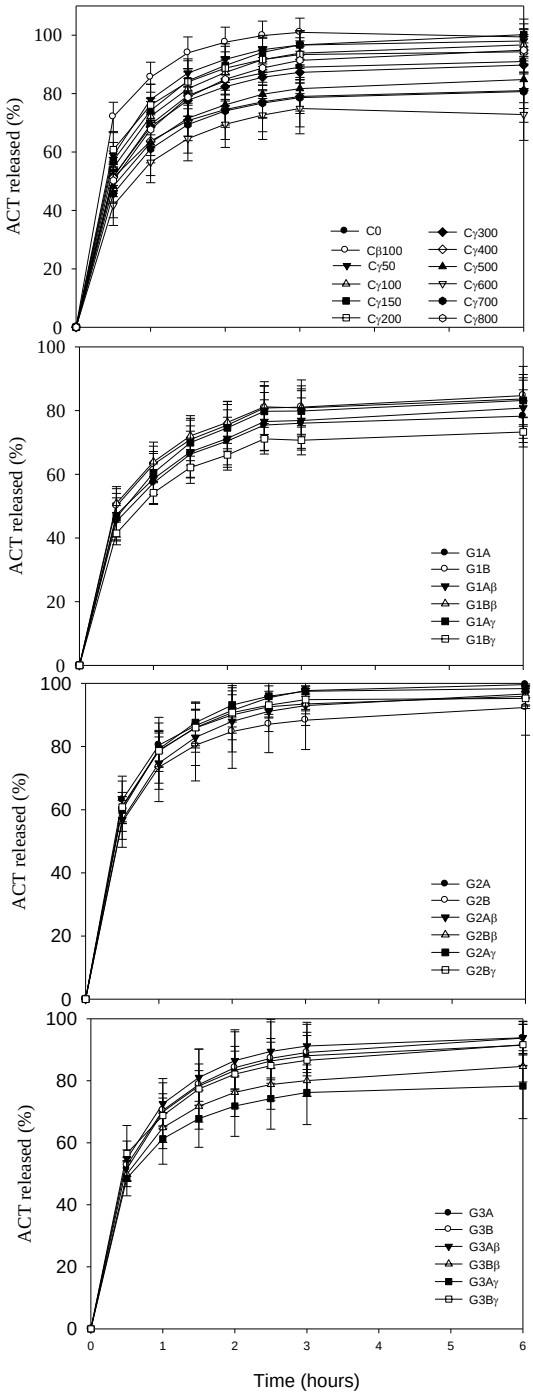


Fig.4.9. ACT release profiles from SCLs hydrogels formulations. (n=6)

4.3.8. Ethoxzolamide loading and release

ETOX loading (Table 4.4) in the hydrogels was carried out by immersion in a drug suspension. Although all hydrogels showed a similar capability to host ETOX, probably due to the prevalence of unspecific hydrophobic interactions with the polymer network has revealed by the high $K_{N/W}$ values in Table 4.3, those copolymerized with the highest proportions of γ-CD-NMA monomers were the ones with more affinity to ETOX. By contrast, the hydrogels prepared without NVP and copolymerized with GMA (codes G1A, G1B and derived from these) were the ones with the lowest uptake ability. These findings suggest that ETOX is more prone to interact with NVP than with DMA. The differences in affinity were more clearly seen when the release was evaluated (Figure 4.10). In the case of hydrogels with built-in CDs, the higher the proportion of γ-CD-NMA, the slower the release. On the other hand, the hydrogels with pendant CDs that sustained more the release were those synthesized with the greater proportion of NVP. Both types of hydrogels sustained the release for almost one week.

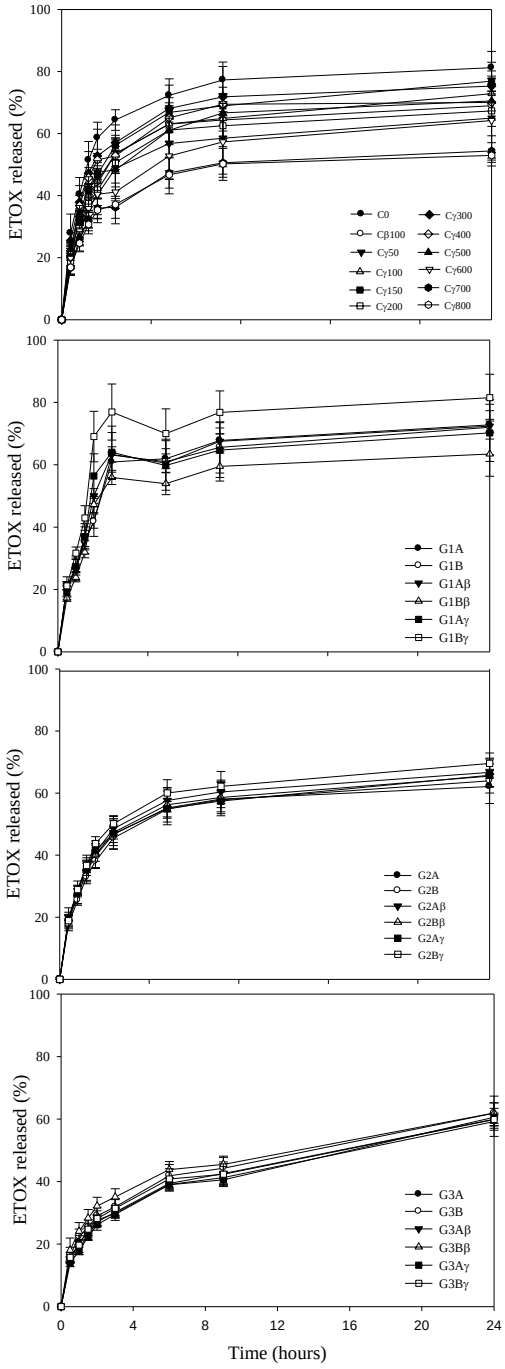


Fig.4.10. ETOX release profiles from hydrogels formulations. (n=6)

4.4. Conclusions

Both β-CD and γ-CD enhanced ACT and, more relevantly, ETOX solubility. Copolymerization of NVP/DMA with GMA followed by grafting of CDs resulted in hydrogels that possess more functional CDs available for interacting through inclusion complex formation that the direct copolymerization of NVP/DMA with β-CD and γ-CD monomers. This effect was noticed for ACT loading. Minor effect of the approach to graft CDs was found for ETOX loading, which resulted to be more dependent on the NVP/DMA ratio, probably because of the hydrophobic interactions with NVP. In the case of hydrogels with built-in CDs, the higher the proportion of γ-CD-NMA, the slower the release, while the hydrogels with pendant CDs that sustained more the release were those synthesized with the greater proportion of NVP.

4.5.References

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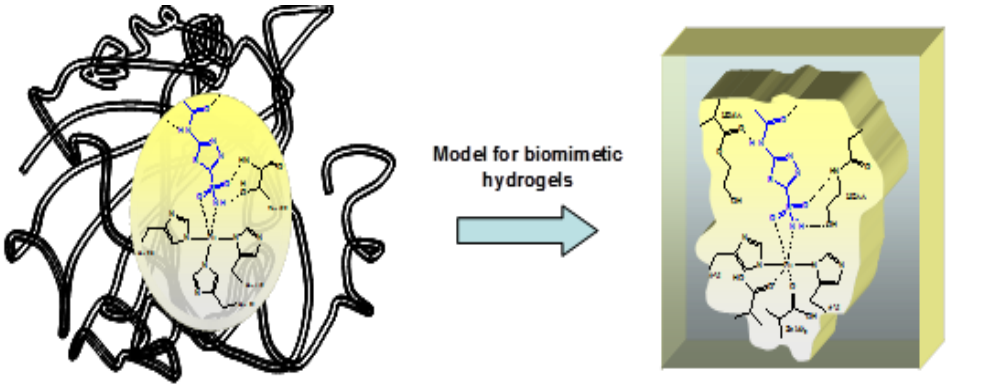
BIOINSPIRED IMPRINTED PHEMA-HYDROGELS FOR OCULAR DELIVERY OF CARBONIC ANHYDRASE INHIBITOR DRUGS



The active site of human carbonic anhydrase II, interacting with acetazolamide

Hydrogels with receptor-like binding sites uptake and control the release of carbonic anhydrase inhibitors

CHAPTER 5

Abstract

Hydrogels with high affinity for carbonic anhydrase (CA) inhibitor drugs have been designed trying to mimic the active site of the physiological metallo-enzyme receptor. Using hydroxyethyl methacrylate (HEMA) as the backbone component, zinc methacrylate, 1 or 4-vinylimidazole (1VI or 4VI) and N-hydroxyethyl acrylamide (HEAA) were combined at different ratios in order to reproduce in the hydrogels the cone-shaped cavity of the CA, which contains a Zn^{2+} ion coordinated to three histidine residues. 4VI resembles histidine functionality better than 1VI and, consequently, pHEMA-$ZnMA_2$ hydrogels bearing 4VI moieties were those with the greatest ability to host acetazolamide or ethoxzolamide (2-3 times greater network/water partition coefficient) and to sustain the release of these antiglaucoma drugs (50% lower release rate estimated by fitting to the square root kinetics). The use of acetazolamide as template during polymerization did not enhance the affinity of the network for the drugs. In addition to the remarkable improvement in the performance as controlled release systems, the biomimetic hydrogels were highly cytocompatible and possessed adequate oxygen permeability to be used as medicated soft contact lenses or inserts. The results obtained highlight the benefits of mimicking the structure of the physiological receptors for the design of advanced drug delivery systems.

Keywords

Biomimetic delivery system, imprinted network, carbonic anhydrase, acetazolamide, ethoxzolamide.

5.1. Introduction

Glaucoma is a progressive disease that causes optic nerve head damage. Currently, its prevalence is a high as 1% in people aged 40-49 years and up to 8% above 80 years old and is one of the most common causes of blindness [1, 2]. The elevation of the intraocular pressure (IOP) is the main risk factor for glaucoma, due to compression of the optic nerve fibers against the lamina cribrosa and/or ischemia associated to the disturbance of the blood supply to the nerve. In open-angle glaucoma there is impaired flow of aqueous humor through the trabecular meshwork-Schlemm's canal venous system [3]. The first choice of glaucoma treatment is the medical therapy with the goal of lowering the IOP to a level at which the damage of the optic nerve ceases to progress. Adrenergic drugs, mainly β-antagonists (such as timolol) alone or combined with β-agonists (epinephrine) or α-agonists (brimonidine), cholinergic drugs (pilocarpine), carbonic anhydrase inhibitors (CAIs; e.g., acetazolamide, ethoxzolamide), cannabinoids and prostaglandins have been shown useful to decrease the IOP [4]. Systemic delivery, mainly through the oral route, is however accompanied by relevant side effects, which are in most cases associated to the doses required to achieve therapeutic concentration at the ocular site. Ophthalmic formulations, such as eye drops, are not exempt of collateral effects either, since less than 5% of the instilled dose is absorbed intraocularly. The rest pass through the conjuntiva or is removed from the eye surface by the defense mechanism that partially leads to naso-lacrimal drainage; both routes may result in systemic absorption [5]. An ideal ocular drug delivery system should be able to increase ocular bioavailability, to prolong the duration of drug action, and to avoid large fluctuations in ocular drug

concentration and ocular and systemic side effects [6]. In this context, soft contact lenses (SCLs) are gaining an increasing attention as combination products able to correct refractive deficiencies and to perform as sustained delivery systems [7-11].

The feasibility of using drug-loaded SCLs depends on whether the drug and the hydrogel material can be matched so that the lens uptakes a sufficient quantity of drug and releases it in a controlled fashion. Most commercially available SCLs show a deficient performance because they release ophthalmic drugs too rapidly [12, 13]. To overcome this drawback, the following approaches are being explored: i) chemically-reversible immobilization of drugs through labile bonds; [7, 14] ii) incorporation of drug-loaded colloidal systems into the lens [15-17]; iii) copolymerization with functional monomers able to interact directly with the drug [18-20]; and iv) molecular imprinting [21-25]. This last technique aims to organize the components of the hydrogel network in such a way that high affinity binding sites for the drug are created. To do that, the drug is added before polymerization and the monomers should arrange as a function of their ability to interact with the drug molecules. After polymerization, the drug molecules that have acted as templates are removed and the polymer network may exhibit "tailored-active sites" or "imprinted pockets" with the size and the most suitable chemical groups to interact again with the drug [26, 27]. A distinguishing key feature of SCLs is their relatively low cross-linking density, compared to common imprinted networks, which can compromise the physical stability of the imprinted cavities due to swelling after synthesis. Only high affinity cavities can memorize the structural features of the drug and undergo an "induced fit" in presence of the drug recovering the same conformation as upon polymerization [22, 28]. In such a way loosely cross-linked imprinted hydrogels try to mimic the recognition capacity of certain biomacromolecules (e.g. receptors, enzymes, antibodies).

Natural evolution has determined the unique details of protein´s native state, such as its shape and charge distribution, that enable it to recognize and interact with specific molecules [29]. Based on biomimetic principles, SCLs endowed with such high affinity imprinted pockets are expected to be able to load the drug and, subsequently, to sustain the release. Ultrathin SCLs synthesized applying the molecular imprinting technology have already demonstrated greater uptake of timolol and better *in vivo* control of drug release to the lacrimal fluid than conventional SCLs and eyedrops, using similar or even lower doses [30]. The selection of the functional monomers responsible for the interaction with the drug can be carried out applying analytical techniques [25] and computational modeling [31, 32] for the screening of monomers libraries, or according to a configurational biomimesis based on the chemical functionality of the natural receptors [27, 33]. Byrne et al. have recently shown that the selection of functional monomers possessing chemical groups similar to those present in the histamine H1-receptor or in the CD44 protein endows the hydrogels with high affinity for the antihistamic drug ketotifen fumarate [23, 34] or for hyaluronic acid [35], respectively. Imprinted hydrogels exhibited higher loading and delayed release, compared to the non-imprinted ones, and it was demonstrated that each functional monomer relating to the biological binding played a role in the delayed release [23, 34, 35].

The aim of this work was to design SCLs with high affinity for CAIs, such as acetazolamide and ethoxzolamide, applying biomimetic principles and the molecular imprinting technology. The idea is to create binding pockets in the network structure that resemble the active site of carbonic anhydrase in order to mime the non-covalent interactions responsible for the docking of the CAIs in the physiological receptor. Carbonic anhydrases are metallo-enzymes that catalyze the conversion of carbon dioxide to bicarbonate ion and protons [36]. Although

there are different isoforms, the active site of most of them consists of a cone-shaped cavity that contains a Zn^{2+} ion coordinated to three histidine residues in a tetrahedral geometry with a solvent molecule as the fourth ligand [37] (Figure 5.1A). There are two main classes of CAIs: i) the metal-complexing anions, and ii) the sulfonamides and their bioisosteres, which bind to the Zn^{2+} ion of the enzyme either by substituting the non-protein zinc ligand to generate a tetrahedral adduct or by addition to the metal coordination sphere, generating trigonal-bipyramidal species [38]. The -NH function of the ionized sulfonamide group replaces the water molecule bound to zinc and the hydrogen bonds to the -OH group of threonine 99. One oxygen atom of the sulfonamide interacts with the -NH group of treonine 199, while another oxygen points toward the zinc ion (Figure 5.1B). Other chemical groups of the CAIs establish van der Waals interactions or hydrogen bonds with neighbor amino acids [37]. Therefore, monomers bearing chemical groups similar to those of the amino acids involved in the active binding site were chosen to prepare biomimetic hydrogels: the zinc ions were introduced as methacrylate salt ($ZnMA_2$); the hydroxyl and amino groups can be supplied by 2-hydroxyethyl methacrylate (HEMA) and N-hydroxyethyl acrylamide (HEAA); and 4-vinylimidazole (4-VI) resembles histidine (Figure 5.1C). 4-VI is not commercially available and thus the first step was to synthesize it. For comparative purpose, 1- vinylimidazole (1-VI) was also included in the study as an alternative for 4-VI in the mimicking of histidine (Figure 5.2). Then, a set of hydrogels with fix content in $ZnMA_2$ and various comonomer combinations was prepared and characterized regarding their ability to load and to sustain the release of acetazolamide and ethoxzolamide. Cytocompatibility, degree of swelling and other relevant features from the point of view of the use of the hydrogels as components of SCLs were also evaluated.

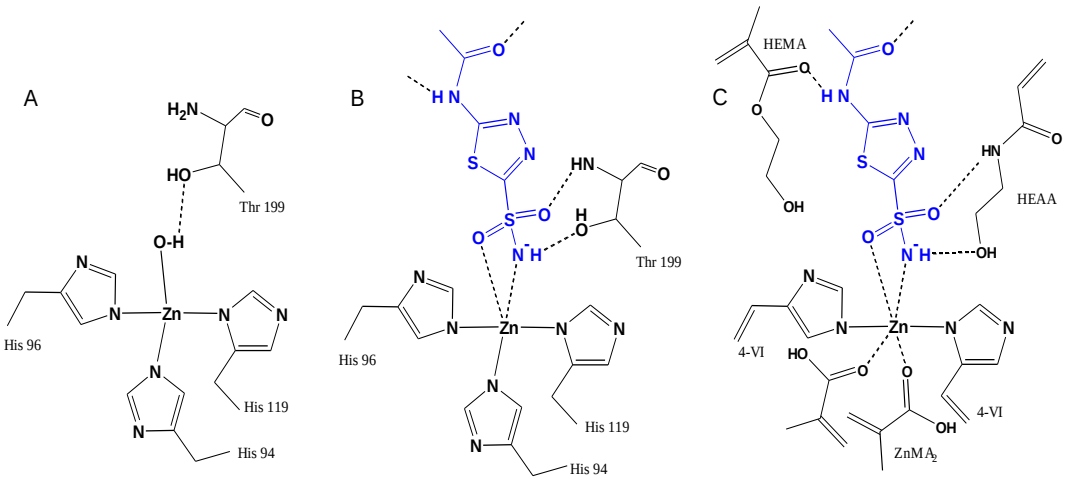


Figure 5.1. Schematic draw of the active site of human carbonic anhydrase II free (A) and after binding acetazolamide (B) as described by Lindskog [37], and of the mimicking binding pockets expected to be created in the biomimetic hydrogels (C).

5.2. Experimental section

5.2.1. Materials

Acetazolamide (ACT), ethoxzolamide (ETOX), 2-hydroxyethyl methacrylate (HEMA), ethyleneglycol dimethacrylate (EGDMA), zinc methacrylate ($ZnMA_2$), 1-vinylimidazole (1VI), urocanic acid, and N-hydroxyethyl acrylamide (HEAA) were from Sigma-Aldrich Chemicals (Madrid, Spain) (Figure 5.2). Azobisisobutyronitrile (AIBN) was from Acros Organic Co. (Geel, Belgium). Zincon monosodium salt (2-carboxy-2'-hydroxy-5'-sulfoformazylbenzene) and zinc nitrate hexahydrate were from Sigma-Aldrich Co. (St. Louis MO, USA). Purified water was obtained by reverse osmosis (MilliQ®, Millipore Ibérica SA, Madrid, Spain). Other reagents were analytical grade.

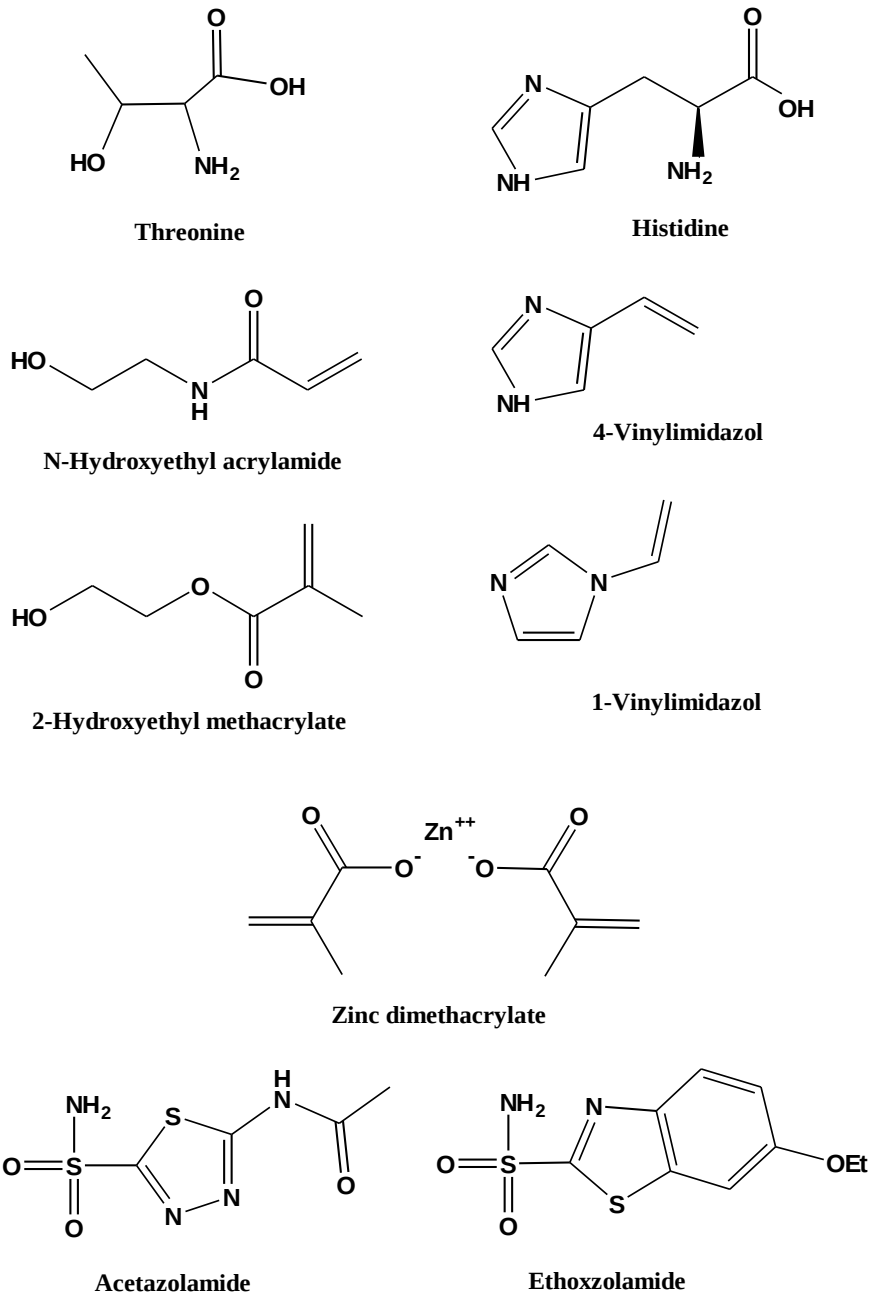


Figure 5.2. Amino acids that form part of the active site of carbonic anhydrase, monomers used to synthesize the hydrogels, and CAI drugs tested.

5.2.2. Synthesis of 4(5)-vinylimidazole (4VI)

4VI was obtained via thermal decarboxylation from urocanic acid according to the literature [39]. Briefly, anhydrous urocanic acid (2g, 0.0145 mol) was placed in a short-necked distilling apparatus. Then the temperature was slowly increased up to 205°C under pressure of 18 torr. The distilled material was trapped using a glass sink (cold finger) cooling system. 4VI was obtained as a crystallized white product in the cold receiver (yield= 12%). Then, it was stored in the fridge at 4ºC. The obtaining of 4VI was confirmed by FTIR-ATR spectroscopy (400–4000 cm^{-1}, Varian-670-FTIR spectrometer equipped with a GladiATR^{TM}, Madison WI, USA) and ^{1}H NMR analysis (Varian Mercury 300 MHz + robot spectrophotometer, Madison WI, USA). The peaks of 4VI in $CDCl_3$ were assigned as follows: δ 5.10 (d, J= 12.15 Hz, 1H), 5.65 (d, J = 17.64 Hz, 1 H), 6.60 (m, 1H), 7.02 (s, 1H), 7.61 (s, 1H), 10.32 (br s, 1H) [40].

5.2.3. Synthesis of pHEMA hydrogels

Various sets of monomer mixtures were prepared with the composition shown in Table 5.1. Acetazolamide was added to some mixtures at a concentration of $7.8 \cdot 10^{-4}$ M in order to attain a molar ratio of 1:1:3 for drug:Zn^{2+}:VI. The monomer solutions were injected into moulds constituted by two glass plates pretreated with dimethyldichlorosilane and separated by a silicone frame 0.9 mm thickness [41]. The moulds were placed in an oven at 50°C for 12 h and then heated at 70°C for 24 h more. The hydrogels were removed from the moulds and immersed in boiling water (600 mL) for 15 min to remove non-reacted components and to facilitate the cutting of the hydrogels as 10 mm discs. The discs were washed in

ultra pure water for 24 h, in NaCl 0.9% solution for 24 h and then immersed in water for 15 days (400 mL). The removal of unreacted monomers was monitored by measuring the absorbance in the 190-800 nm range of the washing solutions. Finally, the discs were dried and stored.

Table 5.1. Composition of the monomer mixtures used to synthesize the hydrogels.

Formulation	HEMA (ml)	$ZnMA_2$ (g)	HEA A (g)	1VI (ml)	4VI (ml)	EGDMA (ml)	AIBN (g)	ACT (g)
0	8	-	-	-	-	0.12	0.0135	-
A	8	0.185	-	-	-	0.12	0.0135	-
B	8	0.185	-	-	-	0.12	0.0135	0.173
C	8	0.185	-	0.22	-	0.12	0.0135	-
D	8	0.185	-	0.22	-	0.12	0.0135	0.173
E	8	0.185	-	-	0.22	0.12	0.0135	-
F	8	0.185	-	-	0.22	0.12	0.0135	0.173
G	8	0.185	0.14	-	-	0.12	0.0135	-
H	8	0.185	0.14	-	-	0.12	0.0135	0.173
I	8	0.185	0.14	-	0.22	0.12	0.0135	-
J	8	0.185	0.14	-	0.22	0.12	0.0135	0.173

5.2.4. Determination of zinc content.

A stock solution of Zincon (1.6 mM) was prepared by dissolving 7.4 mg in 250 µl of 1M NaOH prior to dilution to 10 ml with water. The solution was kept in the fridge and used in few days. Zn^{2+} stock solution (27 mM) was prepared by dissolving zinc nitrate hexahydrate in 50 ml of 0.1M nitric acid. The calibration curve was constructed by adding 25 µl of diluted stock solution (0.31 to 1.25 mM) to 950 µl of USP borate buffer pH 9.0 and 25 µl of the Zincon stock solution [42]. The blank was prepared analogously except for the substitution of the metal sample solution with 0.1M nitric acid. Absorption spectra were recorded from 400

to 750 nm after 5 min of sample incubation at room temperature. The absorbances were measured at 620 nm. The intensity of the blue color was proportional to the zinc concentration [42]. To quantify the amount of zinc present in the hydrogels, dried disks were transferred to test tubes containing 2 ml of 1.0 M nitric acid at kept at 70˚C for approximately 48 hours. Aliquots (100 µl) of the final solution were diluted with 330 µl of 1.0 M nitric acid and then mixed with 70 µl of NaOH 5M to adjust the pH to 4-5. Then, 25 µl of the resulting solution was added to 950 µl of USP borate buffer pH 9.0 and 25 µl of the Zincon stock solution, and incubated at room temperature for 5 min. The absorbance was recorded at 620 nm and the amount of zinc in the sample was estimated from the calibration curve. All experiments were carried out in triplicate.

5.2.5. Physical and structural characterization of pHEMA hydrogels

FTIR-ATR (attenuated total reflection) spectra were recorded over the range 400–4000 cm^{-1}, in a Varian-670-FTIR spectrometer equipped with a GladiATR™ (Madison, WI, USA) with diamond crystal. DSC scans of dried hydrogels (5-10 mg) were recorded by heating from 25ºC to 150ºC, cooling to -10ºC, and then heating again until 300ºC, always at the rate of 10ºC/min, in a DSC Q100 (TA Instruments, New Castle, DE, USA) with a refrigerated cooling accessory. Nitrogen was used as purge gas at a flow rate of 50 ml/min. The calorimeter was calibrated for cell constant and temperature using indium standard (melting point 156.61ºC, enthalpy of fusion 28.71 J/g) and for heat capacity using sapphire standards. All experiments were performed in duplicate. Degree of swelling in water was calculated, in duplicate, as follows:

$$Q(\%) = \frac{(W_t - W_0)}{W_0} \cdot 100 \qquad \text{Eq. (5.1)}$$

where W_0 and W_t represent the weights of a hydrogel disc at the dry state and after being immersed in water for a time *t*. Light transmittance of fully swollen hydrogels was recorded in triplicate at 600 nm (UV-vis spectrophotometer, Agilent 8453, Germany) by mounting a continuous piece of hydrogel on the inside face of a quartz cell. Oxygen permeability (Dk) and transmissibility of hydrogels previously swollen in a 0.9% NaCl solution were measured in triplicate at room temperature using a Createch permeometer (model 210T, Rehder Development Company, Castro Valley, USA) fitted with a flat polarographic cell and in a chamber at 100 % of relative humidity.

5.2.6. Cytocompatibility studies

Dried discs were immersed in phosphate buffer (pH 7.4) and autoclaved (121°C, 20 min). Cytocompatibility tests were carried out in triplicate using the Balb/3T3 Clone A31 cell line (ATCC, LGC Standards S.L.U., Barcelona, Spain) according to the direct contact test of the ISO 10993-5:1999 standard. The discs were added in 24 well plates containing 200,000 cells per well in 2 ml Dulbecco' Modified Eagle's Medium F12HAM supplemented with 10% fetal bovine serum and 13 μg/ml gentamicin. The plates were incubated at 37°C, 5% of CO_2 and 90% of humidity. After 24 hours aliquots (100 μl) of medium were taken in 96 well microplates and mixed with reaction mixture solution (100 μl, Cytotoxicity Detection Kit^{PLUS}, LDH, Roche). The plates were incubated at 20°C for 30 min (protected from light). A stop solution (50 μl) was added to the wells and the absorbance measured at 490 nm (BIORAD Model 680 Microplate reader, USA).

Blank (culture medium), negative (cells in culture medium) and positive (cells in medium with lysis factor) controls were prepared too and the absorbance measured. The cytocompatibility was quantified as follows:

$$Cytocompatibility\,(\%) = \frac{Abs_{\exp} - Abs_{negativecontrol}}{Abs_{positivecontrol} - Abs_{negativecontrol}} \qquad \text{Eq. (5.2)}$$

5.2.7. ACT loading and release

Dried hydrogel discs (six replicates) were placed in 5 ml of ACT 0.2 g/L aqueous solution and kept at room temperature protected from the light for 48 h. The amount of ACT loaded was calculated as the difference between the initial amount of drug in the solution and the amount remaining after loading determined by UV spectrophotometry at 264 nm (Agilent 8453, Germany). Drug-loaded discs were rinsed with water, their surface was carefully wiped and the discs were immediately immersed in 5 ml of NaCl 0.9% solution at room temperature. The amount of drug released was measured spectrophotometrically in samples periodically taken and again placed in the same vessel so that the liquid volume was kept constant. The network/water partition coefficient, $K_{N/W}$, which is an index of the affinity of the drug for the network, was estimated from the total amount loaded per gram of gel [43]:

$$Loading_{(total)} = \left[\frac{(V_s + K_{N/W}V_p)}{W_p}\right]\cdot C_0 \qquad \text{Eq. (5.3)}$$

where V_s is the volume of water sorbed by the hydrogel, W_p is the dried hydrogel weight, C_0 is the concentration of the drug in loading solution and V_p is the volume of dried polymer.

Release profiles up to 60% released were fitted to the square-root kinetics as follows [44]:

$$\frac{M_t}{M_\infty} = K_H \cdot t^{0.5} \qquad \text{Eq. (5.4)}$$

where M_t represents the amount of drug released at time *t* and M_∞ the total amount loaded. Each release profile was fitted to the Higuchi equation and then the mean and the standard deviation calculated from the values obtained for six replicates.

5.2.8. ETOX loading and release

Dried hydrogels discs (six replicates) were placed in 5 ml of ETOX suspension (0.23 g/L) and kept 48 h at room temperature. The ETOX-loaded discs were rinsed with water; their surfaces were carefully wiped and the discs were immediately immersed in 5 ml of NaCl 0.9% at room temperature. The amount of drug released was measured spectrophotometrically at 303 nm in samples periodically taken up and placed again into the same vessel. After 360 h, the discs were removed from the medium, rinsed with ultrapure water, and placed in vials with 5 ml of ethanol:water (70:30) mixture for 48 hours. The drug extracted from the hydrogels was quantified from the absorbance measured at 303 nm (Agilent 8453, Germany). The network/water partition coefficient, $K_{N/W}$, of ETOX was estimated from the total amount released to the aqueous medium plus that

extracted in ethanol:water medium. ETOX solubility in water (21.38 mg/L) was used as C_0 in Equation 5.3, since in the suspension this concentration should remain constant. The release rate constants were estimated using Equation 5.4.

5.3. Results and Discussion

5.3.1. Hydrogels synthesis and structural characterization

The hydrogels were prepared using HEMA as main component due to the recognized biocompatibility of pHEMA and its common use as integrant of SCLs [45]. Several monomers were copolymerized with HEMA in order to mimic the active site of carbonic anhydrase (Table 5.1); the biomimetic level is foreseeable to increase from A to J formulations. A control pHEMA hydrogel without functional comonomers (formulation 0) was used as a reference to quantify the role of the comonomers in the binding of the CAIs. Although 4VI resembles much better than 1VI the functional groups of histidine, both monomers were used to prepare the hydrogels with the purpose of elucidating the incidence of the imidazole structure on the affinity for the CAIs. 4VI was successfully synthesized from urocanic acid and its structure was confirmed by ^{1}H NMR (Figure 5.3). Urocanic acid showed chemical shifts at 2.49 and 3.32 ppm (-OH) and 6.32 ppm (CH=), whereas 4VI showed two singlet peaks at 7.03 and 7.61 ppm for N-CH and N=CH protons (imidazole), respectively. The methylene (CH_2=) and methane (CH=) protons of 4VI appeared at 5.16, 5.67 and 6.63 ppm, according to the literature [46].

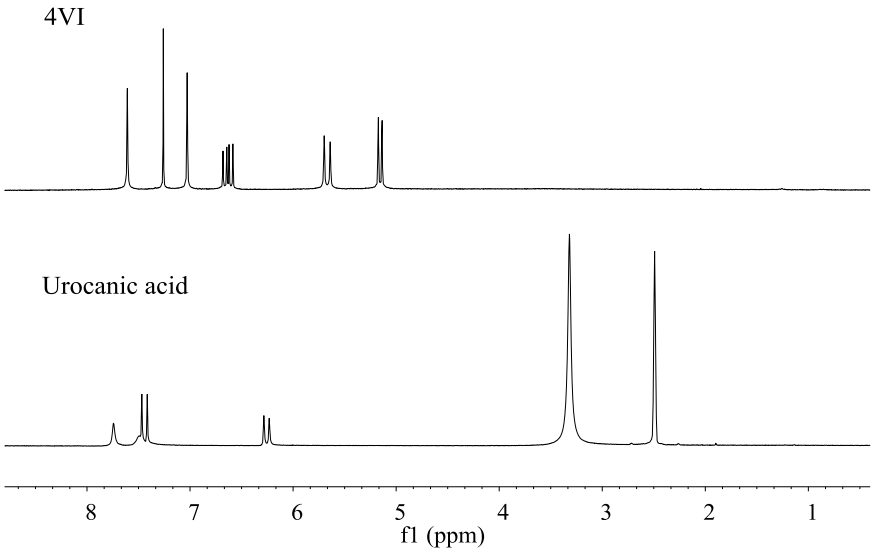


Figure 5.3. ^{1}H NMR spectra of urocanic acid in DMSO-d_6 and 4VI in $CDCl_3$.

The hydrogels were intensively washed after synthesis in order to remove the template molecules. Both imprinted and non-imprinted hydrogels underwent the same cleaning procedure. Since in the natural receptor, zinc directly participates in the binding of the drug (see Figure 5.1B), we first corroborated the presence of zinc ions in the networks. It is interesting to note that, even after boiling and immersion in saline medium, zinc ions were still detected in A to J hydrogel formulations (Table 5.2). Nevertheless, remarkable differences among the hydrogels could be observed; the imidazol monomers being absolutely required to keep significant amounts of zinc in the network. The small amount of zinc ions remaining in the hydrogels without 4-vinylimidazole could be due to that zinc methacrylate is not reacting during polymerization or that zinc ions are replaced by counter ions in the absence of 4-vinylimidazole. Since all hydrogels have a similar monomeric composition (HEMA being the majority monomer) and 100%

zinc complexation can only be achieved if methacrylate mers are effectively copolymerized in the network, it is perfectly plausible that zinc methacrylate is similarly incorporated to all hydrogels. Thus, the decrease in zinc ions can be attributed to the long extraction process in the presence of saline medium. Complete removal of zinc ions by sodium ions is not complete since the affinity of the methacrylic acid mers for divalent ions is larger than for monovalent ones [47]. In sum, the formation of a zinc:methacrylic acid:imidazol coordination complex notably enhances the stability of the zinc bonds to the network. This finding is in agreement with previous papers that reported that polymers containing 1VI or 4VI can bind metal ions, including zinc, with a remarkable affinity even in saline medium [48]. Additionally, we observed that hydrogels bearing 4VI retained twice the amount of zinc ions (71-90%) than those synthesized with 1VI (38-43%). This finding can be related to the fact that 4VI mimics better the coordination of zinc to the amine groups of histidine residues that occurs in the natural carbonic anhydrase enzyme. Since the affinity of zinc ions for methacrylic acid groups is quite high [47] and those zinc ions can still coordinate with two imidazole groups [48], it seems that a quite plausible structure of the binding pocket is that depicted in Figure 4.1C. Nevertheless, other configurations of the receptor, such as that with only one imidazole group, cannot be discarded. The proportion of functional monomers in the hydrogel is too low for being able to gain an insight into this point using common analytical techniques.

The hydrogels were slightly opalescent due to the presence of $ZnMA_2$, particularly those prepared with 4VI (Table 5.2). Nevertheless, it should be noticed that the thickness of the hydrogels is larger (3 to 4-fold) than that of commercial SCLs. Thus, transmittance of thinner hydrogels might make possible to use some formulations as components of SCLs. The lower transmittance of the

hydrogels prepared with 4VI can be also related to their lower degree of swelling in aqueous medium (Figure 5.4). pHEMA-$ZnMA_2$ hydrogels prepared without 1VI or 4VI (formulations A, B, G and H) attained swelling values of 70-76% (Table 5.2), which are in the typical range value of pHEMA hydrogels. Copolymerization with HEAA did not change the degree of swelling. By contrast, those hydrogels containing 1VI or 4VI exhibited a remarkably lower capability to sorb water (Table 5.2). The synthesis in the presence of ACT attenuated to a certain extent the decrease in the degree of swelling; the D, F and J imprinted hydrogels swelled more in water than the corresponding C, E and I non-imprinted networks.

Furthermore, 1VI and 4VI make the networks more rigid as indicated by the increase in the glass transition temperature, Tg, of the hydrogels prepared with these monomers (Table 4.2). The presence of $ZnMA_2$ did not alter the Tg of the pHEMA hydrogels, which is around 110ºC (Table 4.2). It has been reported that poly(1VI) has a Tg around 175ºC [49]. The DSC scans of the hydrogels containing 1VI or 4VI showed only one Tg at an intermediate temperature between the Tg of pHEMA and that of poly(1VI), indicating that the monomers are miscible and confirming that the vinylimidazole monomers are efficiently copolymerized with the methacrylate ones [39, 46, 49]. Macromolecular interactions between the carbonyl group of ethyl methacrylate and the imidazol fragments have been previously shown by FTIR spectroscopy [49]. However, we could not observe relevant shifts in the carbonyl band at 1707 cm^{-1}, probably because the relatively low proportion of 1VI and 4VI in the hydrogels compared to HEMA. The FTIR spectra mainly showed the characteristic bands of pHEMA (data not shown).

Table 5.2. Percentage of zinc ions that remain in the hydrogels after the cleaning step, and physical properties of the networks. Mean values and, in parenthesis, standard deviations.

Formulation	Remaining Zn (%)	Transmittance at 600 nm (%)	Tg (ºC)	Degree of swelling (%)	Dk (barrer)
0	---	85	110	66.0 (3.2)	12.4 (1.1)
A	22.6 (7.1)	76	109	73.0 (0.8)	12.6 (1.5)
B	11.3 (4.8)	70	115	70.6 (1.5)	17.7 (7.6)
C	38.7 (6.6)	68	126	42.8 (5.1)	9.2 (0.4)
D	43.7 (7.4)	64	132	55.5 (0.1)	12.8 (2.3)
E	73.6 (4.5)	29	125	52.7 (0.6)	14.8 (0.3)
F	71.9 (1.1)	29	125	53.4 (1.1)	13.4 (0.4)
G	10.0 (1.9)	73	110	77.5 (1.7)	18.0 (3.3)
H	13.9 (1.5)	77	109	75.2 (4.3)	24.8 (0.2)
I	76.9 (5.1)	22	116	55.5 (0.4)	17.2 (4.9)
J	90.8 (4.5)	30	123	61.0 (1.2)	15.6 (1.5)

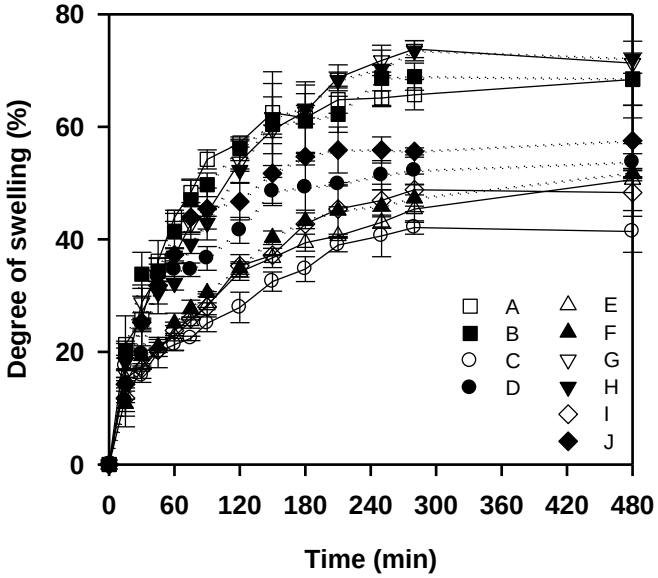


Figure 5.4. Swelling profiles of the pHEMA-$ZnMA_2$ hydrogels (codes as in Table 5.1) in water. Continuous and doted lines correspond to non-imprinted and ACT-imprinted networks.

5.3.2. Oxygen permeability and cytocompatibility

Hydrogels intended as components of SCLs should have enough oxygen permeability for preventing corneal hypoxia and edema. As expected, the higher the degree of swelling, the greater the oxygen permeability of the hydrogels was (Table 5.2). This means that the hydrogels containing 1VI or 4VI are less gas permeable than the other hydrogels. Nevertheless, the differences are not too large and the values are in the range of those recorded for commercially available SCLs [50, 51].

Cytocompatibility studies were carried out according to the direct contact test of the ISO 10993-5:1999 standard for the simultaneous evaluation of the effect of the polymer network and the leached substances, such as unreacted monomers.

Fibroblast showed an excellent viability (96-100%) when cultured on any hydrogel prepared (Figure 5.5), indicating that $ZnMA_2$, 1VI and 4VI do not deteriorate the cytocompatibility of the pHEMA hydrogels and that no toxic substances are leaked from the networks.

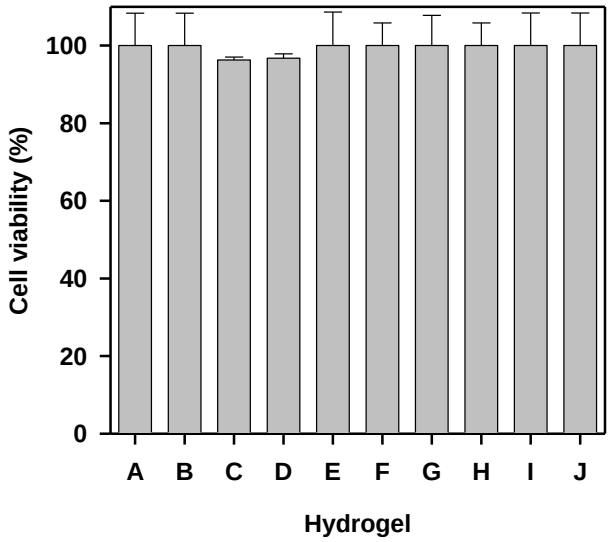


Figure 5.5. Cell viability for different pHEMA-$ZnMA_2$ hydrogels. Mean values and standard deviations (n=3).

5.3.3. ACT loading and release

Although both ACT and ETOX exhibit similar ability to inhibit human carbonic anhydrase isoform II and are active at the nanomolar range [52], relevant physicochemical differences between both molecules can be highlighted. CAIs bind in the active site of the enzyme in deprotonated state, coordinating to zinc while the basic amino acids serve as proton acceptors. Therefore, the lower the pKa, the more favorable the binding is. ACT is more acidic (pKa 7.4) and significantly less lipophilic (LogP = -0.26) than ETOX (pKa 8.0; LogP= 2.01)

[53]. Comparing to ETOX, ACT is more ionized and its solubility is greater at physiological pH 7.4. Although both CAIs obey Lipinski "rule of 5" properties and show good membrane permeability, ACT has 7 hydrogen bond acceptors and 3 hydrogen bond donors, while ETOX has just 5 hydrogen bond acceptors and 2 hydrogen bond donors. Thus, ACT may be anchored to the active site of the enzyme through more hydrogen bonds [53]. Drug loading experiments were designed to have the same number of molecules of ACT or ETOX in the volume of medium in which the hydrogels were immersed. Thus, the first consequence of the physicochemical differences between ACT and ETOX was that the loading experiments with the latter had to be carried out by immersion of the hydrogels in an aqueous suspension due to the low solubility of ETOX. Loading with ACT was carried out by immersion in 0.2 g/L solution.

Copolymerization of HEMA with $ZnMA_2$ solely or with HEAA did not significantly modify the amount of ACT uptaken by the hydrogels (Table 5.3). These hydrogels retained a small proportion of the zinc ions incorporated during synthesis and thus no specific drug-network interactions could be established. A slight improvement in the loading was recorded for formulations having 1VI. By contrast, hydrogels bearing 4VI moities showed a remarkably greater ability (2-fold) to load ACT (Table 5.3). The network/water partition coefficient, $K_{N/W}$, values obtained for formulations E, F, I and J almost triplicate the values recorded for the other hydrogels. No further improvement was achieved by adding ACT as template during polymerization. This means that using the functional monomers that mimic the best the components of the CA receptor, it is feasible to endow the hydrogels with high affinity for ACT. Such a notable increase in affinity was also evidenced in a better control of drug release (Figure 5.6). Control hydrogels (formulation 0) rapidly released the ACT loaded. Copolymerization with functional monomers that resemble the functionalities of the

natural receptor at the active site significantly decreased ACT release rate. Most hydrogels required 15 days to complete the release. Hydrogels A and B showed a certain decrease in ACT release rate compared to control hydrogels, which could be due to a slight increase in the mesh size of the network owing the divalent Zn ions connecting neighbor methacrylic acid mers. The release profiles fitted quite well to the square root kinetics and the hydrogels E, F and I showed the lowest release rate (Table 5.3). Imprinted hydrogels (i.e, formulations B, D, F, H and J) seem to release ACT faster than the corresponding non-imprinted networks (Figure 5.6), although statistically significant differences were only recorded for formulation F compared to E and for formulation J compared to I (t-test, $\alpha < 0.01$).

Table 5.3. ACT loaded, network/water partition coefficients, ACT released in NaCl 0.9% solution, and release rate constants obtained after fitting to the square-root kinetics. Mean values and, in parenthesis, standard deviations (n=6).

Formulation	**Loading (mg/g)**	**$K_{N/W}$**	**ACT released at 48 h (mg/g)**	**K_H (% $h^{-0.5}$)**	**R^2**
0	1.22 (0.10)	5.40 (0.18)	1.17 (0.04)	24.20 (2.05)	0.994
A	1.50 (0.30)	6.67 (1.46)	0.87 (0.04)	10.48 (0.53)	0.962
B	1.18 (0.36)	5.11 (1.77)	0.74 (0.06)	11.67 (0.26)	0.967
C	1.48 (0.20)	6.63 (0.96)	0.92 (0.13)	10.56 (0.47)	0.991
D	1.74 (0.12)	7.79 (0.58)	1.16 (0.02)	14.07 (2.98)	0.946
E	3.37 (0.03)	16.40 (0.16)	1.64 (0.05)	6.86 (0.18)	0.995
F	3.16 (0.25)	15.35 (1.26)	1.87 (0.20)	8.49 (0.45)	0.995
G	1.51 (0.10)	6.52 (0.48)	0.68 (0.04)	8.61 (0.71)	0.948
H	1.38 (0.10)	5.90 (0.50)	0.64 (0.04)	8.99 (0.79)	0.955

I	3.15 (0.29)	15.29 (1.47)	1.61 (0.18)	7.31 (0.04)	0.994
J	3.28 (0.15)	15.64 (0.72)	2.46 (0.30)	13.04 (0.16)	0.993

The reasons behind this behavior are not clear but could be related to small changes in the microstructure of the hydrogels caused by the presence of ACT molecules during polymerization. The removal of the template molecules may have opened paths into the network for an easier entrance/exit of subsequent drug molecules. In fact, the synthesis in the presence of template molecules caused an increase in the degree of swelling of the imprinted networks compared to the non-imprinted ones (Table 5.2).

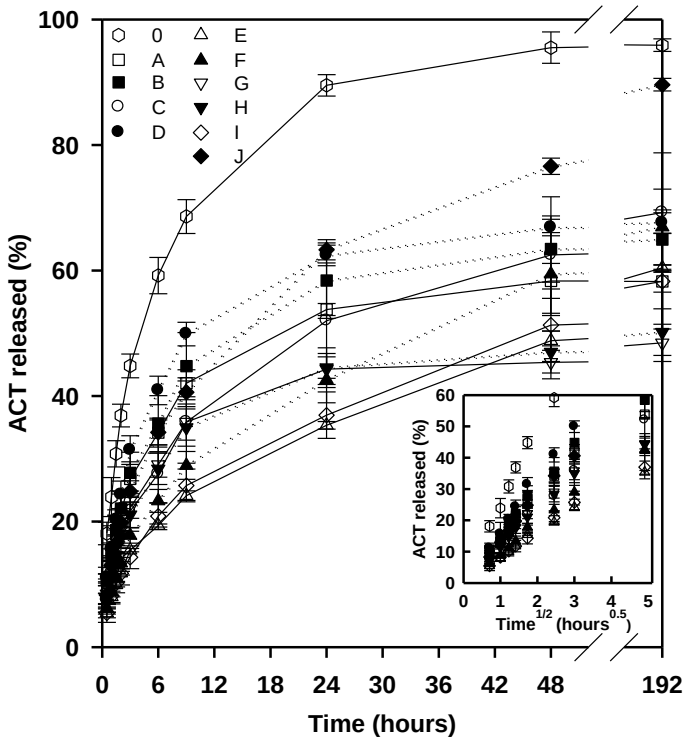


Figure 5.6. ACT release profiles in 0.9% NaCl medium from pHEMA hydrogels containing diverse functional comonomers. Continuous and doted lines correspond to non-imprinted and ACT-imprinted networks. Codes as in Table 5.1. The insert shows data used for the fitting to the square-root kinetics.

5.3.4. ETOX loading and release

The loading of ETOX was carried out by immersion of the hydrogels in aqueous suspensions of the drug because of its limited solubility (21.38 mg/L). The suspensions contained the same number of molecules of ETOX per liter as in the case of ACT. The hydrogels loaded less ETOX than ACT, which may be due to the fact that the drug has firstly to dissolve in order to be available to interact with the network. Nevertheless, the hydrogels (even the control one) showed remarkably high affinity for ETOX (network/water partition coefficients above 36), which suggest unspecific hydrophobic adsorption to the pHEMA network. pHEMA-$ZnMA_2$ hydrogels containing 4VI were again those with greater ability to host ETOX and exhibited 90% greater $K_{N/W}$ values than the other hydrogels (Table 5.4), which indicates the contribution of specific interactions with the biomimetic pockets. The hydrogels sustained the release of ETOX for two weeks, after which the amount released was still below 50% (Figure 5.7). The release rate decreased after 72 hours, which could be due to the attainment of equilibrium between the free drug in the medium and the drug bound to the hydrogel due to the high drug-network affinity. It should be also noticed that the volume of the medium, although enough to dissolve the whole ETOX dose loaded by the hydrogels, was limited to 5 ml to resemble the small volume of lachrymal fluid that could be available on the cornea for the release of the drug from a medicated SCL. The release data obtained in first 48 hours were fitted to the square-root

kinetics (Table 5.4). Hydrogels 0, A and B behaved very similar probably due to the fact that unspecific hydrophobic interactions drive the binding of the drug to the network and small differences in mesh size are less relevant for drug diffusion than in the case of the water-soluble ACT. The most biomimetic hydrogels (formulations E, F, I and J) sustained better the release highlighting the role of an adequate combination of functional groups in the ability of the hydrogels to host the drug with high affinity and to regulate its release rate. Differences in ETOX loading/release between ACT-imprinted and non-imprinted hydrogels were minor and did not follow a clear trend.

Table 5.4. ETOX loaded, network/water partition coefficient, ETOX released in NaCl 0.9% solution, and release rate constants obtained after fitting to the square-root kinetics. Mean values and, in parenthesis, standard deviations (n=6).

Formulation	**Loading (mg/g)**	**$K_{N/W}$**	**ETOX released at 48h (mg/g)**	**K_H (% $h^{-0.5}$)**	**R^2**
0	0.99 (0.17)	45.70 (7.82)	0.43 (0.06)	7.03 (0.66)	0.969
A	0.91 (0.12)	42.11 (5.64)	0.34 (0.04)	7.03 (0.46)	0.957
B	0.80 (0.16)	36.86 (7.55)	0.28 (0.04)	6.70 (0.48)	0.945
C	0.81 (0.14)	37.38 (6.40)	0.28 (0.05)	7.29 (0.97)	0.926
D	1.01 (0.04)	46.73 (1.92)	0.42 (0.02)	7.86 (0.27)	0.954
E	1.51 (0.07)	70.36 (3.45)	0.35 (0.03)	4.81 (0.18)	0.954
F	1.50 (0.18)	69.84 (8.27)	0.36 (0.03)	4.98 (0.45)	0.907
G	1.00 (0.02)	46.26 (1.08)	0.42 (0.03)	8.53 (0.22)	0.971
H	0.95 (0.11)	44.04 (5.18)	0.36 (0.01)	7.61 (0.99)	0.956
I	1.55 (0.12)	72.32 (5.44)	0.35 (0.03)	4.02 (0.63)	0.921
J	1.71 (0.18)	79.38 (7.33)	0.48 (0.06)	4.92 (0.44)	0.962

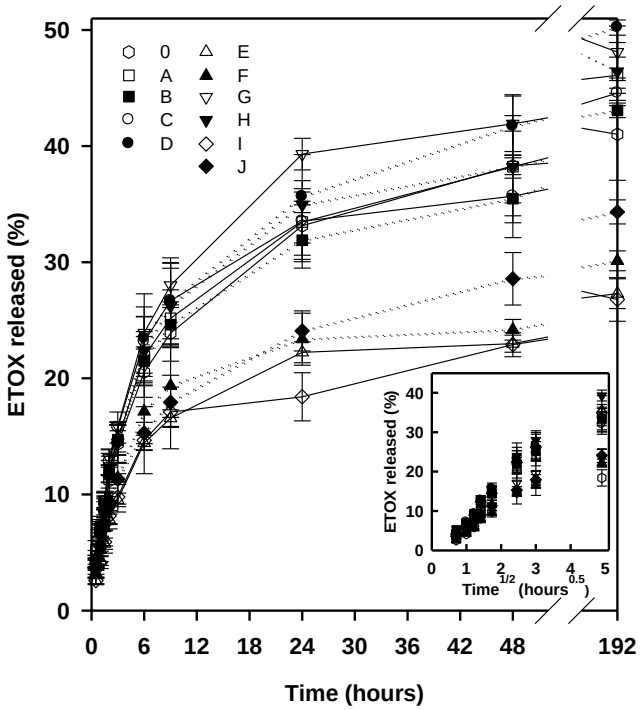


Figure 5.7. ETOX release profiles in 0.9% NaCl medium from pHEMA hydrogels containing diverse functional comonomers. Continuous and doted lines correspond to non-imprinted and ACT-imprinted networks. Codes as in Table 5.1. The insert shows data used for the fitting to the square-root kinetics.

5.4. Conclusions

The knowledge of the physiological receptors with which drugs interact to exert the therapeutic effect has been used so far for the chemical optimization of the drugs or the search of new candidates with improved pharmacological efficacy and safety. Although still few, previous works have suggested that the structure of

the physiological receptor can also be used as the model to follow in the design of optimized drug delivery systems [23, 24, 35]. We have here demonstrated that mimicking the active site of carbonic anhydrase, networks with high affinity for inhibitor drugs (CAIs) can be created. Biomimetic networks can load more drug and control better drug release than conventionally synthesized pHEMA hydrogels, being useful for the development of advanced controlled release systems. Nevertheless, aspects such as optical transparency (for application as drug-eluting SCLs), the effect of thickness on drug release length, and long-term durability of the biomimetic receptors (both from the point of view of time between preparation and use, or of any application that involves loading/release cycles) require further studies in order to fully elucidate the practical potential of enzyme-mimicking networks.

5.5. References

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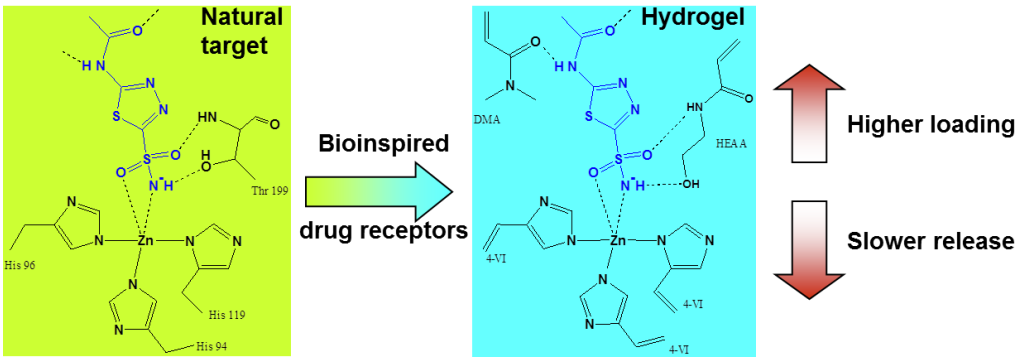
RECEPTOR-BASED BIOMIMETIC NVP/DMA CONTACT LENSES FOR LOADING/ELUTING CARBONIC ANHYDRASE INHIBITORS



CHAPTER 6

Abstract

Biomimetic principles were applied to design N,N-dimethylacrylamide (DMA) and N-vinylpyrrolidone (NVP) hydrogels with enhanced affinity for the antiglaucoma drugs acetazolamide (ACT) and ethoxzolamide (ETOX). These inhibitors of carbonic anhydrase are orally given to decrease intraocular pressure, but their systemic side effects prompt the development of devices for ocular delivery. Receptors for ACT and ETOX were created in the hydrogels by mimicking the active site of the metallo-enzyme, using 1- or 4-vinylimidazole (1VI or 4VI) and N-hydroxyethyl acrylamide (HEAA) as functional monomers. To some hydrogels, zinc salt and ACT (imprinted networks) were incorporated before polymerization for a closer mimicking of the natural receptor. Viscoelasticity, water uptake, light transmissibility, cytocompatibility, zinc content, and drug loading and release were evaluated. 4VI retained the non-polymerizable zinc salt better than 1VI and rendered visible light transparent hydrogels. NVP-co-DMA hydrogels bearing 4VI, HEAA and Zn^{2+} showed 2-fold increase in drug affinity (estimated as network/water partition coefficient) and more sustained delivery. ACT-imprinted networks achieved the highest loading and controlled ACT release for 9 hours. ETOX release was sustained for more than one week. Favorable physicochemical, mechanical and cytocompatibility features suggest that receptor-inspired hydrogels are promising candidates for the development of biomimetic medicated soft contact lenses as well as other delivery systems.

Keywords

Bioinspired drug delivery; CAI; molecular imprinting; combination product; soft contact lens.

6.1. Introduction

Biomimetics has been recently defined as an emerging field of science that comprises the study of how Nature designs, processes and assembles/disassembles molecular building blocks to fabricate high performance soft materials and mineral-polymer composites, and then applies these designs and processes to engineer new molecules and materials with unique properties [1, 2]. Integration of biomimetic principles in the design of drug delivery systems is greatly impacting the therapeutic field [3]. Carriers with camouflage coatings for silent movement in the body, with surface elements that recognize specific cell ligands (bio-addressed) for active targeting, or with switchable components that regulate the delivery as a function of certain variables have shown outstanding performance [4-13]. So far, the mimicking process has mainly focused on how the carrier can deal with the physiological environment and overcome the barriers and compartments of the body, drive the drug to the its receptor and regulate its delivery by imitating common processes in the body. By contrast, biomimetic principles have been barely applied to the design of delivery systems with improved affinity for a given drug and, consequently, with optimized loading and controlled release performance [9].

Receptor-based or ligand-based strategy is routinely applied for the rational optimization of drug candidates since various decades ago, but only recently is attracting attention for the design of drug delivery systems [14, 15]. The knowledge about the structure of the drug target (receptor) is currently utilized for the modelling of lead compounds in drug discovery [16]. Such an information could be also useful for recreating, in the structure of the delivery system, pseudo-receptors able to interact with the drug in an specific way; the strength of the binding drives the loading process and the release rate. Attempts to prepare

networks with specific binding points for drug molecules have focussed on the application of the molecular imprinting technology [17-20]. This approach is based on the *in vitro* or *in silico* screening of monomers (functional monomers) that can interact with a given molecule (the template), followed by polymerization and cross-linking of the monomers in the presence of that template. Removal of the template molecules after polymerization should render networks with cavities (receptors) possessing the shape and size of the template and chemical groups suitable for the interaction [21, 22]. Although this synthetic approach has been shown to provide hydrogels with enhanced affinity for certain drugs, the design of the networks could greatly benefit from the information available about the *in vivo* receptor. Particularly, functional monomers possessing chemical groups similar to those present in the histamine H1-receptor or in the CD44 protein have been recently shown to endow hydrogels with high affinity for the antihistamic drug ketotifen fumarate [14, 23] or for hyaluronic acid [15], respectively, and that sustain better the drug release process.

The active site of carbonic anhydrase is another target particularly attractive to be mimicked searching for a way to design hydrogels with enhanced affinity for carbonic anhydrase inhibitor drugs (CAIs). Acetazolamide and ethoxzolamide are known to inhibit the activity of this enzyme reducing intraocular pressure, being useful for the treatment of glaucoma [24]. However, when orally administered they cause relevant untoward effects (namely fatigue, paresthesias, and kidney stones) due to the almost ubiquitous presence of carbonic anhydrase in the body [24]. Ocular sustained release of CAIs may enhance the drug efficacy/safety ratio. As observed for other drugs [19, 20, 25, 26], drug-loaded inserts or soft contact lenses (SCLs) may prolong the drug residence time on the precorneal area, enhancing ocular bioavailability and, consequently, be more effective in the treatment of glaucoma. Although there are different isoforms, the active site of

most of carbonic anhydrases consists of a cone-shaped cavity that contains a Zn^{2+} ion coordinated to three histidine residues (His-94, His-96 and His-116) in a tetrahedral geometry with a solvent molecule as the fourth ligand [27]. Sulfonamides bind to the Zn^{2+} ion of the enzyme either by substituting the non-protein zinc ligand to generate a tetrahedral adduct or by addition to the metal coordination sphere, generating trigonal-bipyramidal species [28]. The -NH group of the ionized sulfonamide group replaces the water molecule bound to zinc, while other groups establish van der Waals interactions or hydrogen bonds with neighbor amino acids (e.g., Thr-99). One oxygen atom of the sulfonamide interacts with the -NH group of treonine 199, while another oxygen points toward the zinc ion [27]. In a previous work [29], we have tried to mimic the structure of this active site in poly(hydroxyethyl methacrylate) (pHEMA) hydrogels using a fix proportion of zinc methacrylate as the source of Zn^{2+} ions for the complexation. Although the hydrogels exhibited 2-3 times greater network/water drug partition coefficient than those lacking of pseudo-receptors [29], the hydrogels that performed better as delivery systems resulted to be opalescent, which limits their applicability for ocular drug delivery [19, 20].

The aim of the present work was to design novel hydrophilic and optically-transparent biomimetic hydrogels with microdomains that resemble the active site of carbonic anhydrase and possess adequate cytocompatibility for being used as drug-eluting SCLs. Thus, various sets of hydrogels were synthesized using N,N-dimethylacrylamide (DMA) and N-vinylpyrrolidone (NVP) as backbone monomers that were copolymerized with functional monomers bearing groups similar to those of the amino acids involved in the active binding site of the metallo-enzyme. NVP-co-DMA hydrogels are more hydrophilic than those of pHEMA and, consequently, their oxygen permeability and comfort on the eye are greater [30]. These advantages are, however, a challenge for loading of so

hydrophobic drugs as CAIs and therefore the biomimetic receptors may play a relevant role. Various combinations of functional monomers and zinc ions were tested in order to obtain structures of growing biomimicry and to gain an insight into the contribution of each component to the drug recognition. Namely, Zn^{2+} ions were provided as a soluble salt (zinc nitrate hexahydrate) instead of the polymerizable monomer zinc methacrylate previously tested [29]. 4-vinylimidazole (4VI) was chosen to resemble histidine (Figure 6.1) and to form complexes with Zn^{2+} ions. However, since 4VI is not commercially available and thus it has to be synthesized from its precursor urocanic acid, we also evaluated the possibility of replacement of 4VI by the common 1-vinylimidazole (1VI), which also forms complexes with zinc ions [31]. Influence of small binding changes in the receptor structure could be then evaluated depending on using 4VI or 1VI. Furthermore, N-hydroxyethyl acrylamide (HEAA) was tested as a suitable component for forming hydrogen bonds with the CAIs (Figure 6.1). The application of the molecular imprinting technology was also considered and some hydrogels were synthesized in the presence of acetazolamide. All hydrogels were characterized regarding their ability to uptake acetazolamide and ethoxzolamide and to sustain their release.

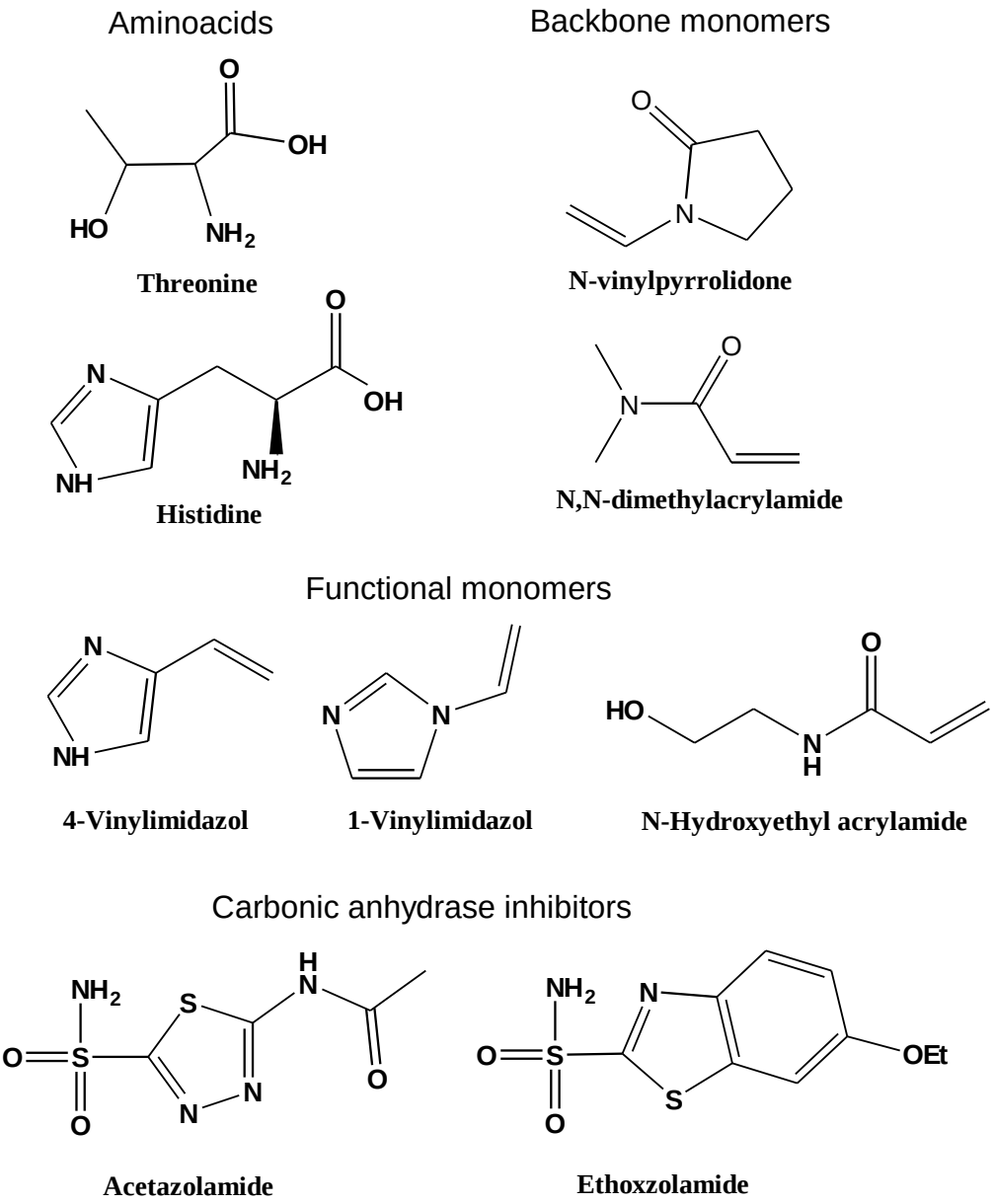


Figure 6.1. Aminoacids that form part of the active site of carbonic anhydrase, monomers used to synthesize the hydrogels, and drugs tested.

6.2. Experimental

6.2.1. Materials

N,N-Dimethylacrylamide (DMA), N-vinylpyrrolidone (NVP), N-hydroxyethyl acrylamide (HEAA), ethylene glycol dimethacrylate (EGDMA), zinc nitrate hexahydrate, 1-vinylimidazole (1VI), urocanic acid, acetazolamide (ACT, 222.25 MW) and ethoxzolamide (ETOX, 258.32 MW) were from Sigma-Aldrich Chemicals (Madrid, Spain). 4(5)-vinylimidazole (4VI) was synthesized as previously described [29, 32]. Azobisisobutyronitrile (AIBN) was from Acros Organic Co. (Geel, Belgium). Nitric acid (65%) was from Panreac (Barcelona, Spain). Other reagents were analytical grade. Purified water was obtained by reverse osmosis (MilliQ®, Millipore Ibérica S.A., Madrid-Spain).

6.2.2. Hydrogels synthesis

NVP/DMA 20/80 molar ratio monomeric mixtures were prepared with the compositions shown in Table 6.1. The hydrogels were designated by the abbreviation of the functional monomers polymerized with NVP and DMA, followed by a number that indicates if zinc ions solely (code 2) or zinc ions plus acetazolamide (code 3) were added to the monomers solution. Hydrogels without functional monomers (H-00) were used as non-bioinspired control. Hydrogels prepared with 1VI or 4VI solely or combined with HEAA are designated as H-1VI, H-4VI, H-1VI-HEAA and H-4VI-HEAA series, respectively. Once the monomers and the other components, if any, were totally dissolved, EGDMA (80 mM, i.e., 0.12 mL for 8 mL of monomer solution) and AIBN (10 mM, i.e., 0.0135 g for 8 mL of monomer solution) were added under stirring. The solutions were

injected into moulds constituted by two glass plates pre-treated with dimethyldichlorosilane and separated by a silicone frame of 0.9 mm thickness [33]. The moulds were kept at 50°C for 12 h and then at 70°C for 24 h. The hydrogels were removed from the moulds and immersed in boiling water for 15 min. Discs (10 mm in diameter) were cut from the wet hydrogels and washed in water for 24 h, in 0.9% NaCl for other 24 h, and again in water for 15 days replacing the medium everyday. The removal of unreacted monomers was monitored by recoding the absorbance of the washing solutions in the 190-800 nm range. Finally, the hydrogels were dried at 70ºC for 24 h.

Table 6.1. Composition of the monomer mixtures used to synthesize the hydrogels.

Hydrogel	NVP (mL)	DMA (mL)	1VI (mL)	4VI (g)	HEAA (g)	Zinc salt (g)	ACT (g)
H-00	1.65	6.35	-	-	-	-	-
H-1VI-1	1.65	6.35	0.22	-	-	-	-
H-1VI-2	1.65	6.35	0.22	-	-	0.23	-
H-1VI-3	1.65	6.35	0.22	-	-	0.23	0.173
H-4VI-1	1.65	6.35	-	0.22	-	-	-
H-4VI-2	1.65	6.35	-	0.22	-	0.23	-
H-4VI-3	1.65	6.35	-	0.22	-	0.23	0.173
H-1VI-HEAA-1	1.65	6.35	0.22	-	0.14	-	-
H-1VI-HEAA-2	1.65	6.35	0.22	-	0.14	0.23	-
H-1VI-HEAA-3	1.65	6.35	0.22	-	0.14	0.23	0.173
H-4VI-HEAA-1	1.65	6.35	-	0.22	0.14	-	-
H-4VI-HEAA-2	1.65	6.35	-	0.22	0.14	0.23	-
H-4VI-HEAA-3	1.65	6.35	-	0.22	0.14	0.23	0.173

6.2.3. Structural and mechanical characterization of hydrogels

6.2.3.1. Content in Zn

Dried discs were weight and placed in plastic tubes with stoppers that were previously cleaned by immersion into 5% (w/v) nitric acid for 24 h and rinsed with ultra-pure water. Then 3 mL of 25% (w/v) nitric acid at 70˚C was added and the tubes were kept at this temperature for 3 hours and vortexed every hour for few minutes [34]. Then the tubes were stored at room temperature until analysis. Zinc quantification was carried out in triplicate using an inductively coupled plasma quadrupole mass spectrometer (ICP-MS, 820-MS Varian, Mulgrave, Australia) equipped with an SPS3 autosampler and a MicroMist nebulizer type. Calibration was carried out using solutions of zinc prepared by step-wise dilution of a 1000 mg/L standard solution (Merck, Darmstadt, Germany). Briefly, the operating conditions were as follows: radiofrequency power 1.40 kW, pump rate 5 rpm, solubilization delay 30 s, and plasma, auxiliary, sheath and nebulizer gas flows 17, 1.65, 0.19, and 1.0 L/min, respectively.

6.2.3.2. FTIR analysis

FTIR-ATR (attenuated total reflection) spectra of urocanic acid, 4VI, and dried hydrogels were recorded over the range 400–4000 cm^{-1}, in a Varian-670-FTIR spectrophotometer equipped with a GladiATR^{TM} (Madison, WI, USA) with diamond crystal.

6.2.3.3. Differential scanning calorimetry (DSC)

DSC experiments were carried out in duplicate using a DSC Q100 (TA Instruments, New Castle, DE, USA) with a refrigerated cooling accessory. Nitrogen was used as purge gas at a flow rate of 50 mL/min. The calorimeter was calibrated for cell constant and temperature using indium standard (melting point 156.61ºC, enthalpy of fusion 28.71 J/g) and for heat capacity using sapphire standards. All experiments were performed using non-hermetic aluminium pans, in which 5-10 mg samples were accurately weighed, and then just covered with the lid. The samples were program-heated from 25ºC to 150ºC, cooled to -10ºC, and then heated again until 300ºC, always at 10ºC/min.

6.2.3.4. Degree of swelling

Dried hydrogel discs were weighed (W_0) and immersed in water at room temperature. At pre-established time intervals, the discs were removed from the aqueous medium, their surface carefully wiped and the weight recorded (W_t). The degree of swelling was estimated as follows:

$$Swelling\ ratio(\%) = \left(\frac{Wt-Wo}{Wt}\right) \cdot 100 \qquad \text{Eq. (6.1)}$$

6.2.3.5. Transmittance

Fully hydrated hydrogels were mounted on the inside face of a quartz cell and the transmittance was recorded in triplicate in the 200-600 nm range (UV-vis spectrophotometer, Agilent 8453, Boeblingen, Germany).

6.3.6. Rheological behaviour

The storage or elastic (G´) and the loss or viscous (G´´) moduli of swollen hydrogels (two replicates) were evaluated at 20°C, applying 0.5% strain and angular frequencies of 0.1-50 rad/s in a Rheolyst AR1000N rheometer (TA Instruments, Surrey, UK) equipped with an AR2500 data analyzer, an environmental test chamber and a solid torsion kit. The sample was fixed between two clamps separated 6.0±0.1 mm.

6.3.7. Cytocompatibility tests

The cytocompatibility of the hydrogels was evaluated using the Balb/3T3 Clone A31 cell line (ATCC, LGC Standards S.L.U., Barcelona, Spain) according to the direct contact test of ISO 10993-5:1999 standard. The discs were immersed in USP phosphate buffer pH 7.4, autoclaved (121°C, 20 min) and placed in 24 well plates containing 200,000 cells per well in 2 mL Dulbecco Modified Eagle's Medium F12HAM (Sigma-Aldrich Chemicals, Madrid, Spain). The medium was supplemented with fetal bovine serum (10%) and gentamicin (0.1 mg/mL). The plates were incubated at 37°C, 5% CO_2 and 90% RH. After 24 hours aliquots (100 µl) of medium were taken in 96 well microplates and mixed with reaction solution (100 µL, Cytotoxicity Detection Kit^{PLUS} LDH, Roche, Barcelona, Spain). The plates were incubated at 20°C for 30 min protected from light. A stop solution (50 µL) was added to the wells and the absorbance measured at 490 nm (BIORAD Model 680 Microplate reader, USA). All experiments were carried out in triplicate. Blank (culture medium), negative (cells in culture medium) and positive (cells in culture medium with lysis factor) controls were prepared too. The cytocompatibility was quantified applying the following equation:

$$\text{Cytocompatibility (\%)} = \frac{Abs_{exp} - Abs_{negative\ control}}{Abs_{positive\ control} - Abs_{negative\ control}} \cdot 100 \qquad \text{Eq. (6.2)}$$

6.2.3.8. Hen's Egg Test-Chorioallantoic membrane (HET-CAM) Test Method

Fertilized broiler chicken eggs (not older than 3 days; Avirojo, Pontevedra, Spain) were incubated with the large end upwards in an Ineltec CCSP0150 climatic chamber (Tona, Barcelona, Spain) at 37±0.3 °C and 60±3% relative humidity. Eggs were rotated (five times per day) for 8 days to prevent the attachment of the embryo to one side of the egg. Then, the ICCVAM-recommended test method protocol was followed [35]. The upper part of the eggshell (air cell) was removed using a Dremel 300 equipped with a rotary saw (Breda, Netherlands). The intact inner membrane was moistened with 0.9% NaCl solution and the eggs were placed in the climatic chamber for a maximum of 30 minutes. The 0.9% NaCl solution was aspired and the inner membrane was removed with a forceps. One disc of hydrogel (previously swollen in 0.9% NaCl solution) was applied on the chorioallantoic membrane over a period of 300 seconds and the irritation potential (hemorrhage, vascular lysis and coagulation) was assessed as a function of time. The experiments were carried out in triplicate. Negative (0.9% NaCl solution) and positive (0.1 N NaOH) controls were tested under the same conditions. Irritation scores (IS) were calculated from the time (in seconds) at which hemorrhage (H), lysis (L) or coagulation (C) started, as follows [35]:

$$IS = \left[\left(\frac{301 - H_{time}}{300}\right)\cdot 5\right] + \left[\left(\frac{301 - L_{time}}{300}\right)\cdot 7\right] + \left[\left(\frac{301 - C_{time}}{300}\right)\cdot 9\right] \qquad \text{Eq. (5.3)}$$

According to the IS values, the hydrogels can be classified as non-irritating (0-0.9), weakly irritating (1-4.9), moderately irritating (5-8.9), severely irritating (9-21) [35].

6.2.3.9. ACT loading and release

Dried hydrogel discs (six replicates, 0.03-0.04 g each) were placed in ACT aqueous solution (0.20 g/L, 5 mL) for 48 h. The amount of ACT loaded was calculated as the difference between the initial amount of drug in the solution and the amount remaining after loading, determined by UV spectrophotometry (Agilent 8453, Boeblingen, Germany) at 264 nm. Drug-loaded discs were rinsed with water, wiped with a piece of paper, and immediately immersed in 0.9% NaCl solution (10 mL) at room temperature. Samples of the medium were periodically taken, the amount of drug released measured spectrophotometrically at 264 nm, and the samples returned again to the corresponding vessel. Since complete release could be achieved in the release medium, ANOVA test of the percentages released at 3 and 6 hours was performed in order to detect statistical differences in ACT release rate (Statgraphics plus 5.1, Warrenton, Virginia USA).

6.2.3.10. ETOX loading and release

Dried hydrogel discs (six replicates) were placed in an ETOX suspension (0.23 g/L, 5 mL) for 48 h. The ETOX-loaded discs were rinsed with water; their surfaces were carefully wiped and the discs immediately immersed in 0.9% NaCl solution (5 mL) at room temperature. The amount of drug released was measured spectrophotometrically at 303 nm in samples periodically taken up and that were reintegrated to the corresponding vessel. At the end of the release experiment (360

h) the discs were removed from the medium, rinsed with ultrapure water, and immersed in vials with 5 mL of ethanol:water (70:30) mixture for 48 hours. The drug extracted from the hydrogels was quantified from the absorbance measured at 303 nm (Agilent 8453, Boeblingen, Germany).

6.3. Results and discussion

6.3.1. Synthesis of biomimetic hydrogels

The structure of the active site of carbonic anhydrase was mimicked by combining monomers bearing chemical groups that resemble those of the amino acids involved in the complexation of zinc ions and in the binding of inhibitor drugs. To achieve more hydrophilic and highly transparent networks suitable as contact lenses and differently from previous attempts [29], zinc ions were incorporated as a soluble salt (without polymerizable moities), which may enable a facile removal of the excess of zinc not forming part of the binding receptors by simple washing in aqueous medium. The backbone monomers chosen to synthesize the hydrogels (NVP and DMA) may render more hydrophilic SCLs than pHEMA. Since natural ligands use amino acid histidine to bind to different ions and molecules, monomers bearing the 4-imidazoyl group are expected to biomimic the binding ability and, in fact, 4VI can efficiently bind metal ions, such as Zn^{2+} or Cu^{2+} [36]. However, 4-imidazoyl monomers are not common components of synthetic polymers because their synthesis is more difficult than that of the most usual 1-imidazoyl ones; therefore in most papers 1VI is used as an accessible alternative [36]. In the present work, for a comparative purpose we tested both 4VI and 1VI as mimickers of histidine. The synthesis of 4VI was carried out by decarboxylation of urocanic acid under vacuum distillation, according to a

procedure well established in literature [29, 32]. The obtaining of 4VI was confirmed by ^{1}H-NMR as previously described [29]. Compared to that of urocanic acid, the FTIR spectrum of 4VI showed the strong absorption peaks of vinyl compounds at 984 (C-H bend), 1286, 1644 and 3085 cm^{-1} (C=C stretch) and of the imidazole group at 832 and 939 cm^{-1}, with a broad absorption band at 2300-3200 cm^{-1} (Figure 6.2). The -C-N-C- and -C-N- groups exhibited peaks at 1252 and 1351 cm^{-1}.

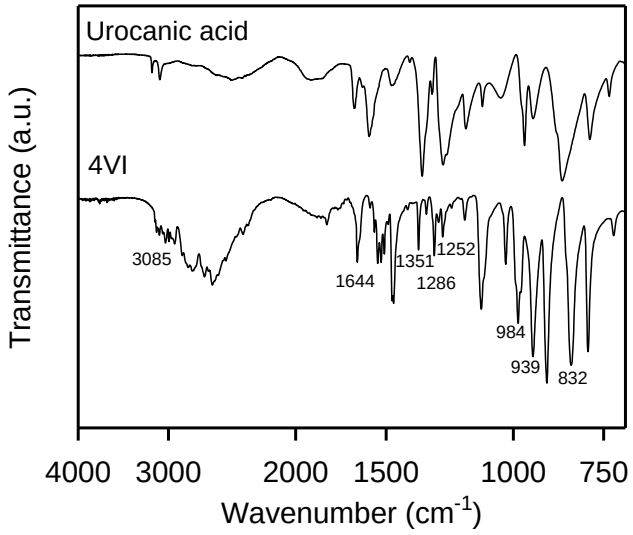


Figure 6.2. FTIR spectra of urocanic acid and 4VI.

Four series of hydrogels were prepared (Table 6.1): i) non-biomimetic control hydrogels, made solely of backbone monomers DMA and NVP (H-00), ii) hydrogels that incorporated the functional monomer 1VI (H-1VI series); iii) hydrogels with the functional monomer 4VI (H-4VI series); and iv) hydrogels with HEAA combined with 1VI or 4VI (H-1VI-HEAA and H-4VI-HEAA series) in a 1:2 molar ratio similar to the 1-2 threonines and 3 histidines involved in the binding of CAIs. Some of these hydrogels were prepared by adding zinc ions to the monomers solution before polymerization (series with code 2). The content in

zinc was 1/3 of the content in imidazole monomers, since this is the molar ratio at the active site of carbonic anhydrase. Some hydrogels were also synthesized in the presence of ACT (imprinted networks, code 3), starting from monomers solution with imidazole monomer:zinc:drug 3:1:1 molar ratio. It is expected that the monomers, the zinc ions and the drug molecules in the solution arrange in the most favourable conformation to render stable complexes and that conformation can be made permanent upon polymerization. All components dissolved easily in the DMA/NVP mixture and no precipitation was observed along the time. Commercial contact lenses do not have homogeneous thickness along the diameter; the centre thickness being smaller (ranging from 0.09 to 0.5 mm depending on the specific application and brand) than the average lens thickness. Since it is not easy to prepare thin hydrogels with a thickness gradient, we prepared slab hydrogels of 0.9 mm thick, which is on average 3 to 4-fold larger than that of a commercially available contact lens. The hydrogels were boiled after synthesis in order to remove unreacted substances and, in the imprinted networks, the drug. Since zinc ions were incorporated as a soluble salt (without polymerizable moities), only those that can effectively form complexes with the imidazole groups could stand the purification step (including the washing in monovalent saline medium) and remain in the hydrogel. Only hydrogels bearing 4VI were able to keep significant amounts of zinc ions (Table 6.2). It should be noted that vinyl monomers are highly activated by conjugation with metal ions, increasing their reactivity [37]. Thus, the presence of Zn^{2+} does not negatively affect the polymerization.

Normalized FTIR-ATR spectra of the hydrogels were quite similar disregarding the presence of the functional monomers (Figure 6.3a and 6.3b). The characteristic absorption band of C=O stretching appeared at 1640 cm^{-1} in hydrogel H-00 and slightly shifted towards lower wavenumbers in the hydrogels

with 1VI and 4VI. Compared to other hydrogels, those combining 4VI and Zn^{2+} ions evidenced as a shoulder at 1680 cm^{-1}, which is characteristic of the complex formation [38]. DSC runs of the H-00 hydrogel showed one glass transition at 139ºC, which is in between that reported for polyNVP (167ºC; [39]) and that of polyDMA (124ºC; [40]). The single glass transition step suggests that both components are perfectly miscible [39]. Hydrogels bearing functional monomers also had one Tg with values in the 122-138ºC range and no clear effects of zinc ions or ACT during polymerization were found.

Table 6.2. Content in zinc ions, amounts of ACT and ETOX loaded by the hydrogels and network/water partition coefficients. Mean values and, in parenthesis, standard deviations (n=6).

Hydrogel	Zn^{2+} content (mg/g)	ACT loaded (mg/g)	ACT $K_{N/W}$	ETOX loaded (mg/g)	ETOX $K_{N/W}$
H-00	0	2.47 (0.02)	6.69 (0.16)	0.92 (0.14)	38 (6.67)
H-1VI-1	0	2.92 (0.05)	8.40 (0.19)	1.05 (0.19)	43 (8.59)
H-1VI-2	n.d.*	3.12 (0.22)	9.50 (0.98)	0.91 (0.13)	37 (5.88)
H-1VI-3	n.d.*	3.14 (0.48)	9.56 (2.26)	0.85 (0.14)	34 (6.51)
H-4VI-1	0	2.90 (0.28)	7.62 (1.23)	0.86 (0.08)	34 (3.56)
H-4VI-2	0.031 (0.001)	3.54 (0.36)	10.51 (1.71)	0.99 (0.15)	40 (6.98)
H-4VI-3	0.036 (0.001)	3.62 (0.26)	10.95 (1.14)	1.05 (0.29)	43 (13.72)
H-1VI-HEAA-1	0	2.82 (0.16)	8.18 (0.80)	0.76 (0.31)	30 (14.45)
H-1VI-HEAA-2	n.d.*	3.19 (0.14)	10.15 (0.67)	0.70 (0.03)	27 (1.41)
H-1VI-HEAA-3	n.d.*	3.34 (0.33)	10.42 (1.47)	0.86 (0.11)	34 (5.31)
H-4VI-HEAA-1	0	3.74 (0.04)	11.63 (0.21)	1.12 (0.32)	54 (6.83)
H-4VI-HEAA-2	0.018 (0.010)	3.81 (0.14)	11.85 (0.14)	1.47 (0.28)	56 (11.14)
H-4VI-HEAA-3	0.010 (0.001)	4.11 (0.36)	13.11 (1.53)	1.34 (0.45)	44 (4.73)

* n.d.: not detectable

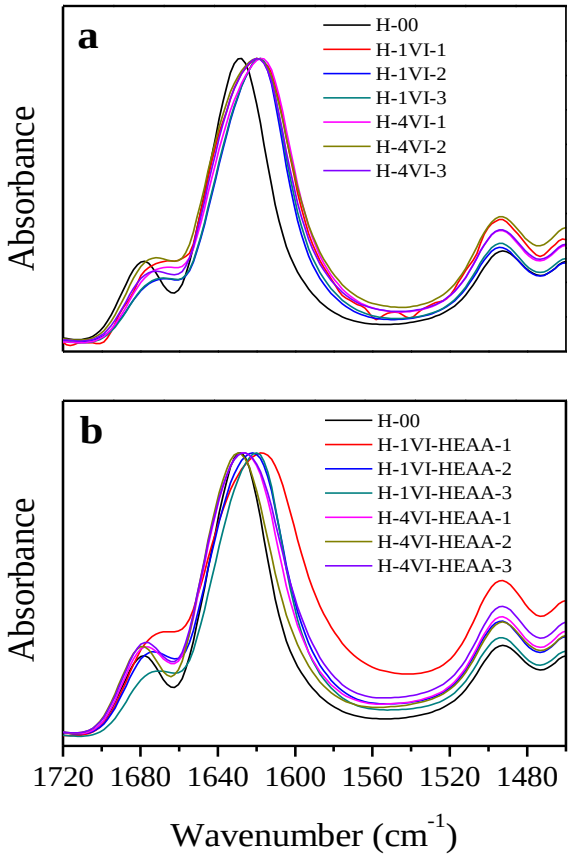


Figure 6.3. FTIR spectra of NVP/DMA hydrogels copolymerized with 1VI or 4VI (a) and with HEAA (b).

6.3.2. Water sorption and light transmission

The hydrogels were highly hydrophilic and rapidly swelled when immersed in water; the sorption profiles being practically superimposable for all formulations (Figure 6.4). It should be noticed that the degree of swelling (which ranged between 80 and 85%) is referred to the total weight of the wet hydrogel (according to Eq. 6.1). This means that each hydrogel can absorb several times

their weigh in water, specifically 4.5-5.5 times. No significant changes were observed when Zn^{+2} or the functional monomers were added. Thus, SCLs prepared with DMA and NVP should be considered of FDA Group 2; i.e., hydrophilic, non-ionic and with high water content [41]. All hydrogels, disregarding the presence of zinc ions, were transparent and, despite the increase in thickness due to swelling (1.10-1.35 mm thick), the transmittance at 600 nm was above 95% (Figure 6.5). Interestingly, the hydrogels that retained zinc ions (namely H-4VI-2, H-4VI-3, H-4VI-HEAA-2 and H-4VI-HEAA-3) showed greater ability to block UV-B radiation (280-315 nm), which may be favourable for protecting eye tissue from the effects of sun light exposure [42].

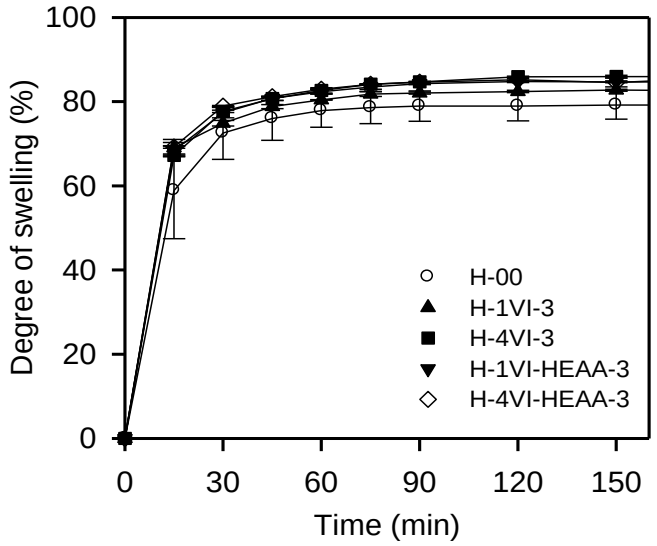


Figure 6.4. Swelling profiles of NVP/DMA hydrogels. Codes as in Table 6.1.

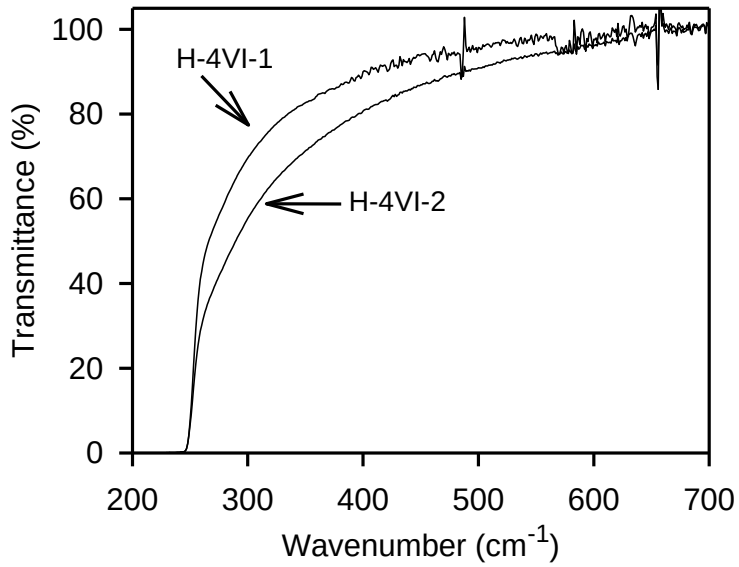


Figure 6.5. Transmittance of water-swollen hydrogels copolymerized with 4VI containing (H-4VI-2) or not (H-4VI-1) Zn^{2+} ions.

6.3.3. Rheological behaviour

Fully swollen hydrogels exhibited *G'* values one order of magnitude larger than *G''* and, in all cases, the moduli were practically independent of the angular frequency (Figure 6.6). These features are characteristic of well-structured polymer networks, which means that a relevantly high number of cross-linking points were formed and that the network can store energy. *G'* and *G''* values of control hydrogel (H-00) were slightly greater than those of the other formulations, but the differences were minor. Hydrogels synthesized in the presence of the template (ACT) showed smaller moduli, although not a clear trend was observed. Hydrogels combining 4VI and HEAA (data not shown) exhibited *G'* and *G''* values similar to those of H-4VI-1. The 10^4-10^5 Pa range of the storage modulus

obtained for all hydrogels is appropriate for SCLs combining comfort and visual performance with the required physical strength [43].

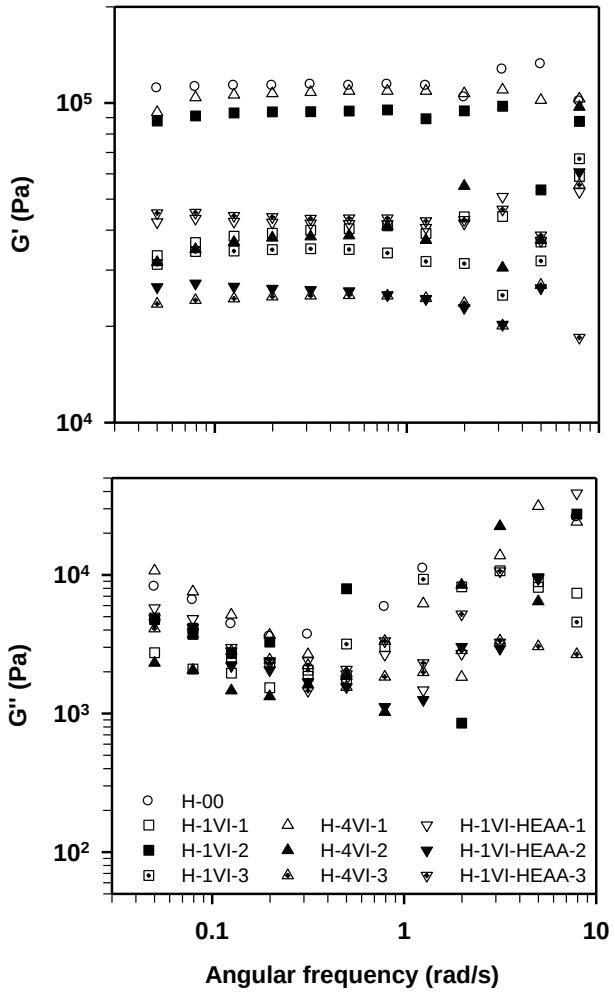


Figure 6.6. Dependence of the storage (*G'*) and the loss (*G''*) moduli of fully swollen hydrogels on the angular frequency. Codes as in Table 6.1.

6.3.4. Compatibility with fibroblasts and chorioallantoic membrane

Polymers of NVP and DMA are widely used for biomedical applications interfacing with living tissues due to their excellent biocompatibility and extremely low cytotoxicity [44]. In order to evaluate the biocompatibility of the hydrogels, *in vitro* experiments were carried out using a fibroblast cell line, according to the direct contact test for the simultaneous evaluation of the effect of the network and the leached substances. No mechanical damage was observed on the cells because of the hydrogel disc, which may be related to the fact that the density of the swollen disc is close to that of the culture medium. All hydrogels rendered cell viability values in the range of 94-100%. Additionally, the potential ocular irritancy was evaluated according to the HET-CAM test according to the NICEATM-ICCVAM protocol [35]. The response of avian chorioallantoic membrane has been also proposed to be a feasible method to predict the response of mammalian tissues to biomaterials, particularly those to be applied in ophthalmology [45,46]. During the time of the test (300 s), the hydrogel discs did not induce haemorrhage, lysis or coagulation. Thus the IS of all hydrogels was 0.0, as it was also that of the negative control (0.9% NaCl). Oppositely, the positive control caused an IS of 19.7 (s.d. 0.1), fulfilling the criteria for an acceptable test [35]. Therefore, the copolymerization with 1VI, 4VI or HEAA and the presence of Zn^{2+} does not have a deleterious effect on the cytocompatibility of the hydrogels and renders non irritating networks.

6.3.5. Loading of CAIs

Formulation of hydrophobic drugs (as most CAIs are) in hydrophilic networks is particularly challenging owing to their opposite polarity [47]. The amount of drug

that can be hosted in the aqueous phase is limited by the drug solubility and its low concentration in the loading solution. To overcome this limitation and to achieve sufficient loading, the drug should show enough affinity for the polymer network itself [40, 47, 48]. An increase in affinity for the inhibitor drugs is expected to be achieved by mimicking carbonic anhydrase receptors in the hydrogel network. It has been previously shown that copolymers of 1-VI and methylmethacrylate forming complexes with metal ions, such as copper or zinc, can modulate the release of antifouling agents able to interact with those metal ions [38].

ACT and ETOX exhibit relevant physicochemical differences, both regarding pKa (7.4 for ACT and 8.0 for ETOX) and lipophilicity (LogP = -0.26 for ACT and 2.01 for ETOX) [49]. At physiological pH, ACT is partially ionized and its solubility is greater (0.72 g/L) than that of ETOX (0.0214 g/L). The loading studies were carried out in ACT solutions (0.20 g/L) and in ETOX suspensions (0.23 g/L), containing a similar number of drug molecules in the system. The limited solubility of ETOX precluded the application of the molecular imprinting technology using this drug as template.

The total amounts of ACT and ETOX loaded by each hydrogel are shown in Table 6.2. The amount of drug that can be hosted in the aqueous phase of the network by a simple equilibrium with the drug solution can be estimated using the following equation [47]:

$$Loading\ (aqueous\ phase) = (Vs/Wp)\cdot C_0 \qquad \text{Eq. (6.4)}$$

where *Vs* is the volume of water sorbed by the hydrogel, *Wp* the dried hydrogel weight, and C_0 the concentration of drug in the loading solution. The amount of ACT loaded in the aqueous phase should range between 1.2 and 1.3 mg/g. In the

case of ETOX, since its solubility is 21.4 mg/L (this concentration remains constant in the suspension), the maximum amount of drug that can be hosted in the aqueous phase of the hydrogels is 0.10-0.12 mg/g. Control DMA-NVP hydrogels without functional monomers (H-00) loaded 2-fold the amount of ACT and 8-fold the amount of ETOX that can be hosted in the aqueous phase. This means that the DMA-NVP copolymer has by itself a certain affinity for the CAIs, particularly for ETOX, which prefers the less polar copolymer environment to the aqueous medium. The lactam and amide groups of DMA-co-NVP polymers have been reported to establish π-σ and π-π interactions with aromatic compounds, leading to charge-transfer complexes or electron donor-acceptor interactions [44].
To estimate the affinity of the drugs for the networks, the partition coefficient, $K_{N/W}$, between the hydrogel and the loading medium was calculated as follows [47]:

$$Loading\ (total) = [(Vs + K_{N/W}\ Vp)/Wp] \cdot C_0 \qquad \text{Eq. (6.5)}$$

where *Vp* is the volume of dried polymer and the other symbols maintain the same meaning as in equation 6.4. The values of $K_{N/W}$ are summarized in Table 6.2. Control hydrogels (H-00) exhibited values of around 6.7 for ACT and 38 for ETOX. Hydrogels bearing functional monomers showed improved ability to host ACT, particularly those containing 4VI and zinc ions. The total amount of ACT loaded by H-4VI-2 and H-4VI-3 was 50% greater than that taken up by H-00, which indicates almost 2-fold increase in affinity for the drug. It should be noticed that the presence of Zn^{2+} contributed to the enhancement in the loading, i.e., to create receptors suitable to host ACT. The synthesis in the presence of the drug only improved the ability of loading ACT in the case of the most biomimetic hydrogels, namely H-4VI-HEAA-3. Copolymerization with HEAA provides an

additional source of hydrogen bonds for interacting with the drug, creating optimally performing networks. Hydrogels possessing 1VI showed less affinity for ACT than those bearing 4VI, disregarding the presence of zinc ions and the application of the molecular imprinting technique, which means that the conformation of the bioreceptor was worse mimicked in the network structure, probably because 1VI does not resemble histidine as well as 4VI does. As a consequence, H-1VI and H-1VI-HEAA hydrogels could not retain zinc ions and did not interact with the drug as well as H-4VI series.

In the case of ETOX, the data variability between replicates was larger (Table 6.2) probably because the loading was carried out in a suspension of the drug. The total amount loaded was indirectly quantified as the sum of the amount released in water for 360 hours plus that extracted with ethanol:water 70:30 (a medium in which ETOX is readily soluble). Removal of small drug particles physically adsorbed to the hydrogels was carefully made before immersion in the release medium. Only hydrogels bearing 4VI and HEAA as functional monomers showed an improvement in drug affinity. The notably high non-specific adsorption of ETOX to the DMA-NVP networks makes the contribution of the specific receptors to be less evident. It should be noted that the content in functional monomers is much lower than the proportion of backbone monomers.

Differences in the specific binding of the two drugs tested can be also related to their different structural features. It is known that the CAIs bind in the active site of the enzyme in deprotonated state, coordinating to zinc while the basic amino acids serve as proton acceptors [50, 51]. Therefore, the lower pKa of ACT should make the binding more favorable. On the other hand, ACT has 7 hydrogen bond acceptors and 3 hydrogen bond donors, while ETOX has just 5 hydrogen bond acceptors and 2 hydrogen bond donors. Thus, each ACT molecule can interact with the receptor through more hydrogen bonds. All these features, together with

a greater drug concentration in solution, may explain the preferential binding of ACT to the carbonic anhydrase-mimicked receptors.

Compared to previous attempts to mimic carbonic anhydrase active site in pHEMA networks [29], the DMA-NVP based hydrogels were able to uptake as much ACT and ETOX (although with lower $K_{N/W}$) despite of being remarkably more hydrophilic. Furthermore, a noteworthy advantage of the hydrogels developed in the present work is that they remained completely transparent to the visible light before and after loading. Although there are not commercially available eye-drops of ACT or ETOX, it has been experimentally shown for these and other CAIs that eye-drops and suspensions at 1% drug decrease the ocular pressure [52]. An eye-drop of 50 µL contains 50 µg of drug. Such amount of drug is similar to that loaded by each hydrogel disc. The difference in performance between the eye-drops and the drug-loaded contact lenses should be the greater ocular bioavailability that can be achieved with the latter, since the absorption from eye-drops is in most cases limited to only 10% of the drug.

6.3.6. Release of CAIs

Drug release profiles were obtained in 0.9% NaCl solution, which has an ionic strength similar to that of the lacrimal fluid. ACT-loaded hydrogels delivered ca. 50% in the first hour and then sustained the release for at least 9 hours (Figure 6.7). Some hydrogels required more than two days and the replacement of the release medium for fresh one in order to achieve 100% release. Thus, the affinity of the drug for the receptors delayed to some extent the release. Although the differences are small, it should be noticed that for hydrogels copolymerized with 4VI (Figure 6.7a) or 4VI and HEAA (Figure 6.7b) the release rate decreased in the following order: series 1 (no zinc, non-imprinted) > series 2 (with zinc, non-

imprinted) > series 3 (with zinc, ACT-imprinted). The non-imprinted hydrogels prepared with 1VI or 4VI but without HEAA released the drug slightly faster than the control hydrogel H-00 and than those bearing HEAA (ANOVA test for % released at 3 hours, $F_{12,38\ d.f.}=3.21$, $\alpha<0.01$; ANOVA test for % released at 6 hours, $F_{12,38\ d.f.}=3.49$, $\alpha<0.01$). Therefore, the receptors with biomimetic structure play a relevant role in the sustaining of ACT release.

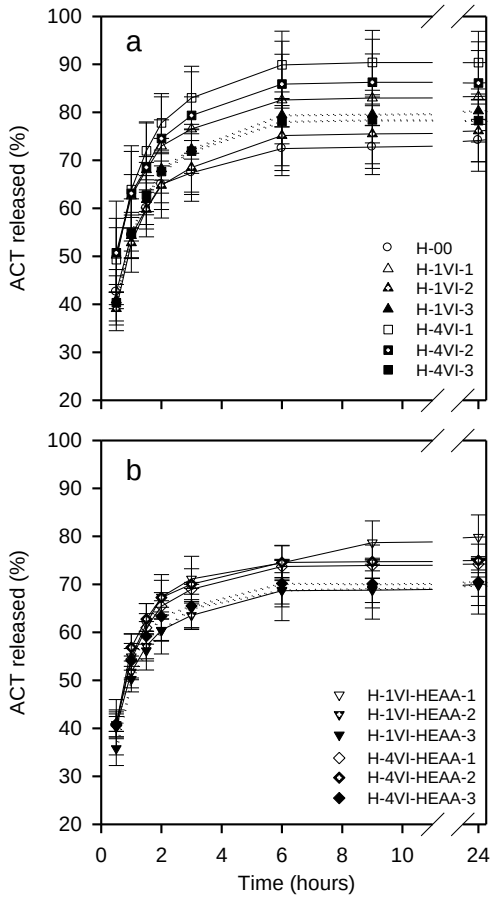


Figure 6.7. ACT release profiles in 0.9% NaCl medium from hydrogels without (a) or with (b) HEAA. Dotted lines correspond to the acetazolamide-imprinted networks.

ETOX was released (Figure 6.8) at slower rate than ACT (Figure 6.7), probably due to the more hydrophobic character of the former. In the first 12 hours, only 50-70% amount loaded was released to the 0.9% NaCl medium. After 7 days, the release completely stopped although 10-20% of ETOX dose still remained in the networks. Compared to ACT, the volume of the release medium for ETOX was smaller (5 vs. 10 ml) to be able to precisely monitor the early period of the release. Nevertheless, that volume was enough to ensure almost *sink* conditions; namely if the whole drug was released, the concentration would be less than 25% drug solubility. The stop in the release confirms the high non-specific affinity of ETOX for the DMA-NVP network. ACT-imprinted hydrogels loaded with ETOX (dotted lines in Figure 6.8a and b) released this drug somehow faster than the non-imprinted networks. Extraction with ethanol:water 70:30 vol/vol enabled the complete removal of the drug from the hydrogels. The mass balance of this drug was satisfied too. As explained above, the thickness of the hydrogels is 3 to 4-fold larger than the average thickness of a commercial contact lens. Therefore, if adapted to the real dimensions of use, the decrease in diffusion time might be of 9-16 times. However, other factors such as the volume and the flow of release medium (smaller under *in vivo* conditions) should be also considered, since they can compensate the decrease in thickness [22].

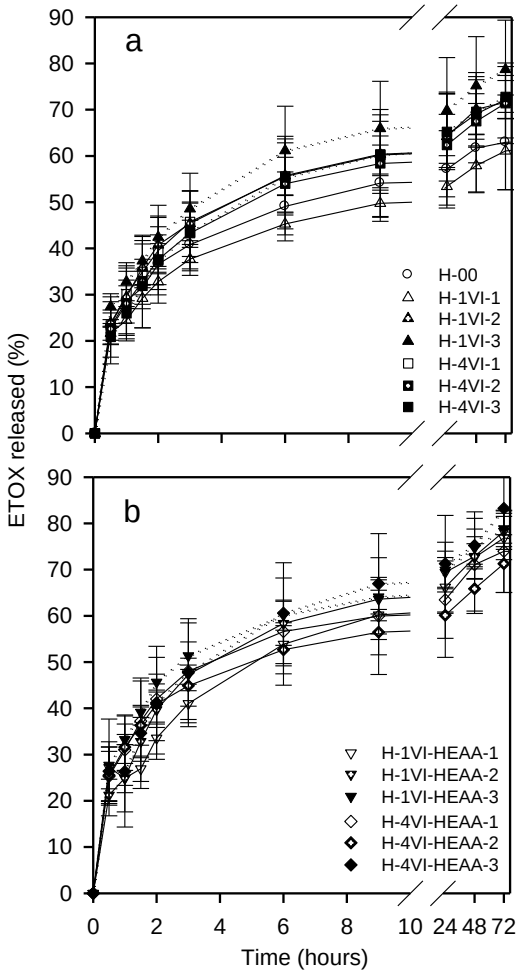


Figure 6.8. ETOX release profiles in 0.9% NaCl medium from hydrogels without (a) or with (b) HEAA. Dotted lines correspond to the acetazolamide-imprinted networks.

6.4. Conclusions

The affinity of NVP-co-DMA (20/80) hydrogels for antiglaucoma drugs of CAIs family was improved by creating artificial receptors in the network that mimic the active site of the metallo-enzyme. Hydrogels that combined 4VI, HEAA and zinc ions resembled better the natural receptor and thus were able to uptake more drug and to control better the release process. Furthermore, the synthesis in the presence of ACT molecules acting as templates (imprinting technology) led to provide the networks with the highest affinity for this drug. The mimicking approach tested in the present work can be useful to develop antiglaucoma drug-eluting hydrophilic SCLs that maintain the cytocompatibility and optical transparency required for being applied on the eye. Additionally, since CAIs have been found useful for the treatment of other diseases (e.g. as anticonvulsants, antiobesity, antipain or antitumor agents [51]), the developed biomimetic networks can be envisioned as suitable components of optimized delivery systems for various therapeutic applications.

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CAPÍTULO 7

Catedral de Santiago de Compostela (Espanha)

CONCLUSÕES E PERSPECTIVAS FUTURAS

7.1. Conclusões gerais

Um sistema ideal de cedência de fármacos para uso ocular, deve assegurar uma concentração efetiva do fármaco no tecido afetado por um período de tempo, com pouca ou nenhuma exposição sistémica. Para, além disto, os sistemas devem ser de fácil utilização, confortáveis e com a possibilidade de ser fabricados à escala industrial. A análise dos resultados obtidos ao longo deste trabalho permitiu-nos obter as seguintes conclusões gerais:

1. O encapsulamento da etoxzolamida em micelas poliméricas e a sua libertação *in vitro* foram alcançados. A utilização do Tetronic® 904 na preparação das micelas foi capaz de aumentar a solubilidade do fármaco até 50 vezes. As micelas mistas, que combinam o Tetronic® 904 com o Tetronic® 1107 ou Tetronic® 1307, apresentou uma maior capacidade de solubilização do que quando o Tetronic® 1107 ou o Tetronic® 1307, foram utilizados individualmente. A incorporação da etoxzolamida nas micelas não modificou a sua organização estrutural, e estes sistemas não demonstraram qualquer citotoxicidade de acordo com ensaios realizados utilizando o método de irritação ocular (*HET-CAM test*). Além disso, a co-micelização das poloxaminas de diferentes hidrofilicidade tornou os sistemas fisicamente mais estáveis, os quais foram capazes de libertar a etoxzolamida de forma mais sustentada e eficiente do que em micelas de componentes individual. Em suma, a co-micelização de poloxaminas, com um número semelhante de óxido de propileno, mas com diferentes unidades de óxido de etileno e proporções de peso variadas, melhoraram a estabilidade do fármaco presente nas micelas e, permitiu ajustar e controlar a carga do fármaco bem como a sua libertação, demonstrando ser uma ferramenta útil em sistemas de cedência ocular.

2. As ciclodextrinas naturais, β-CD e γ-CD foram capazes de solubilizar os inibidores da anidrase carbónica, acetazolamida e etoxzolamida, utilizadas neste trabalho. A sua utilização na funcionalização de redes poliméricas para a formação de hidrogeles oculares do tipo lente de contacto, teve um papel relevante na libertação da acetazolamida e da etoxzolamida e, não alterou as propriedades de transmissibilidade, permeabilidade ao oxigénio e biocompatibilidade.

3. O conhecimento dos receptores fisiológicos, com o qual os fármacos interagem para exercer o efeito terapêutico, utiliza-se de maneira habitual para a optimização química de fármacos ou para a busca de novos candidatos com uma melhor eficácia farmacológica e segurança. Demonstrou-se que ao imitar o sítio ativo dos receptores fisiológicos da anidrase carbónica, podem ser criadas redes de pHEMA com elevada afinidade por fármacos inibidores da anidrase carbónica. As redes biomiméticas formuladas foram capazes de carregar maior quantidade dos fármacos e controlar melhor a sua libertação do que em hidrogéis de pHEMA convencionalmente sintetizados.

4. A afinidade do hidrogel de NVP-co-DMA (20/80) a fármacos inibidores da anidrase carbónica, foi também melhorada através da criação de receptores artificiais nas redes poliméricas que imitam o sítio ativo da metalo-enzima. Os hidrogeles que contêm combinados os monómeros 4-vinil imidazol, o hidroxietil acrilamida e os íões de zinco assemelham-se melhor ao receptor natural e, portanto, foram capazes de absorver e controlar melhor o processo de libertação dos fármacos. Além disso, a presença de moléculas de acetazolamida, atuando como molécula-molde durante a síntese (tecnologia da impressão molecular), apresentou redes poliméricas com maior afinidade por esse fármaco. Os métodos de mimetização testados demonstraram ser úteis no desenvolvimento de lentes de contacto para libertação de fármacos antiglaucomatosos, mantendo a sua

hidrofília, citocompatibilidade e transparência ótica necessária para poder ser aplicado no olho. Além disso, as redes biomiméticas desenvolvidas podem ser vistas como componentes adequados em sistemas de entrega optimizados para diversas aplicações terapêuticas, já que os inibidores da anidrase carbónica podem ser úteis no tratamento de outras doenças.

7.2. Perspectivas futuras

Que perspectivas se podem ponderar em relação ao glaucoma já que esta é uma das maiores causa de cegueira do mundo, tem uma elevada prevalência de casos e promove maiores cuidados e atenção por parte dos serviços da saúde? No decorrer deste trabalho, não restam dúvidas que a forma de tratamento mais empregada no glaucoma é a utilização de gotas oculares contendo fármacos que diminuem a pressão intraocular. Contudo, sabe-se que o tratamento farmacológico do glaucoma apresenta muitos desafios, devido à difícil adesão do paciente ao tratamento e, seu custo econômico que ainda é considerado substancial [1, 2]. O uso de terapias farmacológicas combinadas tem sido uma alternativa bastante explorada. O foco do tratamento tem deixado de ser apenas relacionado com a diminuição da pressão intraocular, mas também através da terapia gênica [3]. Houve alguns avanços interessantes no que se refere à cirurgia e implante de dispositivos intraoculares na última década. Simplificar a terapia e melhorar a segurança com formulações farmacêuticas diferenciadas pode fornecer uma vantagem para os pacientes e resultar em melhor adesão à terapia. Num futuro próximo, novos fármacos podem ser sintetizados e utilizados no tratamento do glaucoma, não prevendo-se que sejam melhores que os utilizados atualmente em relação à eficácia, mas sim em relação à segurança clínica. Os avanços científicos na compreensão da regulação da pressão intraocular têm permitido o desenvolvimento de novas formulações e veículos com melhor biodisponibilidade farmacológica [2]. Atualmente o uso destas ferramentas menos dispendiosas

como adjuvantes no tratamento do glaucoma é um elemento ideal na optimização do tratamento [4, 5]. A criação de sistemas de entrega de fármacos que sejam eficazes e minimamente invasivos é o mais indicado.

As micelas poliméricas são capazes de aumentar a solubilidade de fármacos hidrofóbicos utilizados no glaucoma, e podem dessa forma promover uma maior biodisponibilidade ocular. Uma interessante propriedade das poloxaminas é a sua capacidade de modular a atividade de bombas de efluxo envolvidas na resistência a múltiplos fármacos (MDR), fazendo desses excipientes interessantes candidatos no tratamento de doenças oculares que tem normalmente o seu tratamento reduzido devido a atividades das bombas de efluxo [6].

As lentes de contacto medicamentosas são de grande interesse para o tratamento do glaucoma. A forma com que o fármaco é entregue pode vir a mudar consideravelmente as estratégias do tratamento do glaucoma em longo prazo. Por mais que os colírios não tenham uma tendência a desaparecer, os sistemas como as lentes de contacto seriam muito úteis para conseguir uma maior biodisponibilidade ocular e simplificar a posologia. O estudo e preparação de matrizes poliméricas combinando a técnica da biomimética e de *molecular imprinting* que combina a eficiência das lentes de contacto como corretoras da visão e como dispositivos para a libertação de fármacos oculares é uma estratégia promissora. Os estudos de permeação *in vivo* seriam uma próxima etapa de grande importância para elucidar o potencial destes sistemas.

A combinação de estratégias é uma possível alternativa na terapia ocular. Um exemplo disso pode ser o uso de nanopartículas carregadas com fármacos combinadas com lentes de contatos ou dispositivos intraoculares e que sejam capazes de melhorar a cedência de fármacos. São poucos os sistemas inovadores que têm atingido o circuito comercial, mas é possível afirmar que nos próximos

anos, apesar de um longo caminho ainda ter de ser percorrido em paralelo com a introdução de novos medicamentos, teremos novas formas farmacêuticas que melhoram o desempenho de moléculas de difícil forma de administração, como o caso dos inibidores da anidrase carbónica.

7.1 General conclusions

An ideal drug dosage form for ocular delivery of therapeutics should ensure an effective concentration in the tissue affected for a certain period of time, and with little or none systemic effects. Besides, the system must be easy to use, comfortable, and able to be manufactured on an industrial scale. From the analysis of the results obtained in this work our general conclusions are:

1. The encapsulation of ethoxzolamide in polymeric micelles and their release *in vitro* was achieved. The use of Tetronic® 904 in preparation of the micelles was able to increase the solubility of the drug up to 50 times. The mixed micelles, which combined Tetronic® 904 with Tetronic® 1107 or Tetronic® 1307 showed higher solubilization capacity than Tetronic® 1107 or Tetronic® 1307 individually. The incorporation of ethoxzolamide in the micelles did not change its structural organization, and these systems showed no cytotoxicity according to the method of eye irritation (HET-CAM test) carried out. In addition, co-micellization of poloxamines of different hydrophilicity led to micelles higher physical stability, which were able to release ethoxzolamide in a more sustained and effective way than micelles of individual components. In summary, the co-micellization of poloxamine with a similar number of propylene oxide units, but with different units of ethylene oxide and varying proportions of molecular weight, improved the stability of the drug in the micelles, and also allowed adjustment of the drug loading and release rate. Thus the micellar systems may be a useful for ocular delivery.

2. The natural cyclodextrins, β-CD and γ-CD, were able to solubilize the carbonic anhydrase inhibitors acetazolamide and ethoxzolamide used in this work. Its use

in the functionalization of polymer networks to form hydrogels adequate for eye contact lens played a significant role in the release of acetazolamide and ethoxzolamide and did not alter the properties of transmissibility, oxygen permeability and biocompatibility.

3. Knowledge of the physiological receptor, with which the drugs interact to exert a therapeutic effect, is regularly applied for the optimization of chemical drug candidates that can improve pharmacological efficacy and safety. We have demonstrated that by mimicking the active site of carbonic anhydrase, pHEMA networks can be created with high affinity for carbonic anhydrase inhibitors. The biomimetic networks were able to load a larger quantity of drug and control better the release than conventionally synthesized pHEMA hydrogels.

4. The affinity of hydrogel NVP-co-DMA (20/80) for inhibitors of carbonic anhydrase has been improved by the use of artificial receptors in the polymer networks mimicking the active site of metallo-enzyme. The hydrogels containing monomers combining 4-vinyl imidazole, hydroxyethyl acrylamide and zinc ions are similar to the natural target and thus were able to absorb and control the drug release. Moreover, the presence of acetazolamide molecules acting as template molecule during synthesis process (molecular imprinting technology) led to networks with a greater affinity for this drug. The mimicking methods tested proved to be useful in the development of contact lenses anti-glaucoma drugs, able to elute retaining the hydrophilicity, cytocompatibility and optical transparency necessary to be applied to the eyes. Also, the biomimetic networks developed can be seen as suitable delivery systems for various therapeutic applications, since carbonic anhydrase inhibitors may be useful in the treatment of other diseases.

7.2. Future perspectives

Glaucoma is a major cause of blindness in the world, has a high prevalence of cases and requires increasing attention and costs from health services. There is no doubt that the most used form of treatment of glaucoma involves eye drops containing drugs able to lower the intraocular pressure. Nevertheless, the pharmacological treatment of glaucoma presents is very challenging due to difficult patient compliance, and the economic burden resulted from this disease is still considered significant [1, 2]. Combination of various drugs and even gene therapy have been also explored. [3]. Improvements in surgery and intraocular devices implants have been tested in the last decade. Simplifying therapy and improving safety with patient-friendly formulations may facilitate the adherence to chronic treatment. In the near future, new drugs can be synthesized and used to treat glaucoma, but most probably they will overcome the existing drugs in terms of clinical safety but not efficacy. Scientific advances in understanding the regulation of intraocular pressure are prompting the development of new formulations with improved bioavailability and drug vehicles [2]. Less expensive optimized drug delivery systems may behave as efficient coadjuvant tools in the treatment of glaucoma [4, 5].

The polymeric micelles increase the solubility of hydrophobic drugs used in glaucoma and can thus promote a greater ocular bioavailability of these therapeutics. An interesting additional property of poloxamines is their capability to inhibit the activity of efflux pumps involved in multidrug resistance (MDR), making these excipients interesting components of ocular delivery systems [6].

Drug-loaded contact lenses could change considerably the strategies of glaucoma therapy in the long term. As much as the eye drops do not have a tendency to disappear, platforms such as contact lenses would be very useful to achieve

greater ocular bioavailability and simplify dosage. The study and preparation of polymeric matrices combining biomimetics and molecular imprinting is expected to render efficient contact lenses useful both for vision correction and for ocular drug delivery. *In vivo* permeability studies would be a step of great importance for elucidating the potential as drug eluting systems.

The combination of strategies is a possible alternative therapy in the eye. As an example drug-loaded nanoparticles can be combined with contact lenses or intraocular devices for a better control of drug release. Few innovative systems have reached the trade, but we can say that in the coming years, one can expect that new pharmaceutical forms will improved performance of for molecules of limited ocular bioavailability, as the case of carbonic anhydrase inhibitors, will appear.

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