

**DEVELOPMENT OF TOPICAL DRUG FORMULATIONS FOR THE
TREATMENT OF OCULAR NEOVASCULARIZATION**

by

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ABSTRACT

DEVELOPMENT OF TOPICAL DRUG FORMULATIONS FOR THE TREATMENT OF OCULAR NEOVASCULARIZATION

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Keywords: Angiogenesis, polymersome, self-assembly, amphiphilic polymer
hyaluronan, anti-angiogenic, ocular drug delivery, sphingosine

Ocular neovascularization is a hallmark of several sight-threatening diseases, including diabetic retinopathy and age-related macular degeneration. Currently, available treatments are limited and often associated with side effects. Therefore, we present a novel approach to ocular neovascularization treatment through utilization of polymersomes from self-assembled sphingosine-grafted hyaluronic acid (HA-Sph) amphiphilic polymers. The polymersomes are generated in spherical morphologies, had bilayers, and average sizes between 97,95 nm – 161,9 nm with homogenous size distributions. Our experiments revealed that HA-Sph polymersomes, with concentrations $\geq 150 \mu\text{g/mL}$, significantly inhibit the proliferation of human umbilical vein endothelial cells (HUVECs), while concurrently promoting the proliferation of retinal pigment epithelial (ARPE-19) cells. The efficacy of HA-Sph polymersomes in inhibiting angiogenesis was confirmed through tube formation assay, revealing a substantial reduction in total tube length compared to the control group. Our findings also validated the ocular penetration capability of polymersomes through an *ex vivo* whole porcine eye ocular penetration study, indicating their suitability for topical administration. Potentially, HA-Sph polymersomes can be harnessed to develop intricate drug delivery systems that protect the retina and effectively treat ocular diseases. Moreover, a novel HA-50Sph-Chol blend polymersomes, which harness the synergistic anti-angiogenic potential of HRH peptide, an anti-VEGF peptide, and sphingosine in addressing ocular neovascularization were developed. Optimized blend polymersomes for encapsulating HRH peptide were designed with a three-factor, three-level Box-Behnken design approach. This approach allowed for the systematic optimization of design parameters, resulting in maximized encapsulation efficiency and minimized size, PDI, and zeta potential. Additionally, the sustained release of the HRH peptide from these polymersomes was observed for 7 days with $35,2 \pm 6,5\%$ and $33,4 \pm 4,6\%$ peptide released from HA-50Sph-Chol and HA-100Sph-Chol, respectively, and enzymatic degradation of the blend polymersomes resulted in a twofold increase in peptide release. These optimized blend polymersomes demonstrated enhanced efficacy as ocular drug carriers.

In vitro experiments confirmed the biological activity of the HRH peptide encapsulated HA-50Sph-Chol polymersomes, revealing a synergistic anti-angiogenic effect of the dual drug loading. Nonetheless, more comprehensive investigations are needed to fully elucidate the potential of these peptide-loaded polymersomes as a novel therapeutic avenue for ocular neovascularization-associated diseases, such as wet AMD.



ÖZET

GÖZ NEOVASKULARİZASYONUNUN TEDAVİSİ İÇİN TOPİKAL İLAÇ FORMÜLASYONLARI GELİŞTİRİLMESİ

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Göz neovaskularizasyonu, diyabetik retinopati ve yaşa bağlı maküler dejenerasyon gibi görüşü tehdit eden birkaç hastalığın ayırt edici özelliğidir. Günümüzde sınırlı tedaviler mevcuttur ve genellikle yan etkileri bulunmaktadır. Bu nedenle, sfingosine-greftli hiyalüronik asit (HA-Sph) amfifilik polimerlerden kendiliğinden oluşabilen polimerzomlar kullanarak göz neovaskularizasyonu tedavisine yeni bir yaklaşım sunulmuştur. Polimerzomlar, küresel morfolojilerde oluşabilen, iki katmanlı ve ortalama boyutları 97,95 nm – 161,9 nm arasında homojen boyut dağılımlarına sahiptir. Deneyler, konsantrasyonları ≥ 150 $\mu\text{g/mL}$ olan HA-Sph polimerzomların, insan göbek kordonu endotel hücrelerinin (HUVEC) çoğalmasını önemli derecede engellediğini, aynı zamanda retinal pigment epitelyal (ARPE-19) hücrelerinin çoğalmasını desteklediğini ortaya koymuştur. HA-Sph polimerzomlarının anjiyojenizi inhibe etmedeki etkinliği, tüp oluşum testi ile doğrulanmış ve kontrol grubuna kıyasla toplam tüp uzunluğunda önemli bir azalma ortaya konulmuştur. Bulgularımız ayrıca, *ex vivo* tüm domuz gözü penetrasyonu çalışması yoluyla polimerzomların göz penetrasyon kabiliyetini doğrulayarak topikal uygulama için uygun olduklarını gösterdi. Potansiyel olarak, HA-Sph polimerzomları, retinanın korunmasını ve göz hastalıklarının etkili bir şekilde tedavi edilmesini sağlayan karmaşık ilaç dağıtım sistemleri geliştirmek için kullanılabilir. Sonrasında, göz neovaskularizasyonunu ele alan, anti-VEGF peptit olan HRH peptit ve sfingozinin sinerjik anti-anjiyojenik potansiyelinden yararlanan yeni HA-50Sph-Chol blend polimerzomları geliştirilmiştir. HRH peptitin enkapsülasyonu için optimize edilmiş harman polimerzomlar, üç faktörlü, üç seviyeli Box-Behnken tasarımı yaklaşımı ile tasarlanmıştır. Bu yaklaşım, tasarım parametrelerinin sistematik olarak optimize edilmesine izin vermekte ve maksimum kapsülleme verimliliği ve minimum boyut, PDI ve zeta potansiyeli ile sonuçlanmaktadır. Ek olarak, HRH peptitinin bu polimerzomlardan sürekli salınması 7 gün boyunca gözlemlenmiştir, $35,2 \pm 6,5$ ve $33,4 \pm 4,6$ peptit, sırasıyla HA-50Sph-Chol ve HA-100Sph-Chol'dan salınmış ve blend polimerzomlarının enzimatik degradasyonu, peptit salınımında iki kat artışa neden olmuştur. Bu şekilde optimize edilen blend polimerzomlar, göze ilaç taşıyıcılar olarak gelişmiş etkinlik göstermiştir. *In vitro* deneyler, HRH peptitinin enkapsüllendiği HA-50Sph-Chol

polimerzomlarının artan biyolojik aktivitesini doğruladı ve çift ilaç yüklemesinin sinerjik anti-anjiyojenik etkisini ortaya koydu. Bununla birlikte, bu peptit yüklü polimerzomların yaş AMD gibi gözdeki neovaskularizasyon ile ilişkili hastalıklar için yeni bir terapötik yaklaşım olarak potansiyelini tümüyle açıklayabilmek için daha kapsamlı araştırmalara ihtiyaç vardır.



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To my precious love Ceren...

To my dearest family...

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ARPE-19	Retinal pigment epithelial cells
ATR	attenuated total reflectance
BBD	Box-Behnken design
CAC	Critical aggregation concentration
Chol	Cholesterol
DLS	Dynamic light scattering
DoE	Design of Experiment
DR%	Percentage drug released
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide)
EE%	Encapsulation efficiency
FTIR	Fourier-Transform infrared
HA	Hyaluronan
HUVEC	Human umbilical vein endothelial cells
LC%	Loading capacity
NHS	N-hydroxysulfosuccinimide
NMR	Nuclear magnetic resonance
PDI	Polydispersity index
RSM	Response surface method
SEM	Scanning electron microscope
Sph	Sphingosine
TEM	Transmission electron microscope

INTRODUCTION

Current State of Polymersomes as Ocular Drug Carriers

1. The Eye and Polymersomes

The human eye is anatomically divided into two segments: the anterior segment and the posterior segment (**Figure 1**) [1, 2]. The anterior segment comprises several parts such as the cornea, iris, conjunctiva, ciliary body, lens, lachrymal apparatus, and aqueous humor [3]. The posterior segment encompasses the vitreous humor, retina, choroid, sclera, and optic nerves [1, 3]. Ocular tissues are notably intricate and susceptible to external damage, necessitating a robust defense system composed of various ocular barriers. These ocular barriers, which protect the eye from potential harm, can be categorized into three types: static, metabolic, and dynamic barriers. They encompass vital components such as the blood-aqueous barrier (BAB), blood-retinal barrier (BRB), corneal epithelium and stroma, lymphatic clearance, tear turnover, enzymes, and efflux pumps [3-5]. Despite these protective mechanisms, delivering drugs to the posterior segment of the eye and providing therapeutic drug doses, remains a formidable challenge.

Numerous eye diseases, while not typically life-threatening, significantly diminish patients' quality of life. To address these conditions, drugs have been developed for the prevention and/or treatment of diseases [4, 6]. Unfortunately, the bioavailability of commercial ocular drugs at the disease site is often insufficient, necessitating frequent administrations [7]. Moreover, the route of drug administration varies depending on the disease's location. The preferred methods include topical, intravitreal, subconjunctival, and systemic routes [1, 3]. For diseases affecting the posterior segment, such as age-related macular degeneration, diabetic retinopathy, and choroidal neovascularization,

intravitreal injections are the primary choice in clinical practice [2, 8]. These injections involve the direct introduction of drugs into the vitreous humor, enabling the administration of high drug concentrations directly to the retina [7, 9]. However, due to current dosing practices and the absence of sustained-release systems, patients typically require monthly or bimonthly injections. This frequent injection regimen increases the risk of patient noncompliance and post-administration complications, including retinal toxicity, retinal detachment, and intraocular infections such as endophthalmitis, which can have devastating consequences [2, 7]. In addition to intravitreal injections, subconjunctival or subretinal injections, topical instillations, and systemic routes are also employed. Topical administration offers the advantage of lower post-injection complication risks compared to intravitreal methods. However, less than 5% of the drugs administered via topical instillation reach the retina, primarily due to the ocular barriers [2].

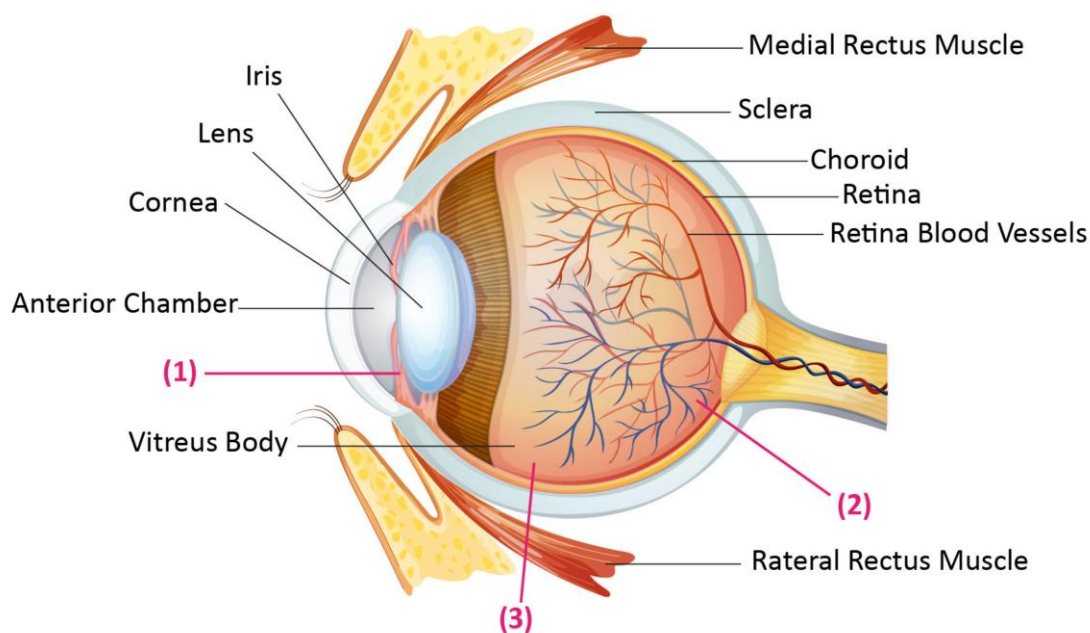


Figure 1. Illustration of the human eye and the routes of ocular drug delivery. (1) Topical route for eye drops, (2) systemic route through retinal capillaries, and (3) intravitreal drug injections (Reprinted under terms of the CC-BY license from [1]. Copyright 2020)

Within the drug delivery systems, vesicular nanocarriers, especially polymersomes have gained significant attention due to their thick membranes, precise control over drug release, and their ability to navigate through biological barriers [3, 7, 10]. Polymersomes, also known as polymeric vesicles, have emerged as a focal point in drug carriers research, capable of encapsulating numerous types of drug molecules including peptides,

antibodies, proteins, enzymes, nucleic acids, and small molecules, regardless of their size, and hydrophobic or hydrophilic nature, as seen in **Figure 2** [11, 12]. To tailor drug release, achieve targeted distribution, enhance bioavailability at specific sites, and protect drugs from degradation within the body, polymersomes offer versatile options for modifications, including adjustments to the size, surface properties, and composition [13].

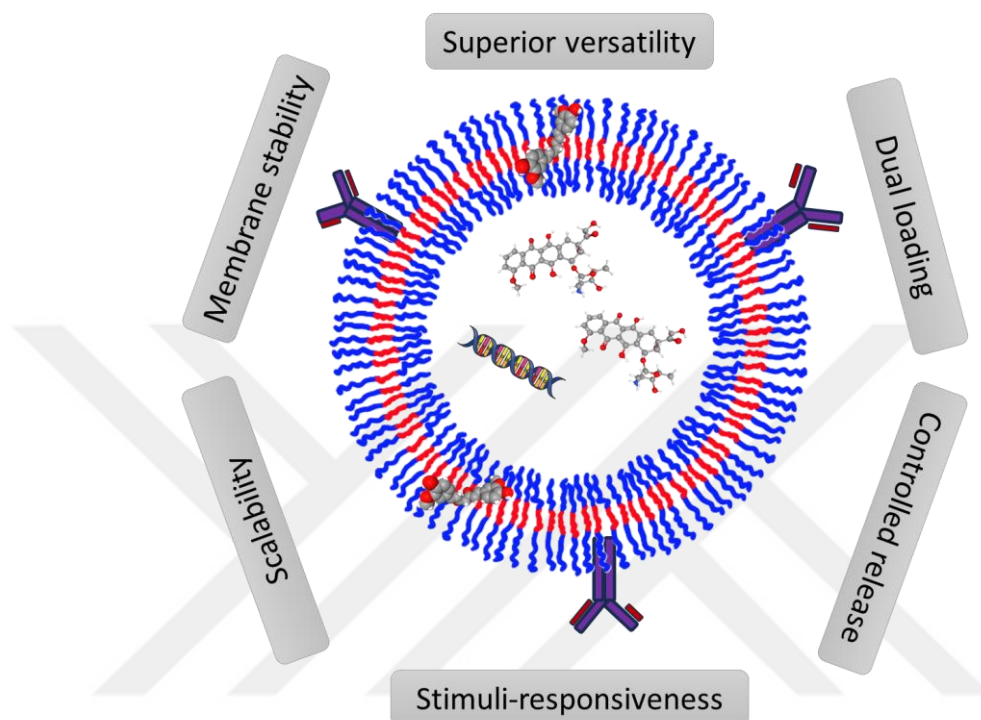


Figure 2. Schematic representation of polymersomes with advantages including superior physical and/or chemical versatility, dual loading of hydrophilic and hydrophobic drugs, high control over release kinetics, stimuli-responsive drug delivery, few scalable production methods, and a high stable membrane structure.

Extensive research and a myriad of drug formulations have been devised to enhance drug bioavailability within the posterior segments of the eye by circumventing ocular barriers. Notably, polymersomes distinguish themselves due to their exceptional attributes, including prolonged and sustained drug release, heightened ocular retention, remarkable permeability, and adaptable preparation processes. In this thesis, we investigated polymersomes as promising carriers for ocular drug delivery.

2. Ocular neovascularization and current treatments

Abnormal ocular neovascularization, triggered by intense angiogenesis, stands as the primary factor behind prevalent ocular conditions like age-related macular degeneration (AMD), diabetic retinopathy (DR), and retinopathy of prematurity (ROP) [14].

Worldwide, an estimated 196 million people grapple with AMD, while approximately 146 million suffer from diabetic eye diseases [15]. While various factors contribute to angiogenesis, including platelet-derived growth factors, fibroblast growth factors, and angiopoietins, the focus in treatment studies predominantly rests on antagonists against vascular endothelial growth factor (VEGF) [16].

Neovascular age-related macular degeneration, characterized by structural changes in the macula (the yellow spot) [17]. In the AMD patients, vision loss occurs during its advanced stages, mainly due to neovascular ("wet") AMD and geographic atrophy ("late dry") [17, 18]. Neovascular AMD involves the invasion of choroidal neovascularization into the neural retina, resulting in fluid, lipid, and blood leakage, finally leading to fibrous scarring. On the other hand, geographic atrophy causes a progressive degeneration of the retinal pigment epithelium, choriocapillaris, and photoreceptors [18]. These advanced stages of AMD are accountable for the most severe visual impairments associated with the disease. While there's no treatment available to reverse the damage, medications exist to inhibit or decelerate the process. Monoclonal antibody-based anti-VEGF therapeutics, administered via intravitreal injections, represent the most widely used among these medications [19].

Diabetic retinopathy emerges as a complication of diabetes mellitus, involving pathways such as the polyol flux pathway, protein kinase C activation, advanced glycation end products (AGEs), oxidative stress, and inflammation [20, 21]. Prolonged high blood glucose levels inflict damage on the retina's blood vessels. The elevated glucose levels lead to vessel occlusion, impeding the supply of vital nourishment to the retina. Consequently, angiogenesis in DR patients is disrupted, failing to meet the nourishment demands of the retina [22]. Additionally, patients with DR exhibit vascular hyperpermeability, endothelial dysfunction, retinal hemorrhage, and macular edema in their retinal blood vessels, all contributing to vision impairment in the disease's advanced stages [21].

Presently, clinic-approved therapies harness diverse anti-inflammatory and anti-VEGF agents like Ranibizumab (Lucentis®), Bevacizumab (Avastin®), pegaptanib sodium (Macugen®), and Aflibercept (Eylea®), with the recent FDA approval of brolucizumab (Beovu®), an effective VEGF inhibitor in wet AMD, diabetic macular edema, and macular edema secondary to retinal vein occlusion (Markham, 2019; Suri et al., 2020).

Research indicates that brolocizumab exhibits a high binding affinity to various isoforms of VEGF-A, effectively hindering its interaction with receptors, specifically receptor 1 and 2 [23]. Intravitreal injections are the primary mode of delivering anti-VEGF agents to the retina in clinical practice, although subconjunctival, subretinal, topical, and systemic routes are also feasible for retinal drug administration [24]. Despite the effectiveness of intravitreal injections, the necessity for monthly or bimonthly injections due to current dosages and the absence of sustained-release systems raises concerns regarding post-administrative complications such as retinal toxicity, retinal detachment, intraocular infections (including potentially severe endophthalmitis) [8]. Innovative treatment approaches should prioritize strategies that reduce injection frequency to mitigate post-administrative risks or explore alternate administration routes like topical formulations [17]. In this context, nanoscale delivery vehicles hold promise in enhancing biodistribution, enabling controlled release, and ensuring targeted delivery of drug molecules to the anterior or posterior segment of the eye [25]. For instance, Formica *et al.* developed lipid nanocapsules (LNC) that feature bevacizumab (BVZ) on the surface and triamcinolone acetonide (TA) in the core [26]. BVZ was covalently bound to the nanocapsule surface using a bifunctional polymer 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(maleimide(polyethylene glycol)-2000) (DSPE-PEG2000-m). Their study highlighted the efficacy of BVZ-TA-LNC in preventing *in vitro* angiogenic tube formation, both in the presence and absence of VEGF, a stimulant for cell migration and angiogenesis. This nanoformulation significantly impeded VEGF-induced angiogenic tube formation, indicating that the BVZ's bioactivity was preserved even after conjugation with LNC [26].

3. What is “Polymersome”?

3.1. Discovery of polymersomes

Polymersomes, or polymeric vesicles, have been known for the last three decades. Comparable to liposomes, polymersomes are known as micro-/nano-sized vesicles, which have hydrophilic core enclosed by hydrophobic membrane that made from self-assembled amphiphilic copolymers [27, 28]. In 1995, two papers were published in *Science*, which could be considered first to mention about polymeric vesicles [28]. One of the study was synthesized amphiphilic polymers by combining polystyrene (PS) and poly(propylene) imine (PPI) dendrimers with varying ratios, and the investigations made for the

conformations of self-assembled aggregates in aqueous phase by transmission electron microscopy (TEM), by van Hest *et al.* [29]. Dendrimers were highly hydrophilic in nature hence, the structures of aggregates in aqueous solutions dependent on the number of amine head groups e.g., PS-dendr-(NH₂)₃₂, PS-dendr-(NH₂)₁₆, PS-dendr-(NH₂)₈. Amphiphilic copolymers were formed spherical micelles, rod-like micelles, and spherical vesicles, respectively [29]. The same year, Zhang and Eisenberg reported their work on diverse morphological assemblies prepared from PS-b-poly(acrylic acid) (PS-b-PAA) diblock copolymers in water/N,N-dimethyl formamide (DMF) mixture [30]. Depending on the length of PAA chains, the morphologies were changed from spherical micelles (26 nm, PS₂₀₀-b-PAA₂₁) to rod-like micelles (23 nm, PS₂₀₀-b-PAA₁₅) and vesicular (100 nm, PS₂₀₀-b-PAA₈) structures [28, 30]. These studies demonstrated the capability of amphiphilic block copolymers to form aggregates with various morphologies depending on the difference in the hydrophobic-to-hydrophilic ratio. Afterwards, Discher *et al.*, demonstrated that the polymersomes made from poly(ethylene oxide) (PEO) (also known as poly(ethylene glycol), PEG) – poly(ethyl ethylene) (PEE) block copolymers, which were approximately an order of magnitude tougher compared to liposomes that made from SOPC (1-stearoyl-2oleoyl phosphatidylcholine) phospholipids [31]. The tougher membranes polymersomes may be beneficial for protecting drugs against dynamic barriers of the eye for topical delivery.

3.2. Mechanisms of the self-assembly of polymersomes

Up to date, numerous polymersomes have been designed and characterized. The principle of the design for determining the morphology of the amphiphilic copolymer assemblies, is known as critical packing parameter ($p = v/a_0l_c$), where v is the volume of the hydrophobic part, a_0 is the area of the head group of hydrophobic part, and l_c is the length of hydrophobic part [32]. By controlling the critical packing parameter of amphiphilic copolymers, spherical micelles ($p < 1/3$), cylindrical micelles or rod-like micelles ($1/3 < p < 1/2$), and spherical vesicles (polymersomes) ($1/2 < p < 1$) have been formed, as seen in **Figure 3** [32-34]. Additionally, in the cases of $p=1$ and $p>1$, morphologies of planar lamellae and inverted structures have been observed [32, 34]. Despite reliability of critical packing parameter, sometimes it may fail to predict self-assembled morphologies of amphiphilic block copolymers [35]. In this regard, Discher *et al.* suggested to use weight fraction of hydrophilic part ($f_{\text{hydrophilic}}$) for determining the self-assembly of amphiphilic

block copolymers [27]. Accordingly, if $f_{\text{hydrophilic}}$ is around $35 \pm 10\%$, these amphiphilic copolymers can form polymersomes whereas, $f_{\text{hydrophilic}} < 50\%$ may formed cylindrical shaped aggregates. In the case of $f_{\text{hydrophilic}} > 45\%$ polymeric micelles could be formed [27, 35]. However, the weight fraction ratio is applicable for linear block copolymers, while, in a more general context, the volume ratio of the hydrophilic part is suitable for a wide range of amphiphilic copolymers, including graft copolymers [36, 37]. Recently, Nishimura *et al.* investigated the effect of structural control factors on self-assembly of graft copolymers [36]. In the study, poly(propylene oxide) (PPO), as hydrophobic group, is grafted onto three different hydrophilic main chains including dextran, poly(2-hydroxypropyl methacrylamide) (PHPMA), and mannan, with comparable degree of substitutions (DS%). The study concluded that morphologies of polymeric self-assemblies have been controlled by the varying persistent length i.e., flexibility of main polymer chain. The persistent length of main chains increased in the order of dextran < PHPMA < mannan. Therefore, polymersomes, rod-like, and spherical micelles were self-assembled from mannan-g-PPO, PHPMA-g-PPO, and dextran-g-PPO, respectively [36].

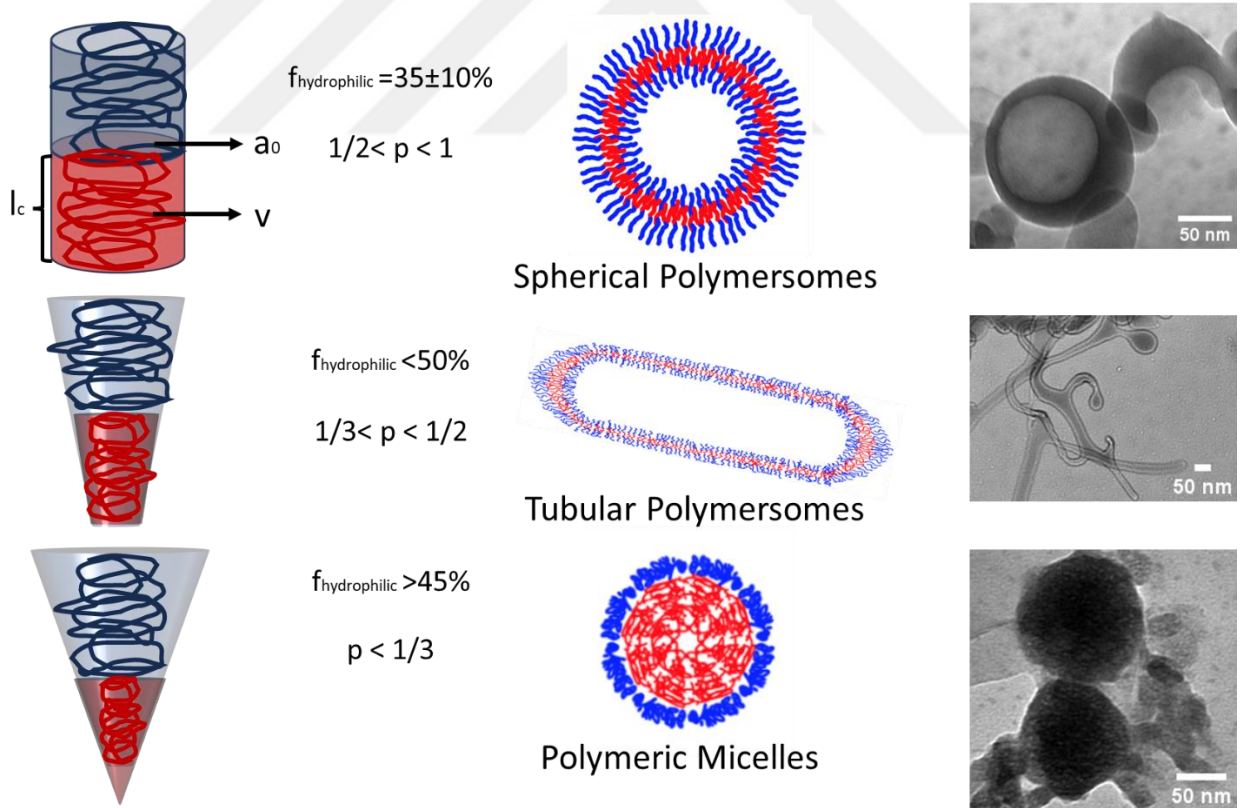


Figure 3. Illustration of critical packing parameter for determination of morphologies of polymeric assemblies and representative cryo-TEM micrographs of amphiphilic hyaluronan-based self-assemblies.

Self-assembly of amphiphilic polymers to polymersomes is driven by hydrophobic interaction i.e., a type of noncovalent interaction, between hydrophobic fractions of block or graft copolymers in aqueous phase [13]. Hydrophilic fractions have an affinity for water molecules due to hydration forces and serve to prevent hydrophobic fractions from encountering to water molecules [13, 35]. So far, studies demonstrated that there are two distinct mechanisms for polymersomes formation, as seen in **Figure 4**. In mechanism I, known as bilayer-to-vesicle model, amphiphilic copolymers initially form spherical micelles in aqueous solutions [38]. As the polymer concentration in the solution increases, copolymers form bilayer sheets. Eventually, the reduced surface energy leads to the closure of the bilayer sheets and the formation of polymersomes [13, 35, 38]. The mechanism I is a widely accepted mechanism, and mathematically and experimentally shown by a few studies [38, 39]. Conversely, in mechanism II, spherical micelles initially form in the aqueous phase and gradually turn into semi-vesicles, because of diffusion of the solvent with reducing bending energies through increasing curvature; finally assemble into polymersomes [13, 35]. Mechanism II was predicted through simulations of external potential dynamics method [13].

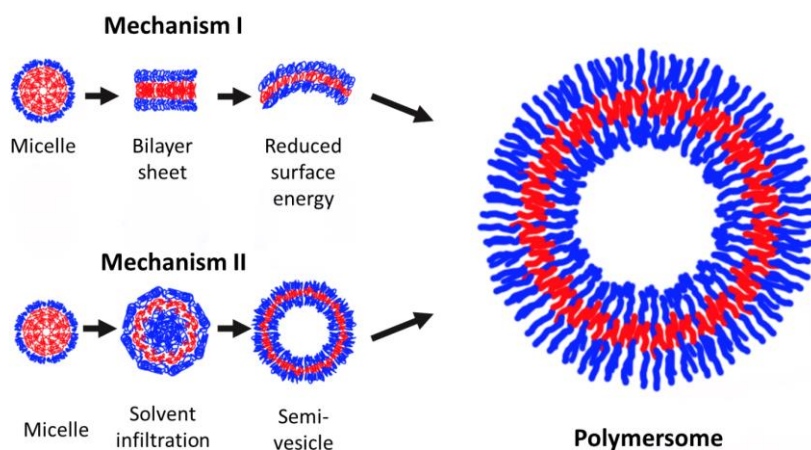


Figure 4. Illustration of proposed self-assembly mechanisms of amphiphilic copolymers into polymersomes. (Red indicates hydrophobic block of copolymer and blue indicates the hydrophilic block of the copolymer).

3.3. Polymersomes as drug carriers

Polymersomes consists of an aqueous core and a thick hydrophobic membrane hence, these drug carriers can encapsulate hydrophilic, lipidic and amphipathic molecules [40]. Additionally, polymersomes can allow dual encapsulation of various types of drugs [41]. Consequently, dual therapeutic formulations and novel theranostic approaches with polymersomes could be achievable for diseases detection and treatment [42]. For instance, Zavvar *et al.* developed theranostic PEG-PCL (PEG-polycaprolactone) polymersomes, which loaded with indium-copper-gadolinium-zinc sulfide hydrophobic quantum dots as contrast agents for magnetic resonance (MR) – fluorescence imaging and loaded with doxorubicin (DOX) for breast cancer treatment [43]. The surface of polymersomes were functionalized with AS1411 DNA aptamer for targeting function [43]. Targeted polymersomes reached to tumor site and was observed at the tumor site via MR images after 24 h post-injection. As a result, tumor growth in BALB/c mice were significantly inhibited by drug loaded polymersomes [43]. D'Angelo *et al.* prepared PEG-PCL (polycaprolactone) polymersomes for dual-encapsulation of cancer chemotherapeutics; doxorubicin (DOX) and vemurafenib (VEM) [44]. They prepared highly stable dual drug loaded polymersomes with encapsulation efficiencies of 55% and 39% for VEM and DOX, respectively [44]. Moreover, dual contrast agents could be loaded for enhanced diagnostic imaging. Previously, gadolinium cations, a paramagnetic probe and Cy5.5, a near-infrared (NIR) fluorophore embedded self-assembled poly(acrylic acid-co-distearin acrylate) polymersomes were demonstrated to significantly enhanced contrasts of NIR and MR images [45].

In recent years, capsosomes and PICsomes (polyion complex vesicles) have been developed as special classes of polymersomes. Capsosomes are nanocarrier systems that contain liposomal sub-compartments together with polymeric membranes [46]. Capsosomes often prepared via layer-by-layer deposition of liposomal and polymeric membranes on a template of sacrificial nanoparticles [46]. Yoo *et al.* designed capsosomes with incorporating hyaluronic acid and chitosan coating on the cationic liposomes as core via layer-by-layer method [47]. Prepared capsosomes had sizes between from 500 nm to 2 μm [47]. PICsomes, on the other hand, are made from polyion complexes. Briefly, PICsomes are formed by mixing oppositely charged copolymers in an aqueous phase [46]. PICsomes showed greater physiological stability, even with the media that contains serum proteins [48]. Recently, robust PICsomes made from PEO-b-poly(amino acids) complexes were prepared by Aydinlioglu *et al.* [12]. The polyion

complexes were formed polymersomes (PICsomes) at near charge equilibrium and successfully encapsulated small interfering ribonucleic acid (siRNA) [12].

Polymersomes offer a multitude of advantages, when compared to other drug delivery systems like liposomes, niosomes, micelles, or nanogels, and they are emerging as a pivotal drug carriers [41]. Polymersomes exhibit superior robustness, wide range of functionalization, and high membrane stability compared to liposomes that made from phospholipids [41, 49]. Size, surface potential, membrane thickness and permeability, biodegradability, and stimuli-responsiveness of polymersomes could be further modified according to requirements of the applications [50, 51]. Moreover, surface of polymersomes could be easily decorated by various targeting molecules including peptides, growth factors, antibodies, and proteins [52]. Additionally, polymersomes can be directly prepared from polysaccharides or proteins, which have intrinsic targeting capabilities, including hyaluronan (HA), collagen, gelatin, and chondroitin sulfate (CS) [52-54]. This targeting strategy, wherein polymersomes inherently acquire, distinguishes them from liposomes and niosomes. In addition to these advantages, there are few concerns for the biomedical applications of polymersomes. Polymersomes prepared from non-biodegradable polymers or degradation products of biodegradable polymers may pose concerns, due to potential dose-dependent toxicity of by-products of polymersomes [28]. Scale-up of polymersome production is another concern for commercialization. Finally, lack of *in vivo* and clinical studies restrict the knowledge on safety profiles of polymersomes [28]. However, polymersomes made from FDA approved polymers might be a possible solution for this issue.

4. Preparation Methods of Polymersomes

4.1. Polymerization Induced Self-Assembly (PISA)

As a recently developed method for polymersomes preparation, polymerization induced self-assembly (PISA) is utilized as a cost-effective and scalable method. The method incorporates polymerization and self-assembly processes in a single step [55, 56]. The PISA method allows large-scale production of a wide variety polymersomes at high concentration [55]. PISA method utilizes solubility variation between monomers and synthesized copolymers in solution [57]. Commonly, reversible addition-fragmentation chain-transfer reaction (RAFT) method is used for polymerization of soluble

homopolymer from first monomer. Then, homopolymer chain extension is continued by second monomer, which is poorly soluble in solvent [55, 57]. As the reaction continues, chain-extended copolymers gradually become insoluble [55, 57]. Finally, amphiphilic copolymer chains self-assemble into various morphologies including micelles, worm-like micelles, and polymersomes to minimize interfacial tension between solvent and the insoluble part of copolymer chain [55]. Morphologies of self-assemblies can be controlled by critical packing parameter, p , which in turn controlled by degree of polymerization (DP) of hydrophobic and hydrophilic blocks of the amphiphilic copolymers [57, 58].

Polymersomes produced by the PISA method do not necessitate post-processing instead, fine-tuning of polymersomes is made by adjusting parameters such as DP of both block, weight fractions of monomers, and solvent type, which makes this method favorable and affordable. Despite the benefits of the PISA method, the use of (meth)acrylates and styrenic compounds in this process often leads to the production of non-biodegradable polymersomes, which limits their potential use in biomedical applications [55]. Recently, Zhang *et al.* demonstrated the effect of reaction temperature on the DP of poly(2-hydroxypropyl methacrylamide) – poly(glycerol monomethacrylate) (PHPMA-PGMA) copolymer and polymersomes morphologies via photo-PISA method [56]. This study suggested that at the higher reaction temperatures, membranes were enlarged both inward and outward during polymerization which increased size and membrane thickness of polymersomes. Moreover, at the lower reaction temperatures, polymersomes were evolved into tubular and donut-like polymersomes [56]. Varlas *et al.* synthesized horse radish peroxidase (HRP) encapsulated and epoxy functionalized polymersomes via RAFT-mediated photo-PISA method using PEG, PHPMA, and poly(glycidyl methacrylate) (PGlyMA) (PEG₁₁₃-b-P(HPMA₃₂₀-co-GlyMA₈₀) [59]. Membrane permeability of polymersomes controlled by epoxide ring opening with diamine crosslinkers or hydrophobic amines. The membrane thickness increased with reduced permeability relative to non-functionalized polymersomes [59]. Among the tested compounds, xylene-based hydrophobic amine (1-naphthylmethylamine) showed highest reduction of relative membrane permeability with >80% reduction [59].

4.2. Rehydration

This method is one of the most commonly used method for liposome preparation and has been adapted for the preparation of polymersomes, as well [28]. The method is considered

solvent-free, however organic solvents such as dimethyl sulfoxide (DMSO) and dichloromethane that could dissolve both hydrophilic and hydrophobic blocks of copolymer, are required [28, 33, 52]. Yet, organic solvent is completely evaporated by rotary evaporator or vacuum oven, and film of copolymers coated inside the container. Appropriate volume of aqueous solvent is added on top of polymers, and vigorously shaken to create dispersion for initiation of self-assembly of polymersomes [28, 33]. Rehydration method produces polymersomes in broad size distribution. Hence, post-processing methods such as sonication, filtering, and extrusion could be applied to narrow the size distribution and decrease the size of polymersomes [57]. In the study by Xu *et al.*, the thin film rehydration method was utilized for preparation of paclitaxel (PTX) encapsulated PEG-b-PCL polymersomes [60]. Copolymers and PTX were dissolved in chloroform as organic solvent, and rotary evaporator was used for evaporation of the solvent to create thin film of copolymers. Drug loaded polymersomes, which have size of 136,5 nm and encapsulation efficiency (EE%) of 39.93%, were self-assembled by rehydration of thin film with milliQ water via sonication [60]. In some studies, polymersomes were prepared via direct hydration method without including prior film preparation step. For instance, Walvekar *et al.* fabricated vancomycin (VCM) loaded oleylamine-grafted hyaluronan (HA-OLA) polymersomes via direct hydration of bulk copolymers with water by probe sonication [61]. Size and EE% of HA-OLA polymersomes were <250 nm and 43.12%, respectively [61].

Recently, Greene *et al.* applied gel-assisted rehydration method, which was used for preparation of lipidic giant unilamellar vesicles (GUVs), for the preparation of polymeric GUVs with PEO-PBD amphiphilic copolymers [62]. Additionally, the size of polymeric GUVs had been controlled by temperature and incorporation of membrane fluidizer molecules e.g., sucrose, during self-assembly. In this regard, increasing the temperature of rehydration significantly increased the size of the polymersomes [62].

4.3. Solvent switch (solvent injection) method

The solvent switch method is another frequently used technique for production of polymersomes, mainly due to its ease of use. The organic solvents such as tetrahydrofuran (THF), dioxane, acetone, DMSO, or dimethylformamide (DMF) that dissolves hydrophilic and hydrophobic groups of amphiphilic copolymers, should be preferred [32, 55, 63, 64]. Then, organic solvent with amphiphilic copolymers slowly injected into

aqueous solution or aqueous solution is injected into organic solvent [55, 63]. The aqueous solution did not dissolve hydrophobic block of amphiphile and the interfacial tension between membrane forming groups of polymer chains with aqueous solution triggers self-assembly. Lastly, the organic solvent must be completely removed from the solution through dialysis or evaporation [32, 57]. The size distribution of prepared polymersomes is usually broad. Thus, extrusion may be included to narrow the size distribution [57]. Moreover, sizes and size distribution of polymersomes are affected by various factors such as choice of organic solvent, and concentration of copolymers in solvent [42]. Wong *et al.*, investigated the self-assembly parameters of ellipsoidal PEG₄₃-b-P(NIPAM₂₁-co-PDMI₉) (poly(ethylene glycol)-b-poly(N-isopropylacrylamide-co-*perylene diester monoimide*) polymersomes, which were fabricated via solvent switch method [65]. This study showed that depending on controlling initial concentration of copolymers in THF and final concentration in aqueous phase, size and shape of aggregates changed from small ellipsoidal micelles to giant ellipsoidal polymersomes and ellipsoidal to tubular polymersomes, respectively [65]. In a study, a modified method of solvent evaporation was suggested for rapid and simple preparation of polymersomes made from various block copolymers [66]. In this method, the size of polymersomes were controlled by modifying the preparation parameters including temperature of the evaporation of THF, aging of polymersomes in mixed solvent, lengths of hydrophilic-hydrophobic blocks of copolymers, and ionic strength of aqueous solution [66]. Polymersomes that consist of same block copolymers with sizes ranging from 200 nm to 2 μ m, was fabricated by this method [66].

4.4. Nanoprecipitation

Nanoprecipitation closely resembles the solvent switch method, with the primary distinction arising from the rapid addition of the aqueous phase to the organic phase (or vice versa). The rapid addition of one phase to the other is attributed to the loss of solvent mixture quality to support copolymer rearrangement [67]. In nanoprecipitation, amphiphilic copolymers are dissolved in water-miscible organic solvent, and aqueous phase dropwise added to organic solvent under constant stirring [41, 68]. The removal of the organic solvent becomes of utmost importance. Thus, additional purification steps, such as dialysis and centrifugation, are incorporated [13, 69]. Any remaining organic solvent could cause toxicity for *in vitro* and *in vivo* studies and obstruct biomedical

applications. Moreover, organic solvents could act as plasticizers that decrease the membrane stability of polymersomes in the solution [28]. There are other challenges associated with this technique including scalability, reproducibility, ease of fabrication, and loading efficiency [70]. Flash nanoprecipitation method has recently been proposed for polymersome preparation to address some of these challenges [28, 70]. In flash nanoprecipitation, water-miscible organic solvent with copolymers mixes with aqueous phase by employing multi-stream mixers, under turbulent conditions then, mixture introduced to aqueous reservoir [28]. However, flash nanoprecipitation failed to address the issue of low-concentration polymersomes, hence, give rise to the requirement of an additional step for concentrating the polymersomes [28]. Alibolandi *et al.* demonstrated inhibitory effects of docetaxel (DTX) loaded folate conjugated dextran-poly lactide-co-glycolide (Dex-PLGA) polymersomes [71]. In the self-assembly of polymersomes nanoprecipitation method was utilized with DMSO as organic solvent and as a result, polymersomes prepared with size, encapsulation efficiency and drug loading of 178.5 nm, 78.85%, and 9.32%, respectively [71]. In a previous study, Sanson *et al.* explained the parameters effecting the size and size distribution of polymersomes, which prepared via nanoprecipitation method [72]. Depending on the parameters of nanoprecipitation including copolymer concentration, type of organic solvent, duration and order (aqueous-to-organic or organic-to-aqueous) of solvent addition, poly(trimethylene carbonate)-b-poly(L-glutamic acid) (PTMC-b-PGA) diblock copolymers assembled into polymersomes with various sizes [72]. In that regard, increased copolymer concentration was shown to increase the size of polymersomes. Additionally, the size of polymersomes increased by evaluating the duration of injection rate [72].

4.5. Microfluidics

A recent method for the preparation of polymersomes involves the use of microfluidic devices for rapid self-assembly [57]. The method offers greater control over the self-assembly of polymersomes [73]. Microfluidics employ the double emulsion system (w/o/w), in which amphiphilic copolymers are dissolved in organic solvent that is injected through 1st channel and aqueous phase (may contain hydrophilic compounds) is injected through 2nd channel, and water-oil-in-water emulsions are formed at the interface in the microchannels [42, 57, 69]. Polymersomes produced by this method have a narrow size distribution [74]. Size of polymersomes can be controlled by flow rates of solvents [42,

57]. Smaller sized polymersomes are produced when a higher flow rate ratio of solvents is used [42]. Furthermore, this method have great potential for commercialization of polymersomes due to large-scale fabrication and elimination of batch-to-batch variations [67]. Recently, Wong *et al.* developed a novel fabrication method for polymersomes called continuous flow method, which utilized micromixers for preparation of polymersomes and downstream processes [75]. As a result of this method, dynamic metastable polymersomes are produced, which allows incorporation of downstream processes that could be used to control polymersome size and shape. The method has advantages including scalability, rapid polymersome production rate (more than 3 g/hr), robustness of method, applicability for variety of copolymers, and plug-and-play type production setup [75]. Martin *et al.*, were fabricated polymersomes from PEG-b-PTMC (PEG-block-poly (trimethylene carbonate)) diblock copolymers via microfluidic approach [76]. Investigations made by study about microfluidic chips with varied flow regimes (micromixer and Herringbone) showed no significant differences of polymersomes fabricated. However, copolymer concentration had an impact on size of polymersomes, which increased from 76 nm to 224 nm by increasing concentration from 0.2 mg/mL to 6 mg/mL [76]. Furthermore, the size of polymersomes were increased from 160 nm to 218 nm by decreasing flow rate from 1000 $\mu\text{L}/\text{min}$ to 100 $\mu\text{L}/\text{min}$ [76].

4.6. Electroformation

The electroformation method is also considered as a solvent-free method, which derived from film rehydration. Thus, this method could also be described as aided-film rehydration method [55, 57]. The method have been used for preparation of giant liposomes, which is utilized for self-assembly of giant polymersomes [32]. Briefly, the film of amphiphilic copolymers is deposited onto the electrodes including gold, indium-titanium oxide (ITO) glass or platinum, by dissolving them in volatile organic solvent and evaporation [33, 57]. Then, alternating current (AC) is applied to electrodes to control rate of water diffusion across copolymer film [33]. Consequently, the assembled polymersomes have narrow size distributions, due to the control over degree of bulging and separation of vesicular structures [57]. Previously, giant unilamellar vesicular (GUV) polymersomes from poly(ethylene oxide)-b-poly(butadiene) (PEO₁₃-PDB₂₂) were fabricated via electroformation technique [77]. The study investigated the effects of the parameters of electroformation, including nanosecond electrical pulses with various

repetition rate and buffer solutions with variable conductivities, on size and shape of polymersomes. Additionally, the polymersomes with concentrations up to 250 $\mu\text{g/mL}$, did not have cytotoxic effect on Chinese hamster ovary (CHO) and B16-F1 murine melanoma cells for 48 h [77].

4.7. Post-processing methods

Most of the polymersome preparation methods yield mixture of polymersomes that consist of polymersomes with various sizes and morphologies. In this regard, post-processing methods could be employed for obtaining preferred fraction of polymersomes. These methods, including extrusion, gel permeation chromatography, centrifugation, sonication, and freeze-thaw cycles, are utilized for adjusting the size of polymersomes [28, 33]. Men *et al.* developed post-processing techniques for producing smaller sized polymersomes (<100 nm) using organic solvent as plasticizer in extrusion and sonication processes [78]. When ratio of organic solvent in solution is 66.7 v/v% (water content of 33.3 v/v%) then, size of PEG-b-PS (PEG-b-polystyrene) polymersomes decreased to 100 nm and below with uniform size distribution with 30 sec. sonication [78]. When water content increased to 50 v/v%, sonication time needed to increase to 5 min., however, only a part of polymersomes' sizes was reduced from >200 nm to ≤ 100 nm. On the other hand, membranes became highly rigid when water content increased to 66.7 v/v% [78].

5. Encapsulation and stimuli-responsive release of polymersomes

5.1. Encapsulation methods

Self-assembled vesicular particles, e.g., liposomes, niosomes and polymersomes, can encapsulate hydrophilic, hydrophobic, and amphipathic compounds [41, 46]. Polymersomes offer the flexibility of loading either individual drug molecules per polymersome or multiple drug molecules per polymersome through various techniques, encompassing active and passive loading, as well as covalent binding of drug molecules [79]. Hydrophilic drugs often encapsulated into aqueous core, while hydrophobic and lipophilic molecules contained in the membranes of polymersomes [80].

Passive and active loading methods are generally preferred for encapsulation of hydrophilic drugs to polymersomes. Passive loading refers to the concurrent encapsulation of drug molecules in the aqueous phase during the preparation of

polymersomes [49]. Predetermined amount of drug is dissolved in aqueous phase, and this solution is incorporated to organic phase during preparation step [42, 81-83]. On the other hand, active (remote) loading methods, which utilize transmembrane gradients such as pH and salt and depend on the drug properties such as ionizability and diffusivity across polymeric membrane i.e. low molecular weight and slightly polar weak acids or bases [28, 49, 79]. The thicker membranes are advantageous for allowing drugs to diffuse rapidly in the active loading [49]. For instance, the principle of transmembrane pH gradient method is ionization of basic drug molecules and penetration to the acidic core of polymersomes [84]. Likewise, in the transmembrane ion gradient method, phosphate or sulfate salts are utilized for loading of drug molecules [85]. Usually, after these drug loading methods, excess drugs are separated from polymersome solutions by dialysis or centrifugation [82, 84, 85]. The integrity of membranes is of utmost importance for active drug loading methods, as leaky membranes may lead to low encapsulation efficiencies and the release of the drug after loading. In this regard, if the membranes are permeable enough for drug loading, the thicker membranes of polymersomes could offer advantages over liposomes or niosomes for active drug loading [28].

Hydrophobic drugs are often encapsulated passively inside the bilayer of the membrane of polymersomes. Hydrophobic drugs are dissolved in organic phase and encapsulated to polymersomes during self-assembly process [86, 87]. Among vesicular drug carriers, polymersomes have thicker membranes (on average 5 – 50 nm) compared to liposomes (3 – 5 nm), which is extremely beneficial for hydrophobic drug encapsulation [33, 49, 73]. Additionally, the thicker membranes of polymersomes lead a larger distance for diffusion, hence resulting in slower drug release rates [33]. A previous study investigated the effects of encapsulating hydrophobic small molecule bromoindirubin-3'-oxime, in core or membrane of polymersomes [88]. As a result, both aqueous core group and membrane group showed encapsulation efficiency around 90%. Furthermore, hydrodynamic sizes and PDI values were also comparable to each other. However, after 14 days, aqueous core encapsulated polymersomes showed significant increase in the size and PDI values compared to membrane loaded polymersomes [88].

Peptides, lipids, antibodies, and small drugs could be conjugated/grafted to amphiphilic copolymer chains for the treatment and targeting purpose of disease site, while avoiding immune system [49, 51, 53, 61, 89]. However, conjugation amount may be limited by

critical packing parameter of amphiphilic copolymers, as that parameter determines the morphology of polymersomes.

5.2. Drug release rates and stimuli-responsive polymersomes

The rates of drug release for both hydrophilic and hydrophobic drugs are considered as the important parameters for drug carrier systems [90]. The drug molecules may rapidly release from polymersomes without reaching the target site. Consequently, rapid clearance and degradation of drug molecules or adverse effects to healthy tissues may significantly reduce the therapeutic efficiency of the formulation [46]. Possible solution for this issue is that the drug molecules could be covalently bound to amphiphilic copolymers with biodegradable linkers to promote drug retention in the polymersomes [49, 91]. Meanwhile, when the rapid release of drug is required, the membrane of polymersomes could be made from biodegradable polymers such as polysaccharides, polypeptides, polyesters, and polycarbonates [90]. Alternatively, drug release could be controlled by responsiveness to internal and external stimuli, regulating properties such as membrane permeability or polymersome disintegration [41, 92, 93]. Depending on chemical and physical factors including pH, temperature, ultrasound, light, enzymes, ionic gradient, and redox potential, drugs can be released at the designated site of action [93, 94]. Recently, pH responsive polymersomes from hyaluronic acid-b-poly (β -amino ester) (HA-PBAE) diblock copolymers that encapsulated with DOX were formulated for the treatment of metastatic breast cancer [95]. HA block of copolymer targeted the CD44 receptors that were found on breast cancer cells. When pH of environment dropped to pH 5.5, hydrophobicity of PBAE blocks changed towards hydrophilicity due to protonation of amines groups [95]. Thus, the DOX release was enhanced by disruption of polymersome membranes [95]. In another study, Ferrero *et al.* fabricated redox-responsive mPEG-PDH-mPEG polymersomes and encapsulated with DOX [96]. Triblock copolymer of mPEG-PDH-mPEG, which contained PEG methyl ether hydrophilic blocks and a hydrophobic block with disulfide bounds. Drug loaded polymersomes had encapsulation efficiency of 98 wt.% and around 34% of DOX was released in 48h [96]. However, at the presence of 50 mM glutathione (GSH), drug release rate was doubled and around 77% drug released, due to severance of disulfide bonds [96].

6. Ocular drug delivery with polymersomes

So far, most of the research studies of ocular drug delivery focused on utilizing nanocarriers such as liposomes and niosomes. Yet, few recent studies were demonstrating the undeniable potential of polymersomes on the ocular drug delivery. Among them, Ridolfo *et al.* investigated the drug release kinetics and vitreous mobility of polymeric micelles and polymersomes with various sizes and morphologies including spherical and tubular polymersomes, as well as spherical and worm-like micelles [97]. Spherical and worm-like micelles were fabricated from copolymers of poly(ethylene glycol) (PEG) as hydrophilic block and various combinations of poly(trimethylene carbonate) (PTMC) and poly(ϵ -caprolactone) (PCL) as hydrophobic blocks. Meanwhile, spherical and tubular polymersomes were assembled from PEG and poly(D,L-lactic acid) (PDLLA) [97]. Apart from morphology and size of self-assemblies, effects of surface functionalization on vitreous mobility were also investigated by blending 10 wt% of amine or carboxylic acid modified PEG incorporated copolymers together with unmodified copolymers [97]. Additionally, dexamethasone (DEX) loaded polymersomes were prepared via solvent switch method and micelles were prepared via direct hydration. Micelles had shown the highest drug loading capacities of 4 wt% for spherical and 10 wt% for worm-like micelles. On the other hand, polymersomes had drug loading capacity of 0.5 wt% [97]. Uptake studies with ARPE-19 (retinal pigment epithelial) cells showed that high aspect ratio particles (tubular polymersome or worm-like micelles) were internalized in higher amount by the cells. Accordingly, self-assembled particles with high aspect ratios demonstrated higher vitreous mobility compared to spherical ones [97]. Vitreous mobility of tubular polymersomes were measured 55% - 75% more than that of spherical polymersomes. Additionally, surface modifications were affected vitreous mobility as well and carboxylic acid modified self-assemblies showed enhanced mobility, which may be due to interactions with anionic polysaccharides e.g., hyaluronic acid and negative surface potential of particles [97]. Later, Junnuthula *et al.* investigated *in vivo* ocular pharmacokinetics of polymersomes and polymeric micelles, which were assembled from poly(ethyleneglycol)-b-poly(ϵ -caprolactone)-g-poly(trimethylenecarbonate) and poly(ethylene glycol)-b-poly(trimethylene carbonate), respectively [98]. Polymersomes with sizes around 100 nm and micelles with sizes around 30 nm were injected intravitreally to rabbit eyes. Half-lives of polymersomes and micelles in vitreous humor

were calculated as 11.4-32.7 days and 4.3-9.5 days with intravitreal clearance values of 1.7-8.7 $\mu\text{L/h}$ and 3.6-5.4 $\mu\text{L/h}$, respectively [98]. Notably, retention of polymersomes at vitreous observed 92 days post-injection, and accumulated 111 days at optic nerve head [98]. This study suggested the potential of polymersomes for long-term drug delivery to the posterior segment of the eye. Additionally, Amir Sadeghi *et al.* compared the *in vivo* ocular pharmacokinetics of polymeric micelles (PEG-b-PTMC) (37.5 nm) with liposomes (48.6 nm) [99]. In rat vitreous, liposomes around 50 nm in size showed retention for 10 days and liposomes around 1000 nm size showed retention for 20 days, larger liposomes showed longer retention due to mesh size of vitreous around 550 nm. The polymeric micelles showed ocular retention in rat eyes for up to 65 days even though the size of micelles notably smaller than micron-sized liposomes. This was explained by the chemical interactions of micelles with the surrounding vitreous.[99].

Sen *et al.* produced self-assembled particles from mPEG-Chol (methoxy-poly(ethylene glycol)-cholesterol) and mPEG-cholane amphiphilic polymers via rehydration method [100]. The self-assembled particles encapsulated with VCP (ATP-driven chaperone valosin-containing protein) inhibitor called ML240. The study demonstrated the retinal neuroprotective effect of ML240 loaded particles for over 21 days *in vivo* [100]. de Oliveira *et al.* suggested that verteporfin-loaded lipo-polymersomes, which prepared from DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine) lipid and Pluronic® F127 (PEO₁₀₆-PPO₇₀-PEO₁₀₆) polymer, as potential vehicles for photodynamic therapy (PDT) to ocular tissues [101]. Lipo-polymersomes prepared via film rehydration method and had size, zeta potential, and EE% of 112.1 nm, 0.97 mV, and 92.8%, respectively. When drug loaded lipo-polymersomes irradiated with blue LED (6.62 J.cm⁻²), the 99% of viability loss were observed on T98G glioblastoma cells [101].

Recently, Banerjee *et al.* designed self-healable and antifouling hydrogels from crosslinking of PEG-DA (polyethylene glycol dialdehyde) and amine functionalized PDMS polymersomes, poly(glycidyl methacrylate)-block-polydimethylsiloxane-block-poly(glycidyl methacrylate (PGMA-b-PDMS-b-PGMA), via Schiff-base reaction [102]. Antimicrobial property was incorporated into the hydrogels by adding curcumin (Cur) loaded zwitterionic PDMS polymersomes of triblock copolymer of poly(N-[3-(dimethylamine)propyl]methacrylamide) and PDMS (PDMAPM-b-PDMS-b-PDMAPM), as well [102]. Both polymersomes showed higher polydispersity values with

similar sizes. Cur-loaded zwitterionic polymersomes had a size of 282 ± 4 nm with a PDI of 0.379 and amine-functionalized polymersomes had a size and PDI values of 255 ± 5 nm and 0.334, respectively. Additionally, curcumin was encapsulated to polymersomes with EE% of 63.4% and loading capacity (LC%) of 4.2% [102]. Hydrogel incorporated with Cur-loaded polymersomes were non-cytotoxic to HaCaT (human keratinocyte cell line) cells and human dermal fibroblasts, even at concentrations up to 50 $\mu\text{g/mL}$ [102]. Moreover, this novel cur-loaded polymersome containing hydrogel showed anti-microbial properties against gram negative (*E. coli*) and gram-positive (*S. aureus*) bacteria with zone inhibitions of 17 ± 1.5 mm and 14 ± 0.5 mm, respectively. They concluded that, self-healable hydrogels have a great potential as contact lens materials [102].

In a recent study, Chen *et al.* prepared ciprofloxacin (CIP) encapsulated self-assembled poly(polyethylene glycol methyl ethermethacrylate)-co-N-benzylacrylamide)-block-poly(2-methylthioethyl methacrylate) (P(PEGMA₁₀-co-PBA₂)-b-PMTEMA₂₅) polymersomes for treatment of ocular infection called bacterial keratitis (BK), as seen in Figure 5 [103]. Hydrophobic block of PMTEMA polymers were utilized for ROS (reactive oxygen species) responsive part of the polymersome that enhance the permeability of membrane upon contacted with H₂O₂, which was observed through enhanced antibacterial effect of polymersomes against *S. aureus* with response to H₂O₂ [103]. Additionally, PBA block had an important role as mucoadhesive agent for enhancing the corneal retention of polymersomes. Solvent switch method was utilized for CIP-loaded polymersomes with size of 247 nm and LC% of 7.04% [103]. Drug-loaded polymersomes topically administered to wild-type C57BL/6j mice eyes twice a day for 7 days in order to see *in vivo* therapeutic efficiency. Results suggested that CIP-loaded polymersomes demonstrated greater antibacterial effects when compared to CIP alone [103].

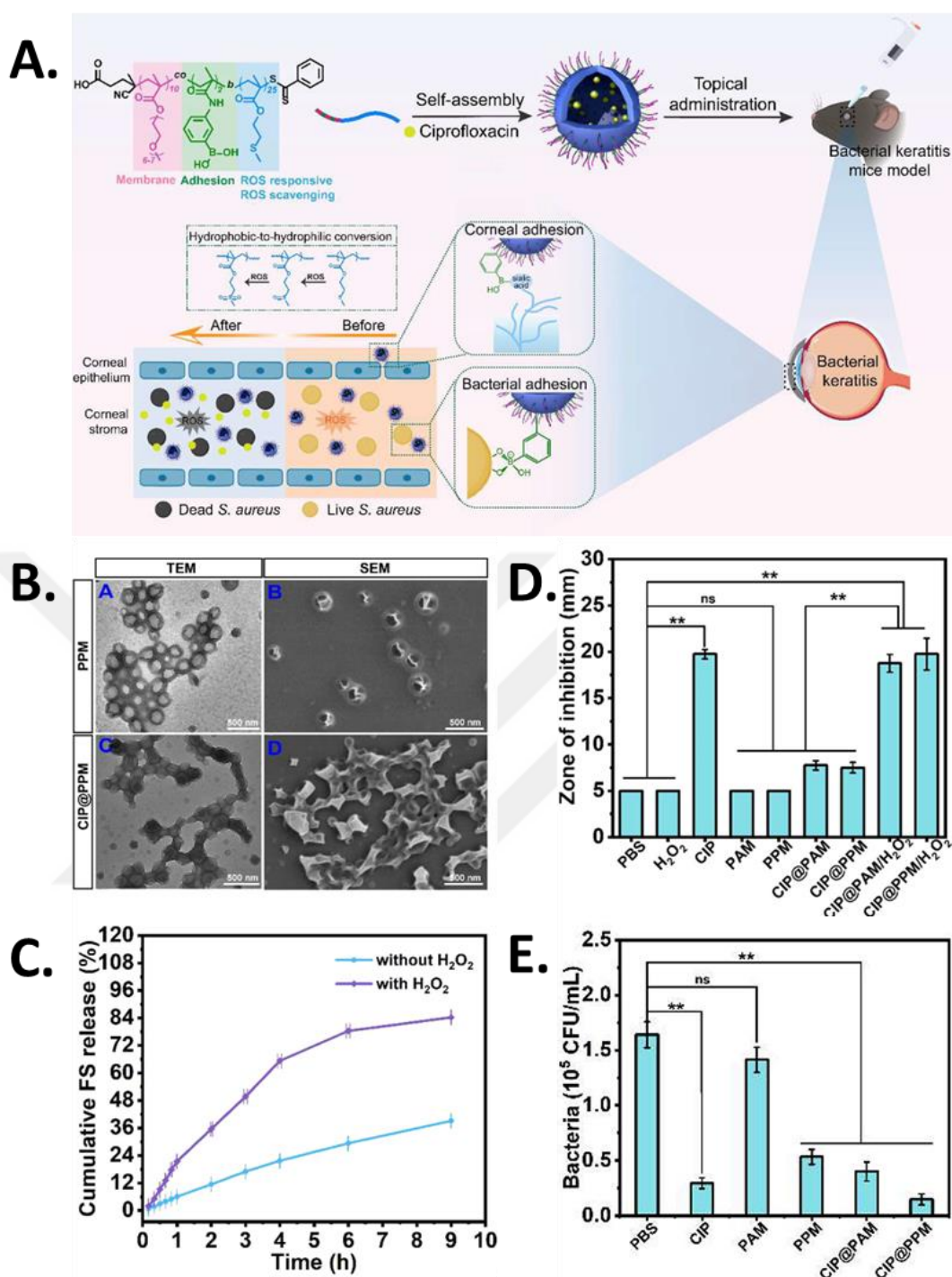


Figure 5. **A.** Representative schematic of the study aimed to develop ciprofloxacin (CIP)-loaded P(PEGMA₁₀-co-PBA₂)-b-PMTEMA₂₅) polymersomes for the treatment of bacterial keratitis. **B.** TEM and SEM micrographs of blank and CIP-loaded polymersomes. **C.** Cumulative release of fluorescein sodium (FS) from polymersomes under stimulation of H₂O₂. **D.** Antibacterial property of CIP loaded polymersomes against *S. aureus* with and without the ROS response, comparing to blank polymersomes and control groups of PBS and H₂O₂. **E.** Quantification of bacterial colonies formed of samples from mouse corneas, taken after the in vivo treatment of polymersomes. (Adapted with the permission from [103]. Copyright 2023, American Chemical Society.)

7. Future perspectives

Drug delivery to the posterior segment of the eye is a great challenge that requires specialized nanocarriers, which have tailored properties including high bioadhesive surfaces and vitreous mobility. The major issue on ocular drug delivery is the existence of anatomical and physiological barriers in the eye that prevent passage of any foreign matter, therefore reducing the success rate of drug delivery. The ocular barriers, including the tear film, nasolacrimal drainage, cornea, conjunctiva, sclera, choroid, and blood-retinal barrier are required to be bypassed by drug molecules and carriers depending on the routes of administration [104, 105]. Despite numerous ocular drug delivery vehicles and strategies, only a few studies have addressed the pharmacokinetics of the drug delivery system [105]. The importance of ocular pharmacokinetics, especially in vitreous humor, arises when the disease occurs at the posterior segment [106]. Therefore, ocular drug carrier formulations should possess the properties including bioadhesion to ocular surfaces, targeted and sustained drug release, ability to pass through barriers, high bioavailability of drug, reduced or no ocular irritation, optimal frequency of treatment, and adequate ocular safety [107, 108].

Various types of nanomaterials were studied for ocular drug delivery such as liposomes, niosomes, micelles, polymersomes, polymeric nanoparticles, and dendrimers [109]. Among them, liposomes and niosomes are most widely studied as ocular drug vehicles [110]. Their physicochemical properties are well-known, and ocular pharmacokinetics are extensively researched [111, 112]. While there are numerous promising studies of polymersomes as drug carriers, most of them have remained in preclinical stage. Commercially available amphiphilic copolymers are also limited, which may reduce the rate of progression of polymersomes research [113]. Nonetheless, there are polymeric micelles (PM) including SP1049C (Pluronic F127-Pluronic L61), Genexol-PM (mPEG-PDLLA) and Nanoxel-PM (mPEG-PDLLA) that are approved by US Food and Drug Administration (FDA) or there are still in the clinical trial phases [113-116]. Meanwhile, there are already several studies, designed and applied these polymers especially PEG-PLA, for preparation of polymersomes [94, 117-119]. FDA approved synthetic and natural polymers are given in Table 1.

Table 1. FDA approved synthetic and natural polymers

	Polymer	Hydrophilicity	Approval for usage in ocular tissues	Ref.
Synthetic polymers	Poly(ethylene glycol) (PEG)	Hydrophilic	Yes	[94, 120]
	Poly(vinyl alcohol) (PVA)	Hydrophilic	Yes	
	Poly(glycolide) (PGA)	Hydrophilic	Yes	
	Poly(glycolide co – lactide) (PLGA)	Hydrophobic	Yes	
	Poly(lactide) (PLA)	Hydrophobic	Yes	
	Poly(methacrylates) (PMMA)	Hydrophilic/hydrophobic	Yes	
	Poly(amidoamine) (PAMAM)	Hydrophilic	No	
	Poly(caprolactone) (PCL)	Hydrophobic	No	
	Poly(acrylic acid) PAA	Hydrophilic/hydrophobic	Yes	
	Poly(orthoester) (POE)	Hydrophobic	No	
Natural polymers	Hyaluronan	Hydrophilic	Yes	[120, 121]
	Chitosan	Hydrophilic	Yes	
	Alginate	Hydrophilic	No	
	Gelatin	Hydrophilic	Yes	
	Collagen	Amphiphilic	Yes	
	Cellulose	Hydrophilic	Yes	
	Carboxymethyl cellulose (CMC)	Hydrophilic	Yes	
	Guar gum	Hydrophilic	Yes	
	Dextran	Hydrophilic	Yes	
	Pullulan	Hydrophilic	Yes	

Aibani *et al.* recently compared polymersomes with liposomes in terms of size, stability, encapsulation efficiency, drug release, cellular uptake and toxicity [122]. Polymersomes

had smaller sizes and greater stability when stored at 25°C for 8 weeks compared to liposomes [122]. Polymersomes and polymeric micelles also similarly compared in a few studies. Recent study investigated the differences between DOX loaded PEG-PDLLA polymeric micelles and polymersomes [123]. Polymersomes showed superior encapsulation efficiency of DOX and higher *in vitro* stability compared to polymeric micelles [123].

So far, numerous approaches have been researched for ocular drug delivery whereas, acquiring sufficient treatment efficiency remains a challenge. Polymersomes are extensively studied for drug delivery to cancerous tissues and results are extremely promising. However, limited studies of polymersomes are found for ocular drug delivery. Polymersomes potentially beneficial in ocular drug delivery, due to the advantages including high concentration of loading for both hydrophilic and hydrophobic drug encapsulation, controllable morphology, size, zeta potential, and membrane thickness and rigidity, superior mucoadhesive properties, higher ocular permeability, ease functionalization for targeted delivery and stimuli-responsive drug release, biodegradable, and tunable ocular pharmacokinetics. Despite these advantages, the toxicity of polymersomes, as well as degradation products, is still an important issue to be considered. Further studies with polymersomes that made from FDA approved polymers may be overcome this drawback.

Synthesis and Evaluation of Hyaluronan-Sphingosine Polymersomes for Treatment of Ocular Neovascularization

1. Introduction

Neovascularization, a vital process for growth and healing, can become problematic when excessive, leading to the formation of abnormal blood vessels. This phenomenon, often observed in conditions such as neovascular age-related macular degeneration, diabetic retinopathy, and corneal neovascularization, originates from dysfunctional blood vessels [14, 124, 125]. Critical cellular pathways are influenced by factors like vascular endothelial growth factor (VEGF) and hypoxia-inducible factor (HIF) thus making modulating VEGF a key strategy in preventing the progression of neovascular ocular diseases [16]. There are clinically utilized anti-VEGF agents such as ranibizumab (Lucentis®), bevacizumab (Avastin®), pegaptanib sodium (Macugen®), aflibercept (Eylea®), and brolucizumab (Beovu®) however, challenges including frequent injections, poor membrane permeability, and high costs are potential limitations for patients [8, 126, 127]. Even though, topical drug administration to the eye holds promise, less than 5% of the applied drug reach the posterior segment of the eye due to ocular barriers [2]. Ocular-targeted drug delivery systems are employed to improve the efficiency of topical eye drop formulations [1].

Advancements in biomaterials and nanotechnology have notably broadened research into polymeric nanoparticles, hydrogels, and ocular implants to enhance effectiveness of drug delivery [126]. One prominently studied polymer is hyaluronan (HA), a glycosaminoglycan consisting of repeating units of D-glucuronic acid and N-acetylglucosamine which present in the vitreous humor of the eye, the umbilical cord, and the extracellular matrix of cartilage and connective tissues [121]. The critical mucoadhesive properties of HA, can potentially limit drug clearance from the cornea, beneficial for topical ocular drug delivery [128, 129]. Additionally, it interacts with

cluster-determined 44 (CD44) receptors on various ocular cells like retinal Müller glia microvilli, corneal epithelium, and endothelial cells [129]. Despite its inherently hydrophilic nature, HA can be chemically modified by combining it with different polymers, bioactive compounds, lipids, and peptides to produce self-assembled nanogels, micelles, and polymersomes [61, 130-134]. Among these, polymersomes stand out due to their ability to encapsulate both hydrophilic and hydrophobic drugs, their ease of functionalization, and their adjustable chemical and mechanical characteristics [51, 135].

Sphingolipids, crucial components of eukaryotic cell lipid membranes, play a pivotal role in regulating multiple cellular functions including proliferation, survival, migration, inflammation, and cell death across various cell types. They are also implicated in numerous ocular conditions including ocular neovascularization [16, 136, 137]. Ceramide (Cer), sphingosine (Sph), sphingosine-1-phosphate (S1P), and ceramide-1-phosphate (C1P) are key signaling molecules among the sphingolipids [138-140]. As early as the mid-1980s, Sph and Cer were identified as significant biosignaling molecules, inducing cell death and suppressing cell proliferation. Conversely, their phosphorylated derivatives have demonstrated the ability to promote cell proliferation, survival, and differentiation [137]. Recent detailed analyses indicate that approximately 11-13 mole% of lipid composition in rat and bovine retinas consist of sphingolipids [136]. However, some of the distinctive roles of individual sphingolipids as biosignal molecules in the ocular tissues are remained unidentified until this day [138]. Previous research has highlighted variations in the roles and metabolism of sphingolipids among different cell types [138]. Previous studies demonstrated that Sph could be utilized to promote cell proliferation and differentiation for cell types such as Swiss 3T3 fibroblasts [141, 142], and neuro2a cells [143]. However, Sph had also inhibited proliferation of HUVECs [144].

2. Aim of the study

In this study, we developed hyaluronan-sphingosine (HA-Sph) polymersomes to deliver Sph specifically to the retina for its anti-angiogenic properties through topical ocular administration. Our primary aim was to improve Sph's solubility and availability by attaching it to hyaluronic acid chains, creating self-assembled polymersomes for targeted delivery to the retina (as depicted in Figure 6-A). The conjugation of Sph to hyaluronic acid polymer chains was accomplished using EDC/NHS chemistry. These resulting amphiphilic HA-Sph polymers then self-assembled into polymersomes through a solvent

evaporation method. Notably, our study is among the first to explore the application of Sph as an anti-angiogenic agent for treating ocular neovascularization.

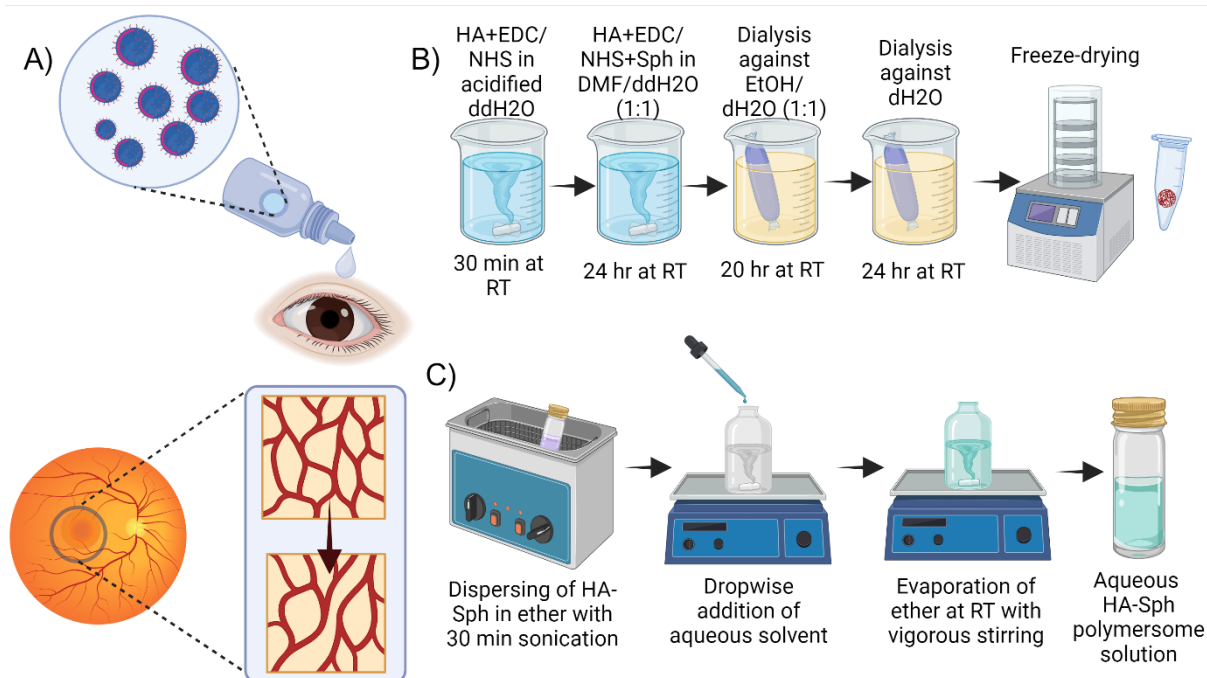


Figure 6. Schematic representation of **A)** the aim of the study, **B)** the preparation steps for HA-Sph amphiphilic polymers, **C)** the preparation of the self-assembled HA-Sph polymersomes

3. Materials and Methods

3.1. Materials

Hyaluronan (<10 kDa) was purchased from Creative PEGWorks (North Carolina, USA). Sphingosine (d18:1) was purchased from Cayman Chemical (Michigan, USA). N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), N,N-dimethylformamide (DMF), ethanol, and pyrene were purchased from Sigma Aldrich (Missouri, USA). Diethyl ether (99,5%) was purchased from Isolab (Eschau, Germany). Cell counting kit-8 (CCK-8) was purchased from Abcam (Cambridge, UK). Matrigel® matrix was purchased from Corning (Arizona, USA). Rhodamine B powder was purchased from Merck KGaA (Darmstadt, Germany). Endopan-3 medium with supplement kit, RPMI 1640, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM F-12), fetal bovine serum (FBS), L-glutamine (200 mM), non-essential amino acids solution (100x) and penicillin-streptomycin were

purchased from PAN-Biotech (Aidenbach, Germany). Fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) were purchased from PeproTech (London, UK).

3.2. Synthesis of amphiphilic HA-Sph polymers

Amphiphilic hyaluronic acid polymers are synthesized by using EDC/NHS coupling reaction, as shown in Figure 1-B [145]. Briefly, 2 mg of 10 kDa hyaluronic acid (HA) was dissolved in acidified milliQ-grade (MQ) water (pH between 3,00-3,50) and 10-times unit mole of HA of EDC and NHS dissolved in acidified MQ water was incorporated into HA solution. The solution was stirred for 30 min at RT for activation of carboxylic groups of HA. Then, the amounts of sphingosine that are calculated as 50%-, 75%-, and 100%-unit mole of HA, were added to solution in DMF (1 mg/mL) with dropwise manner (Table 1). The final ratio of water/DMF of the solution was set to 1/1 (v/v). The coupling reaction was continued with constant stirring for 24 hr. at RT. The solution was quenched with MQ water. Then, solutions were dialyzed against ethanol/water at 1:1 (v/v) ratio for at least 20 hr. with 3500 Da dialysis membranes, and dialysis media changed several times. Dialysis continued for another 24 hr. against dH₂O, with several dialysis media changes. Lastly, amphiphilic HA-Sph solutions were freeze-dried and stored at -20°C. Corresponding to incorporated Sph amounts, the samples were entitled as HA-100Sph, HA-75Sph, and HA-50Sph, respectively (Table 2).

Table 2. Amounts used for the synthesis of amphiphilic HA polymers (HA-Sph) and degree of substitutions

HA-Sph	Unit moles of HA	Moles of Sph	Moles of EDC and NHS	% Sph to the unit moles of HA
HA-50Sph	2,5 µmol	1,25 µmol	25 µmol	50%
HA-75Sph	2,5 µmol	1,875 µmol	25 µmol	75%
HA-100Sph	2,5 µmol	2,5 µmol	25 µmol	100%

3.3. Characterization of amphiphilic HA-Sph polymers

Amphiphilic HA samples were characterized by ^1H NMR and ATR-FTIR to verify conjugation of Sph molecules, and degree of substitutions (DS%) of HA-Sph polymers. FTIR spectrums were measured between $600 - 4000\text{ cm}^{-1}$ by using ATR-FTIR (Shimadzu IRAffinity-1S). Nuclear magnetic resonance (NMR) samples were prepared by dissolving samples in D_2O . Measurements were conducted using Varian VNMRs 600 spectrometer operating at 400 MHz for ^1H resonance frequency and 5 mm NMR sample tubes [146]. Data acquisition and processing were made by using Vnmrj 4.2 software and Bruker TopSpin 4.1.4 software, respectively.

Critical aggregation concentrations (CAC) of amphiphilic polymers were determined by the following the modified method of Saadat *et al.* [131, 147]. A 10 μL solution of pyrene in acetone (10^{-5} M) was introduced into glass vials for each dilution, and the acetone was evaporated at room temperature (RT). The HA-Sph amphiphilic polymer was accurately measured and dissolved in acetone by vortexing and sonicating for 30 minutes in a sonic bath. Once the polymer dispersed, the acetone was removed in a vacuum oven at 37°C for one hour. Then, MQ water was added to the glass vials to yield a final polymer solution of 0.25 mg/mL . The solution was vortexed and sonicated again for 30 minutes in a sonic bath. Diluted solutions of the amphiphilic polymer, along with pyrene, were prepared in glass vials to achieve a final pyrene concentration of $5 \times 10^{-6}\text{ M}$ and a total volume of $100\text{ }\mu\text{L}$. These polymer dilutions containing pyrene were further sonicated for 30 minutes at room temperature. Following sonication, the dilutions were left to incubate at room temperature in the dark for 24 hours. Subsequently, fluorescence spectrums of the pyrene-containing polymer dilutions were recorded for each HA-Sph polymers by using a fluorescence spectrofluorophotometer (RF-6000, Shimadzu) in the wavelength range of 350-500 nm, with an excitation wavelength of 333 nm.

3.4.Preparation of HA-Sph polymersomes via solvent evaporation method

Self-assembled polymersomes from HA-Sph amphiphilic polymers were prepared via solvent evaporation method [96]. Diethyl ether was used as organic phase. HA-Sph polymers were fully dispersed in organic phase by vortexing and sonication for 30 min in a sonic bath. MQ water, as aqueous phase, was introduced dropwise to the polymer dispersions during vigorous stirring and the final ether/water ratio was set to 9/1 (v/v). Vigorous stirring continued until diethyl ether was evaporated completely. Finally,

polymersomes were filtered by 0,45 μm cellulose acetate (CA) filter, freeze-dried, and stored at -20°C until use.

The same method was used for preparation of rhodamine B encapsulated HA-50Sph polymersomes. Briefly, rhodamine B dissolved in MQ water (100 μM) and added dropwise into the HA-50Sph dispersion, as aqueous phase. Ether was completely evaporated, samples were filtered and freeze-dried.

3.5. Characterization of HA-Sph polymersomes

Before the characterization of HA-Sph polymersomes, solutions were filtered through 0,45 μm CA filter to eliminate any dust or aggregates. Hydrodynamic sizes and zeta potentials of polymersomes were investigated by DLS (dynamic light scattering) (Zetasizer Pro, Malvern Panalytical). Measurements were taken in plastic disposable DLS (ZEN0040) and zeta cuvettes (DTS1070) at 25°C in triplicates.

Morphologies of polymersomes were investigated by field emission scanning electron microscopy (FE-SEM). For FE-SEM imaging, 4 μL samples were drop-casted onto glass slides and dried at RT. Then, samples were sputter coated (Sputter Coater 108, Cressington) with Au/Pd for 120 s at 40 mA current. SEM micrographs were taken by using a mixed signal of secondary electron and in-lens detectors (mixing ratio of 0.6) of FE-SEM (Supra 35VP FE-SEM, Zeiss) at 2-3 kV.

Morphologies of polymersomes were further observed under transmission electron microscope (TEM). Polymersome solutions were drop-casted onto 300 mesh carbon-coated copper TEM grids and dried at RT, overnight. TEM (JEM-ARM200CFEG UHR-TEM, JEOL) micrographs of samples were taken at 200 kV accelerating voltage.

3.6. Temperature stability and long-term storage of polymersomes

The temperature stability of polymersomes between 10°C - 90°C was investigated by using pyrene as the fluorescence probe. Polymersomes loaded with 5×10^{-6} M final pyrene were prepared with 100 $\mu\text{g}/\text{mL}$ by solvent evaporation method. Pyrene loaded polymersomes excited at $\lambda_{\text{ex.}} = 333$ nm and fluorescence intensities of 374 nm, 384 nm, and 470 nm (for excimer emission) were collected by a fluorescence spectrophotometer (Cary Eclipse, Agilent), equipped with temperature controller. Fluorescence intensities were collected in the range of 10°C - 90°C - 10°C with rate of $5^{\circ}\text{C}/\text{min}$. Stability of polymersomes was

further observed through DLS (Zetasizer Pro, Malvern Panalytical) in the range of 10°C-70°C-10°C. Sizes were taken at the rate of 10°C/steps, in triplicates.

The storage stability of polymersomes in the long-term was determined by using pyrene. 100 µg/mL polymersomes loaded with 5×10^{-6} M pyrene were stored at temperatures of 4°C, 25°C, and 37°C. Every week, DLS, zeta potential, and fluorescence intensities of the polymersomes were measured for 2 months.

3.7. Cell culture

Human Umbilical Vascular Endothelial cells (HUVECs) which were cultured for cell proliferation assays, were a kind gift from Pelin Saglam Metiner from the Bioengineering Department of Ege University (Izmir, Türkiye). HUVECs were cultured in RPMI media supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 1% non-essential amino acids. HUVECs (CRL-1730, ATCC) used for tube formation assay were cultured in Endopan-3 media containing growth supplement kit, 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. Spontaneously Arising Retinal Pigment Epithelium (ARPE-19) (CRL-2302, ATCC) cells were cultured in DMEM/F12 complete media containing 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin. All cells were incubated at 37°C, 5% CO₂, with media changes every 3-4 days until cells reached 70-80% confluency.

Human corneal epithelial cells (HCEpiC) were a kind gift of Afsun Sahin from Koc University Hospital (Istanbul, Türkiye). HCEpiC was cultured in DMEM/F12 media containing 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 10 ng/mL EGF (epidermal growth factor) (Peprotech). Human corneal endothelial cells (HCEC) (HCEC-B4G12, DSMZ) were cultured in human endothelial-SFM media (Gibco) supplemented with 10 ng/mL FGF-2 (Peprotech) and 1% penicillin/streptomycin. Culture plates were coated with FNC coating mix (Athena Enzyme Systems) prior to cell seeding. All cells were incubated at 37°C, 5% CO₂ and media changes were made every 3-4 days until cells reached 70-80% confluency.

3.8. *In vitro* bioactivity of HA-Sph polymersomes on HUVECs and ARPE-19 cells

The cytotoxicity of HA-50Sph polymersome and its individual components (HA, Sph, amphiphilic polymer) on ARPE-19 cells and HUVECs were investigated by cell counting

kit – 8 (CCK-8) assays. Briefly, ARPE-19 and HUVEC cells were seeded to 96-well plates with 10000 cells/well and 5000 cells/well seeding density, respectively. ARPE-19 cells were incubated overnight and HUVECs were incubated for 6-8 hr, before introducing the HA-Sph and its components. HA-50Sph polymersome, HA-50Sph amphiphilic polymer, and HA (MW of <10 kDa; 150 µg/mL) and sphingosine (55 µM) were prepared with fresh media and put onto the cells. Cells were incubated for 4 days at 37°C, 5% CO₂. Finally, the cell media was replaced with 100 µL fresh media containing CCK-8. Absorbances ($\lambda_{OD} = 460$ nm) were collected with using microplate reader (Infinite M200 Pro, Tecan). Cell viability was calculated by Eq. 1. Experiment was done in triplicate.

$$Cell\ Viability\ (\%) = \frac{OD_{sample} - OD_{Blank}}{OD_{Control} - OD_{Blank}} \times 100 \quad (Eq. 1)$$

The effects of HA-Sph polymersomes (HA-50Sph, HA-75Sph, and HA-100Sph) with various concentrations on cell proliferation of HUVECs and ARPE-19 cells was observed via CCK-8 assay. Cells were treated with polymersomes in fresh media at 50 µg/mL – 250 µg/mL concentrations for 4 days. Cell viability was calculated by Eq.1. Experiment was done in triplicate.

Similarly, CCK-8 assay was utilized for investigation of time-wise effect of self-assembled HA-50Sph polymersome (150 µg/mL), HA-50Sph amphiphilic polymer (150 µg/mL), and sphingosine (final concentration of 55 µM) on cell proliferation. Cells were incubated for 1 day, 2 days, 3 days, and 4 days and cell viability was measured at each day. Cell viability was calculated by Eq. 1. Experiment was done in triplicate.

HCEC and HCEpiC cells were seeded with 10000 cells/well seeding density and cells were treated with polymersomes (HA-50Sph, HA-75Sph, and HA-100Sph) in the fresh media, after overnight incubation. Cells were incubated for 4 days at 37°C, 5% CO₂. Then, media with polymersomes was disposed, and 100 µL fresh media with CCK-8 was added to wells according to manufacturer's manual. After 2-4 hr. incubation, absorbances ($\lambda_{OD} = 460$ nm). were measured by microplate reader (Infinite M200 Pro, Tecan). Cell viability was calculated by Eq. 1. Experiment was done in duplicates.

3.9. Tube formation assay

For tube formation assay, each well of 48-well plate was coated with 100 μ L Matrigel on ice. Matrigel was incubated at 37°C, 5% CO₂ incubator for 30 min then, HUVECs (CRL-1730, ATCC) were seeded onto cured Matrigel® with 2,5x10⁵ cells/well concentration. HA-Sph polymersome was introduced to cells with concentration of 150 μ g/mL. As a control, HUVECs were seeded onto Matrigel® with only fresh media. After 5 hr. incubation, 4% PFA (paraformaldehyde) was used for fixing cells, and tube formations were collected with the bright-field microscope. Quantification of tube formations was made by using Angiogenesis Analyzer module of ImageJ software [148]. The experiment was done in triplicate.

3.10. *Ex vivo* whole porcine eye penetration study

The whole porcine eyes were utilized for investigation of permeation potential of HA-50Sph polymersomes to the retina [149]. Porcine eyes were obtained from Acibadem University Center of Advanced Simulation and Education (CASE; Istanbul, Türkiye) (Eyes from euthanized animals were used in compliance with ethical guidelines; therefore, ethical committee approval was not deemed necessary for the *ex vivo* ocular experiments). The freshly extracted porcine eyes were transported in an insulated box at 4°C and used immediately. Porcine eyes were put into wells of 6-well plate and a gasket was placed at the center of the corneas to prevent the spreading of the polymersome solutions (Figure 7). Freshly prepared Rhodamine B (10 μ M) encapsulated HA-50Sph polymersome solution (10 mg/mL) in PBS or 10 μ M Rhodamine B solution with 50 μ L was dropped in the gasket. Porcine eyes were incubated at 37°C, 5% CO₂ incubator for 24 hr. Finally, porcine eyes were dissected, and the cornea and retina were obtained. The corneas and retinas of porcine eyes were frozen with embedding resin (Cryomatrix®, EpreDia). Cryostat (Cryostar NX50, EpreDia) was utilized for cross-sectioning of corneas and retinas of porcine eyes with 10 μ m thickness. Sections were stained with DAPI (1,5 μ g/mL) for 15 min and thoroughly washed with sterile PBS. Cornea and retina sections were imaged under confocal microscope (LSM 710, Zeiss) for DAPI (blue, $\lambda_{Em.}$ = 467 nm) and Rhod B (red, $\lambda_{Em.}$ = 617 nm). The experiment was done in quadruplets.

The vitreous humors of the porcine eyes were also collected, and fluorescence signal of Rhod B was measured by fluorescence spectrophotometer (RF-6000, Shimadzu). The fluorescence signal was collected between 550 nm – 750 nm ($\lambda_{Ex.}$ = 545 nm).

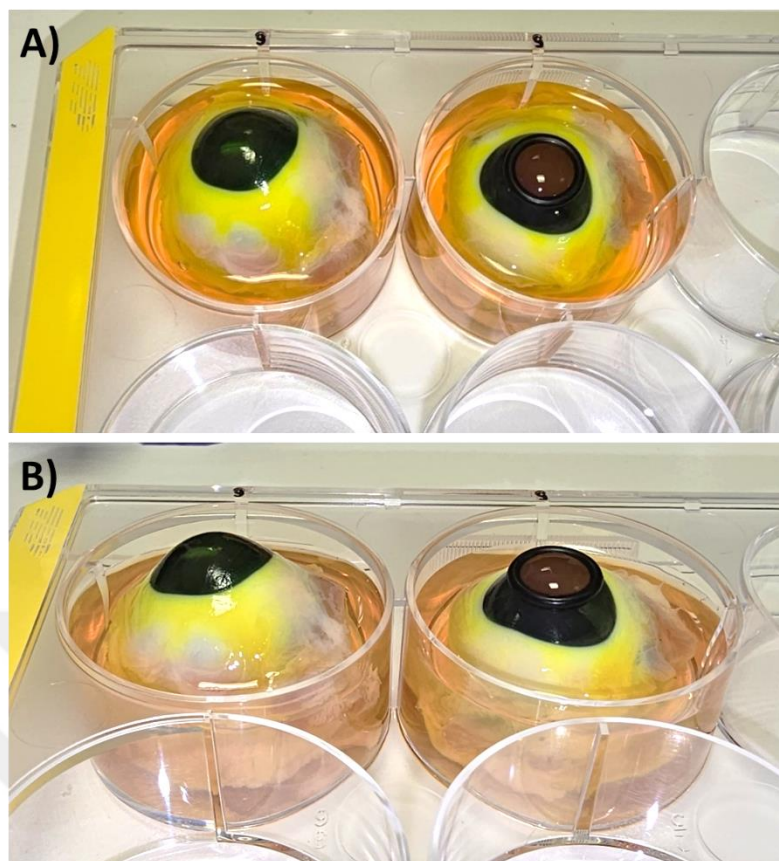


Figure 7. Experimental setup of *ex vivo* whole porcine eye penetration study. Rhod B loaded HA-50Sph polymersome solution in PBS (pinkish solution on the right-hand side) were dropped inside gasket on the porcine eyes. Porcine eye at left-hand side was used as negative control (only PBS) group. A large portion of the porcine eyes were submerged in the media. **A)** Top view, **B)** Side view.

3.11. Statistical analysis

GraphPad Prism 8.0.1 software (GraphPad Software, Massachusetts, USA) was utilized for statistical analyses of the data. The quantitative data are expressed as mean $\pm$ SD for each group. One-way and Two-way ANOVAs were performed to assess differences between means. $p < 0.05$ is represented as *, $p < 0.01$ as **, $p < 0.001$ as ***, and $p < 0.0001$ as ****.

4. Results and Discussion

4.1. Characterization of HA-Sph amphiphilic polymers

Amphiphilic HA-Sph polymers were synthesized via EDC/NHS coupling reaction [145, 150]. This reaction which involves linking the amine ($-\text{NH}_2$) group of sphingosines with the carboxyl ($-\text{COO}^-$) groups present within the hyaluronic acid chain, is initially verified

by the appearance of new peaks in the FTIR spectrums of HA-Sph polymers (Figure 8-B). The C-O-C hemiacetal system saccharide units of HA were observed as sharp peak around 1033 cm^{-1} in all samples. Within hyaluronan, a peak within region II (approximately 1600 cm^{-1}) corresponds to the symmetric vibration of carboxyl (COO^-) groups. In the amphiphilic HA-Sph polymers, this peak splits into two distinct peaks. The peak at around 1640 cm^{-1} indicates the C=O stretch, while the peak at around 1560 cm^{-1} signifies the in-plane N-H bending of secondary amide groups. Moreover, an additional N-H wagging peak is evident in region I for the HA-Sph amphiphilic polymers. Finally, in region III, two distinct sharp peaks were observed in the HA-Sph polymers, indicating the C-H stretch of methylene ($-\text{CH}_2-$) and methyl ($-\text{CH}_3$) groups present in sphingosine molecules.

^1H NMR spectroscopy was employed for further investigation of the chemical structures of hyaluronan and amphiphilic HA-Sph polymers, as seen in Figure 8-C. The glycosidic protons of sugar units of HA were demonstrated at chemical shifts between 3,0-4,1 ppm along with methyl protons of N-acetyl ($-\text{NHCOCH}_3$) groups of HA that observed at 1,9 ppm for all samples [131, 151]. Appearance of two additional peaks at sphingosine conjugated samples (HA-Sph) with chemical shifts of 1,165 ppm and 0,95 ppm were indicated methylene ($-\text{CH}_2-$) protons and terminal methyl ($-\text{CH}_3$) protons of sphingosine molecules, respectively [131, 139]. These peaks verified the successful conjugation of sphingosines to HA chain. Degree of substitution (DS%) of each HA-Sph polymer is defined as the number of sphingosine molecules per 100 units of HA polymer chain [131].

DS% of each HA-Sph polymer was calculated with the relative ratio of integration of the terminal methyl group peak of sphingosine to the N-acetyl peak of HA. The DS% of each HA-Sph amphiphilic polymers were determined as 15,45%, 21,05%, 23,24% for HA-50Sph, HA-75Sph, and HA-100Sph, respectively (Table 3). In the previous study of Saadat *et al.*, they conjugated the HA (10 kDa) with two types of phosphatidylethanolamines (PE), and obtained PE-grafted HA polymers with DS% of 10,2% and 8,1% [131]. In our study, DS% of Sph is notably higher with minimum of 15,45% which might be due the difference in EDC/NHS reaction conditions (Table 3).

Table 3. Results of the characterizations of HA-Sph amphiphilic polymers and self-assembled polymersome (CAC: Critical aggregation concentration; PDI: Polydispersity index)

	Degree of Substitution (DS%)	CAC ($\mu\text{g/mL}$)	Hydrodynamic size (nm)	Zeta potential (mV)	PDI
HA-50Sph	15,445 $\pm$ 0,0071	22,04 $\pm$ 12,7	97,95 $\pm$ 12,23	-30,0 $\pm$ 0,4	0,219 $\pm$ 0,042
HA-75Sph	21,065 $\pm$ 0,0033	15,04 $\pm$ 5,9	150,00 $\pm$ 16,6	-31,1 $\pm$ 1,9	0,199 $\pm$ 0,040
HA-100Sph	25,235 $\pm$ 0,0144	13,01 $\pm$ 3,0	161,90 $\pm$ 31,76	-37,9 $\pm$ 1,6	0,206 $\pm$ 0,064

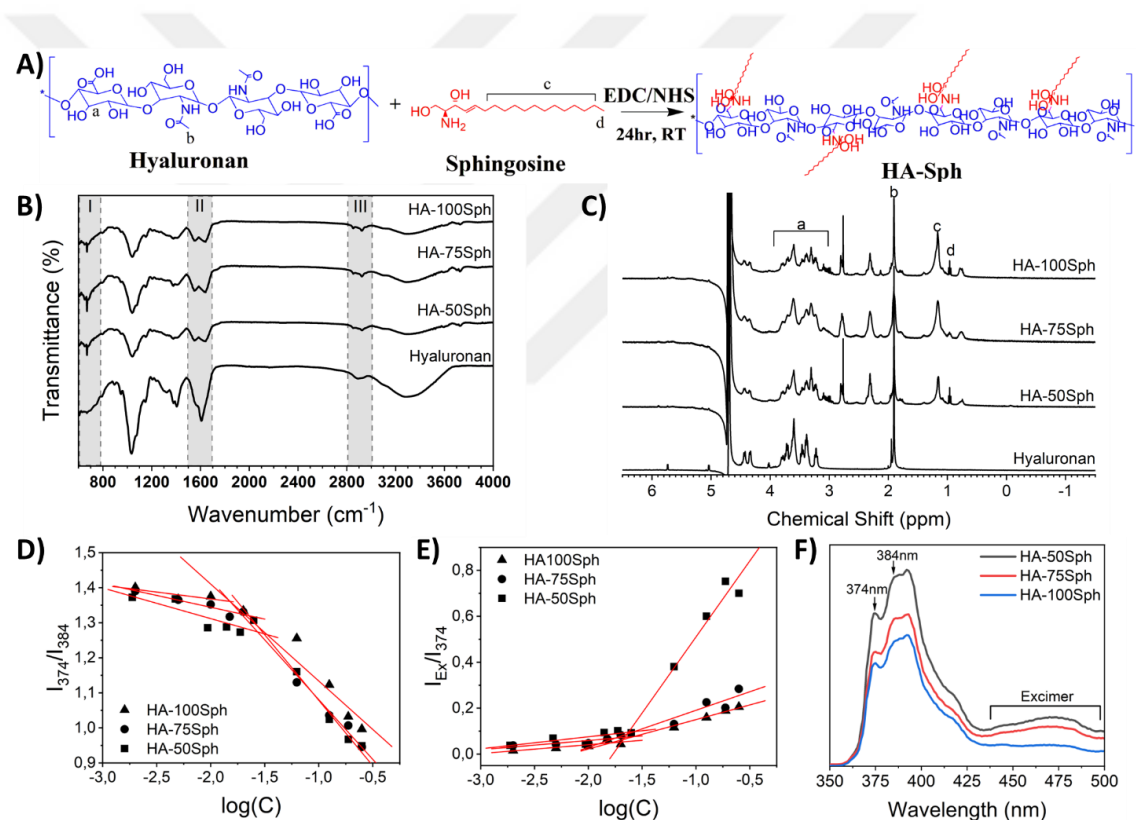


Figure 8. A) Reaction equilibrium for HA-Sph polymer synthesis and chemical structures of hyaluronic acid, d-sphingosine, and HA-Sph polymer. B) FTIR spectrums of HA-100Sph, HA-75Sph, HA-50Sph, and 10kDa HA polymers. C) ^1H NMR spectrums of HA-100Sph, HA-75Sph, HA-50Sph, and 10kDa HA polymers. Here, a, b, c, and d show corresponding peaks at 3,0 – 4,1 ppm, 1,9 ppm, 1,165 ppm, and 0,95 ppm. D and E) Critical aggregation concentrations (CAC) of amphiphilic polymers that were determined by pyrene as fluorescence probe, D) the change of the ratios of pyrene peak intensities of 374 nm and 384 nm against $\log(\text{Concentrations})$ or $\log(C)$, E) the change of the ratios of pyrene peak intensities of excimer peak (~ 470 nm) to 374 nm against $\log(\text{Concentrations})$ or $\log(C)$. F) Corresponding fluorescence spectrums of $5 \times 10^{-6} \text{M}$ pyrene encapsulated in 250 $\mu\text{g/mL}$ HA-50Sph (black), HA-75Sph (red), and HA-100Sph (blue), respectively.

Amphiphilic polymers possess a distinctive characteristic known as the critical aggregation concentration (CAC) [35]. The CAC denoting the specific concentration at which these polymers start to self-assemble into aggregates like micelles or polymersomes. [34, 74]. Pyrene, in its monomeric state, demonstrates five distinct peaks between 374 nm and 410 nm. The fluorescence intensities of these pyrene peaks alter based on the polarity of the molecular surroundings [152]. For instance, peak I intensity (I_{374}) decreases while peak III intensity (I_{384}) increases with a shift from an apolar to a polar environment. Therefore, fluorescence peak intensity ratio (I_{374}/I_{384}) of pyrene decreased when the concentration of HA-Sph polymers increased in aqueous solutions, as seen in Figure 8-D. After reaching the CAC and initiation of self-assembly of polymersomes, the ratios of I_{374}/I_{384} diminish rapidly (as seen in Figure 2-D) [153, 154]. The CACs of HA-50Sph, HA-75Sph, and HA-100Sph were calculated from the intersection points of two linear regression lines of I_{374}/I_{384} , and were measured as $22,04 \pm 12,7 \mu\text{g/mL}$, $15,04 \pm 5,9 \mu\text{g/mL}$, and $13,01 \pm 3,0 \mu\text{g/mL}$ (Table 3), respectively. Notably, in HA-Sph amphiphilic polymers, CAC values exhibit an inverse relationship with the DS% of polymers. It was observed that as the DS% increased, the CAC decreased [35, 155]. Pedro *et al.* have proposed that hydrophobicity of polymer chains increased with the increasing DS% of hydrophobic groups, allowing more compact hydrophobic interactions and consequently fostering stronger self-aggregation [155].

When two pyrene molecules, one in the ground state and the other in the excited state, are at around $10 \text{ \AA}$ from each other, their interaction generates new broad peak within the range of 425-550 nm (centered around 470 nm), namely excimer or dimeric emission of pyrene (as seen in Figure 8-F) [152]. According to Xu *et al.*, the intensity ratios of the excimer peak to 374 nm peak intensity ratios (I_{Ex}/I_{374}) significantly increase upon surpassing the critical aggregation concentration (CAC) when bilayer or vesicular structures form [156]. In our study, pyrene's I_{Ex}/I_{374} peak ratios were increased drastically after passing the CAC of respected HA-100Sph, HA-75Sph, and HA-50Sph polymers. CAC values obtained from the peak ratios of I_{Ex}/I_{374} were consistent with those obtained from peak ratios of I_{374}/I_{384} (as seen in Figures 8-D and 8-E).

4.2.Characterization of HA-Sph polymersomes

The solvent evaporation method was utilized to self-assemble the HA-Sph amphiphiles into polymersomes [96, 157]. The size, surface charge, and structure of these

polymersomes were observed through DLS, SEM, and TEM methods (as depicted in Table 3 and Figure 9). All the PDI (polydispersity index) values of HA-Sph polymersomes are ranging around 0,2 which indicates a highly homogenous particle sizes distribution for all three samples [2]. The hydrodynamic sizes of the polymersomes measured were $97,95 \pm 12,23$ nm, $150,0 \pm 16,6$ nm, and $161,9 \pm 31,76$ nm for HA-50Sph, HA-75Sph, and HA-100Sph, respectively. Contrary to previous findings of studies, the size of polymersomes increased with increasing DS% [11, 158]. However, as suggested by Zhang *et al.*, the bilayer thickness of the polymersome might correspondingly increase with higher DS% [11]. This phenomenon for HA-Sph polymersomes was further confirmed by variations in the fluorescence intensities of pyrene. When the DS% increases, fluorescence intensity of pyrene that encapsulated in polymersomes decreases, as shown in Figure 8-F.

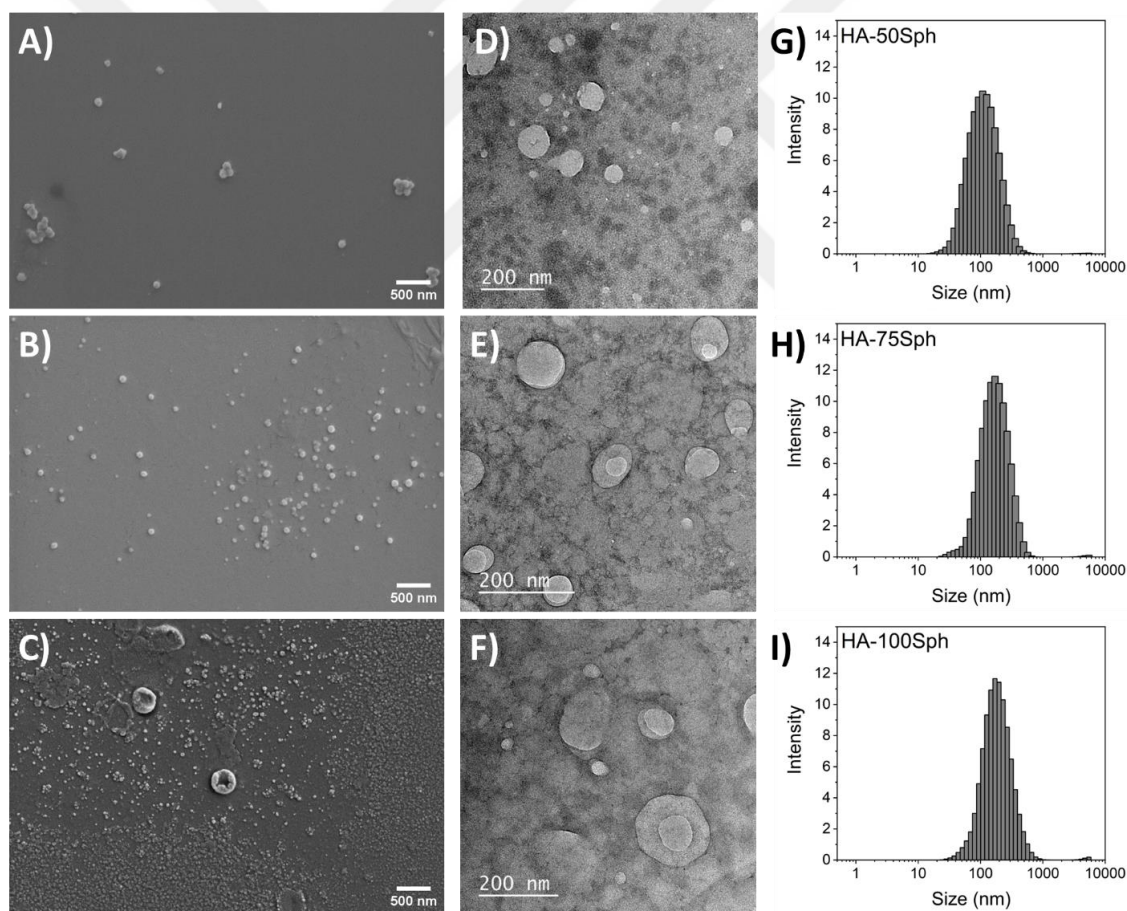


Figure 9. A-C) Scanning electron microscopy images of HA-Sph polymersomes at 50000x magnification. A) HA-50Sph, B) HA-75Sph, and C) HA-100Sph (Scale bar: 500 nm). D-F) TEM micrographs of HA-Sph polymersomes. D) HA-50Sph, E) HA-75Sph, and F) HA-100Sph (Scale bar: 200 nm). G-I) Hydrodynamic size distribution of corresponding HA-Sph polymersomes. G) HA-50Sph, H) HA-75Sph, and I) HA-100Sph.

HA-Sph polymersomes with spherical morphologies were verified by SEM and TEM micrographs, as seen in Figure 9. Most of the particles had sizes ≤ 200 nm with a uniform size distribution of the polymersomes which observed by SEM micrographs. Nonetheless, it's worth noting that micrographs of HA-100Sph polymersomes demonstrated a few larger particles compared to the others. Additionally, the edges of polymersomes in SEM micrographs were brighter than the center which could verify the collapsed surface of vesicular structures [11]., Bilayer structures of polymersomes are demonstrated by TEM micrographs. HA-50Sph had smaller-sized polymersomes compared to other HA-Sph polymersomes in the TEM micrographs, which confirmed the size distribution data of DLS.

4.3. Temperature stability and long-term storage of polymersomes

Polymersomes possess remarkable resilience and stability owing to their low entropy and heightened immiscibility [93]. The stability of polymersomes were investigated by exposing them to temperatures ranging from 10°C to 90°C for one cycle. Pyrene intensity ratios (I_{374}/I_{384}) were tracked throughout the temperature variations. As the temperature increased, the pyrene ratio increased and reverted to its initial state (initial values of I_{374}/I_{384}) upon cooling (Figures 10-A, 10-B, and 10-C) This trend indicated that even at elevated temperatures, the polymersomes maintained their stability. Stability of polymersomes was also confirmed by observing their size through DLS over a temperature range of 10°C to 70°C (Figure 11). The size of HA-50Sph polymersomes increased at the higher temperatures, while the size of HA-100Sph polymersomes decreased. The impact of temperature on lipid membranes is well-known, revealing that the fluidity of these membranes increase with increasing temperatures [159]. In the case of HA-50Sph, the weaker hydrophobic interactions between bilayers might have resulted in the structural integrity loss of some polymersomes as the temperature increased. Conversely, HA-75Sph and HA-100Sph, with higher Sph amounts, exhibited more stable polymersomes. Nevertheless, increased fluidity of HA-50Sph initiated the disruption of their bilayer membranes, leading to the aggregation of a few HA-50Sph polymersomes. Overall, these results highlight the tunable stability of HA-Sph polymersomes with the variations of DS% against high temperatures, primarily attributed to the hydrophobic interactions of Sph molecules within the bilayers.

The stability of HA-Sph polymersomes over long-term storage was investigated using pyrene as a fluorescence probe. HA-Sph polymersomes were stored at +4°C, 25°C, and 37°C for 8 weeks and their stability was assessed weekly by evaluating I_{374}/I_{384} peak ratios. It is important to note that pyrene peak ratios are dependent on the concentration of amphiphilic molecules [160]. As observed in The temperature-dependent stability experiment, peak ratios of I_{374}/I_{384} were increased over time with increased temperature. In this regard, the values obtained from week 1 were considered as baseline for investigation of long-term stability of polymersomes. Across all storage conditions, particularly at 4°C and 37°C, HA-50Sph and HA-75Sph polymersomes demonstrated higher stability, showing minimal changes in the peak ratios of I_{374}/I_{384} , compared to HA-100Sph (Figure 10-D and 10-E). For instance, over 8 weeks, I_{374}/I_{384} of HA-50Sph was slightly decreased from 1,27 to 1,25 at 37°C and at 4°C, increased from 1,17 to 1,20. Conversely, in HA-100Sph, peaks ratios were notably increased at all storage conditions due to disruption of membranes and/or leakage of pyrene from membranes. On the other hand, the sizes of HA-50Sph and HA-100Sph polymersomes reduced over time by around 100 nm while, size of HA-75Sph was decreased only by 15 nm (Figure 12-A). Additionally, zeta potentials for all polymersomes showed an increase after first week of storage at 25°C and 37°C (Figure 12-B). This slight increase may be attributed to the protonation of sphingosine groups in the aqueous solution [139].

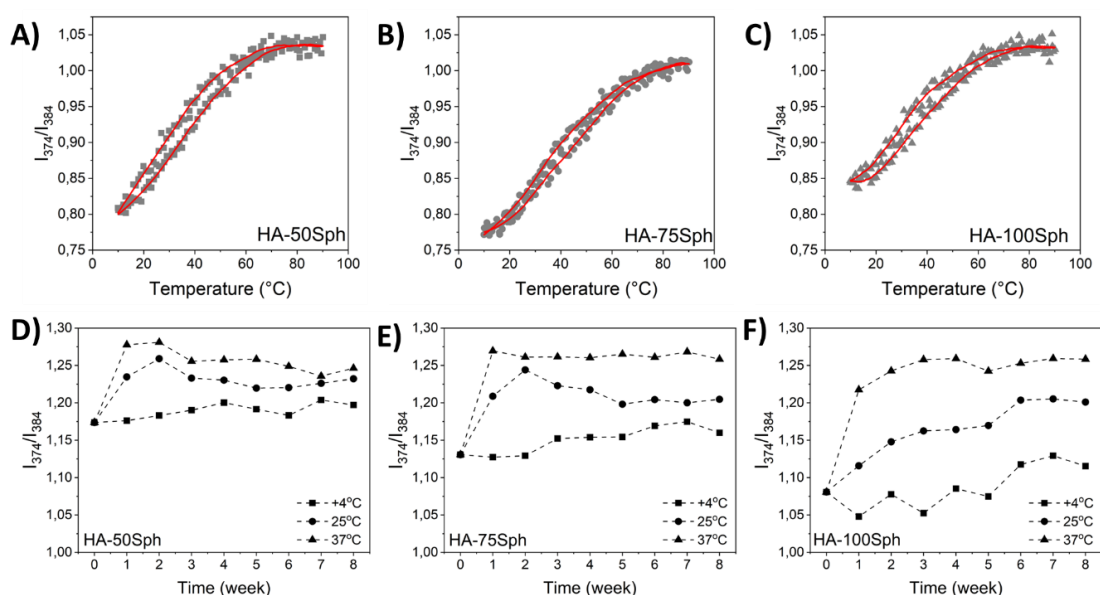


Figure 10. Stability of polymersomes against temperature and long-term storage stability. **A – C)** Fluorescence peak ratios (I_{374}/I_{384}) of 5×10^{-6} M pyrene loaded HA-Sph polymersomes (100 $\mu\text{g/mL}$) for investigation of the stability of polymersomes against temperatures between 10°C -

90°C and 90°C - 10°C. **D – F)** Fluorescence peak ratios (I_{374}/I_{384}) of 5×10^{-6} M pyrene loaded HA-Sph polymersomes solution (100 µg/mL) for long-term storage stability at certain conditions of 4°C, 25°C, and 37°C.

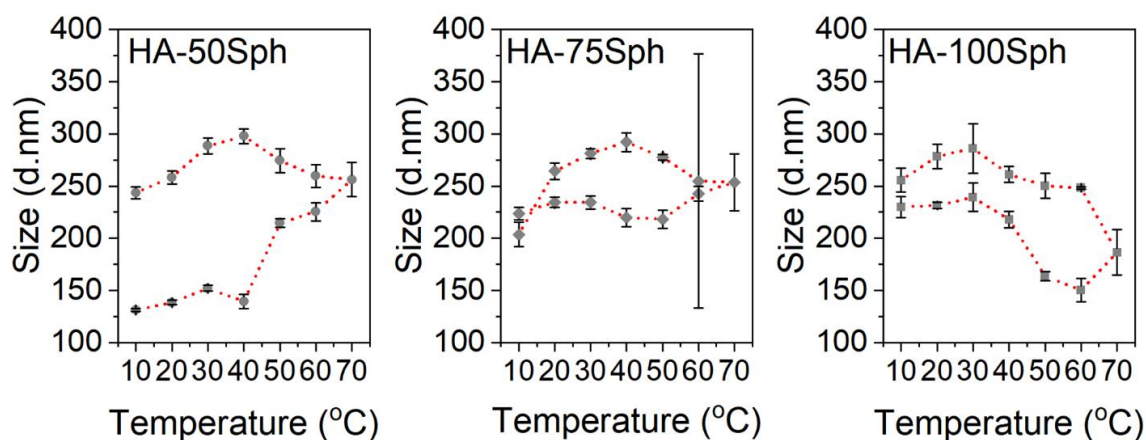


Figure 11. Stability of polymersomes against temperature change. DLS measurements were made between temperatures of 10°C – 70°C – 10°C with 10°C increments.

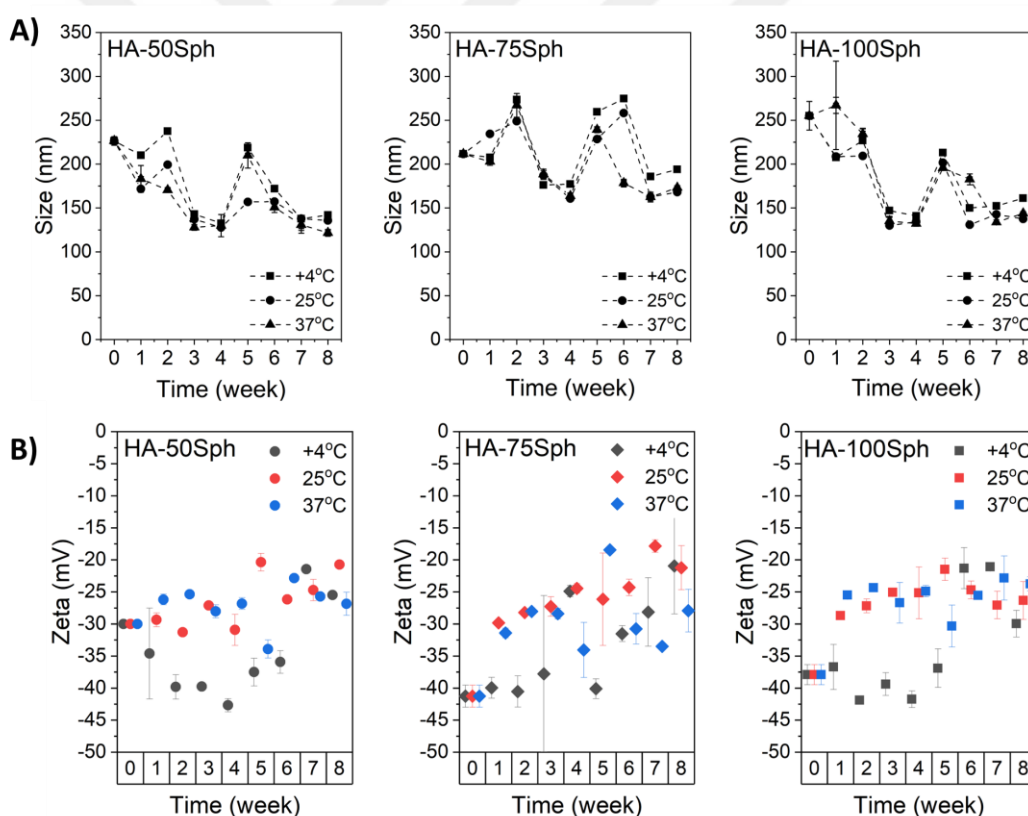


Figure 12. A) Size (nm) response of polymersomes for various storage temperatures including 4°C, 25°C, and 37°C, over the long-term storage. B) Zeta potential (mV) response of polymersomes for various storage temperatures including 4°C, 25°C, and 37°C, over the long-term storage.

4.4. *In vitro* bioactivity of HA-Sph polymersomes on HUVECs and ARPE-19 cells

The effects of HA-Sph polymersomes and their constituents, specifically HA and Sph, were evaluated by a preliminary *in vitro* study on the proliferation of HUVECs, while potential cytotoxic effects against ARPE-19 cells was investigated simultaneously. Compared to control, HUVEC viability was notably decreased upon treatment with both HA-50Sph polymer and polymersomes, as seen in Figure 13-A. However, there was no significant difference in cell viability between the HA-50Sph polymer and polymersome treatments. In contrast, the viability of ARPE-19 cells was significantly increased with HA-50Sph polymer and HA-50Sph polymersomes exposure, compared to the control group. Hyaluronan (10 kDa) did not affect the viability of both ARPE-19 and HUVECs while, free sphingosine (55 μ M) completely eliminated the presence of HUVEC cells and demonstrated a highly cytotoxic effect on ARPE-19 cells [144].

Further assessment of the dose-dependent cytotoxicity of Sph for ARPE-19 and HUVEC cells was examined in the concentration range of 5 - 20 μ M, as seen in Figure 13-B. This concentration range was chosen due according to findings of study by Hisano *et al* [144]. Proliferation of HUVECs was significantly reduced with Sph (20 μ M in media), compared to control. Meanwhile, the viability of ARPE-19 cells non-significantly increased with increasing Sph concentrations, until 20 μ M. According to previous study, the media supplemented with 20 μ M sphingosine was promoted the proliferation of Swiss 3T3 fibroblasts, akin to the effects of epidermal growth factor (EGF) with 10 ng/mL concentration [142]. Another study demonstrated the mitogenic effects of Sph and cytosolic phosphatidic acid on Swiss 3T3 fibroblasts, suggesting that Sph concentrations >30 μ M were highly cytotoxic to these cells [141]. Further observations that made on Sph was suggested to affect the differentiation of Neuro2a (murine neuroblastoma) cells in a concentration-dependent manner [143]. Ceramide production within the cells increased when the culture media was supplemented with 10 nmol/10⁶ cells of Sph, leading to active metabolism by neuro2a cells and differentiation of cell morphology of the neuro2a [143]. Sph significantly inhibited the HUVEC proliferation with increasing concentrations (Figure 13-B). These results, coupled with the findings from prior studies, suggest that

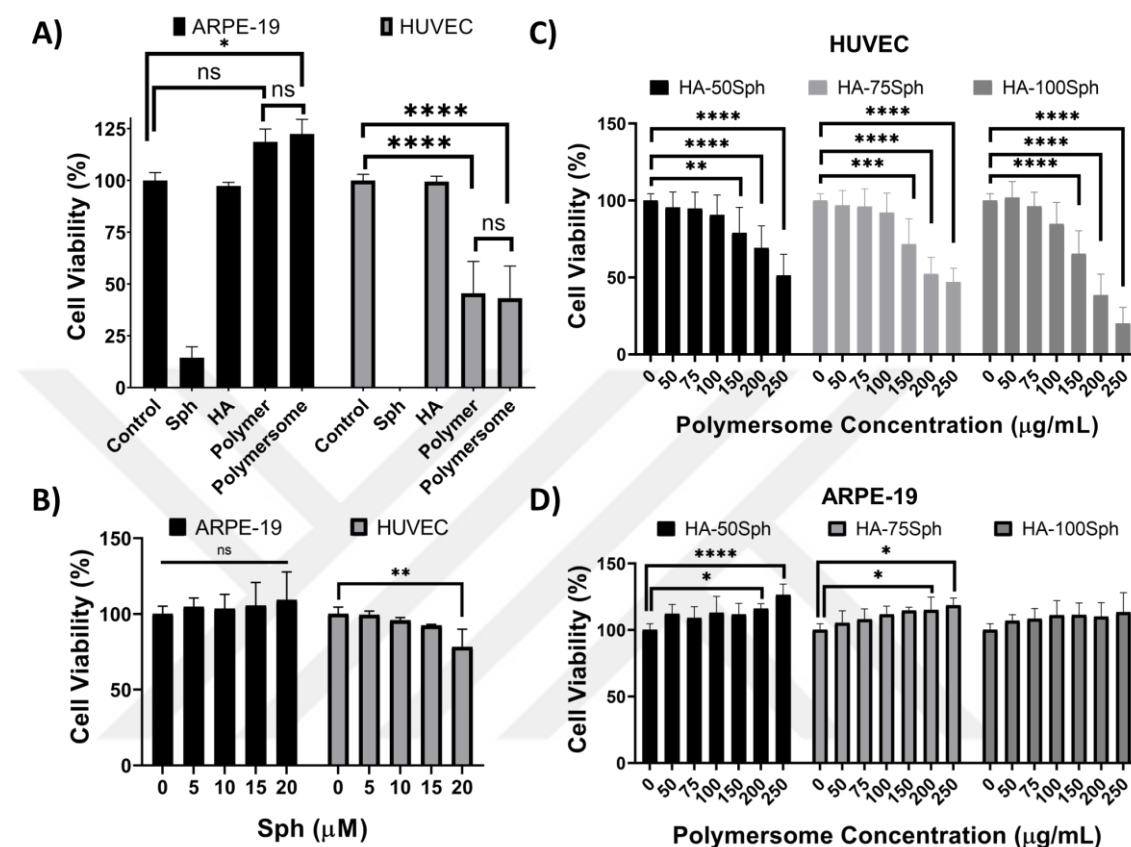
Sph could be potentially utilized as an anti-angiogenic factor for the treatment of ocular neovascularization.

The effect of HA-Sph polymersomes on HUVECs and ARPE-19 cells was shown by CCK-8 assay. The results demonstrated a concentration-dependent reduction of the HUVEC proliferation which corresponded to increasing in both concentration of HA-Sph polymersome and the amount of Sph conjugated to these polymersomes. At concentrations ≥ 150 $\mu\text{g/mL}$, polymersomes showed cytotoxic effects on HUVECs, reducing cell viability to $<80\%$, as shown in Figure 13-C. When ARPE-19 cells were treated with HA-Sph polymersomes for 4 days, results displayed no cytotoxicity. Across nearly all polymersome concentrations, ARPE-19 cells exhibited viability values of more than 100%, as seen in Figure 13-D.

While explored Sph metabolism in HUVECs, Hisano *et al.* revealed that its impact on cell fate (apoptosis or mitosis) depends on cell type [144]. Additionally, the Sph has been presumed to potentially trigger cell death [136]. On the other hand, the overexpression of acid ceramidase in ARPE-19 cells corresponding to elevated Sph levels, conferring protection against oxidative stress [136, 161]. In the recent study of Zhang *et al.*, mesoporous silica nanoparticles (MSNs) loaded with sphingosine-1-phosphate (S1P), encapsulated into aldehyde hyaluronic acid (AHA)/N,O-carboxymethyl chitosan (NOCC) nanocomposite hydrogels [162]. According to the study, S1P significantly enhanced proliferation of HUVECs and promoted *in vivo* vascularization of bone defects [162, 163]. Sph can be phosphorylated by sphingosine kinases to produce sphingosine 1-phosphate that has adverse effects compared to Sph [137]. In this regard, our findings revealed the anti-angiogenic potential of Sph.

The investigations were made for determination of effects of HA-Sph polymersomes against viability of corneal endothelial cells (HCEC) and corneal epithelial cells (HCEpiC). Similar to ARPE-19 cells, HA-Sph polymersomes did not show notable decrease on cell viability of HCEpiCs at concentrations between 50 $\mu\text{g/mL}$ – 250 $\mu\text{g/mL}$ (Figure 14). Meanwhile, there was an apparent cytotoxic effect on HCECs (Figure 15-A). The S1P with sphingosine-1-phosphate receptors (S1PR) modulates the barrier functions of endothelial cells [164]. In our study, the barrier functions of HCECs might be disrupted due to the increased exogenous Sph and possibly reduced the viability *in vitro*. In this regard, IC_{50} values of HA-Sph polymersomes were calculated from cell viability of

HCECs (Figure 15-B). According to the results, IC_{50} of HA-50Sph, HA-75Sph, and HA-100Sph were 205,9 $\mu\text{g/mL}$, 177,8 $\mu\text{g/mL}$, and 118,2 $\mu\text{g/mL}$, respectively. Therefore, further investigations are required for adjusting the safe dosage of potential topical drug formulation.



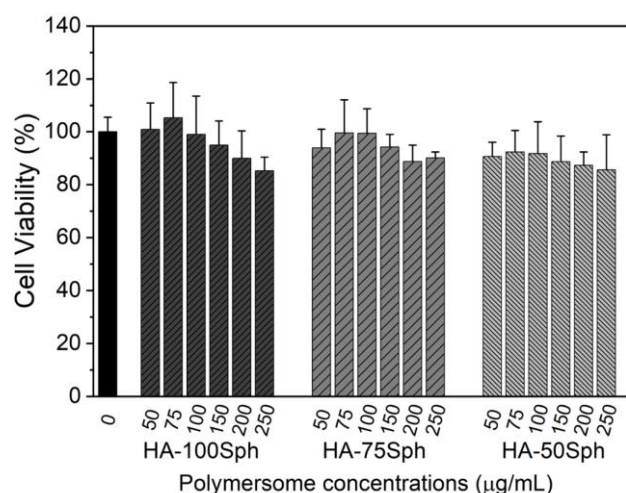


Figure 14. Cell viability of human corneal epithelial cells (HCEpiCs) against HA-Sph polymersome concentrations (n=2).

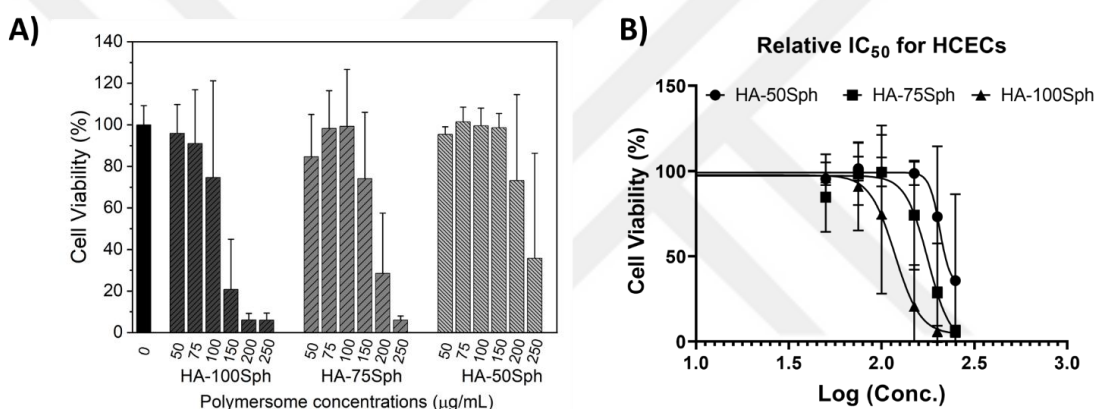


Figure 15. A) Cell viability of human corneal endothelial cells (HCECs) against HA-Sph polymersome concentrations (n=2). **B)** Determination of IC₅₀ values of each HA-Sph polymersomes for HCECs. IC₅₀ values of HA-50Sph, HA-75Sph, and HA-100Sph were 205,9 µg/mL, 177,8 µg/mL, and 118,2 µg/mL, respectively. (*IC₅₀ values were calculated by GraphPad Prism 8.0.1 software.*)

In preliminary experiments, both the HA-50Sph amphiphilic polymer and the self-assembled polymersomes derived from HA-50Sph exhibited a significant restraint to proliferation of HUVECs. These results suggested a potential gradual degradation of both HA-50Sph polymer and polymersomes with time-wise manner. Time-dependent cell proliferation assay was conducted for further investigating this phenomenon. The daily measurement of cell viability of HUVECs showed a steady increase in the inhibitory effect of HA-50Sph polymers and polymersomes on HUVECs, as shown in Figure 16-B. These results align with the hypothesis of polymersome degradation, which resulted in a gradual increase in Sph concentration in the media. In contrast, viability among ARPE-

19 cells did not reduce during the experiment; rather, it showed an slightly increased viability, as shown in Figure 16-A.

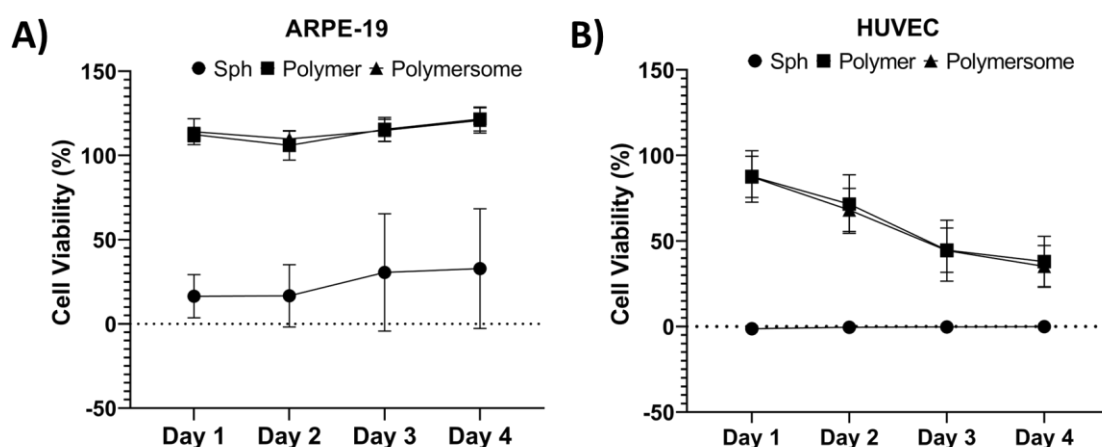


Figure 16. A) Comparison of ARPE-19 cell viability differences of Sph (55 μ M), HA-50Sph polymer, and HA-50Sph polymersome (150 μ g/mL) in a daily manner for 4 days. **B)** Comparison of HUVEC viability differences of Sph (55 μ M), HA-50Sph polymer, and HA-50Sph polymersome (150 μ g/mL) in a daily manner for 4 days. Each experiment was conducted in triplicates, and all results are presented as mean $\pm$ SD.

4.5. Tube formation assay

The tube formation assay is a common method used to evaluate the angiogenic potential of endothelial cells, which form the inner lining of blood vessels [165]. When seeded onto a thin matrix gel, the endothelial cells rapidly organize into capillary-like tubular structures [165]. This assay is valuable for screening potential therapeutic agents that can regulate angiogenesis, offering insights into addressing ocular neovascularization [166].

The images captured under a brightfield microscope demonstrated fewer tube formations in the groups treated with HA-Sph polymersomes compared to the control group, as shown in Figure 17-A. Measurement of the total tube length from these images demonstrated a significant inhibition of tube formation in groups treated with 150 μ g/mL HA-Sph polymersomes, as depicted in Figure 17-B. Additionally, the findings suggested that the influence of polymersomes on tube formation heightened with higher DS%, particularly with an increased concentration of Sph in HA-Sph polymersomes.

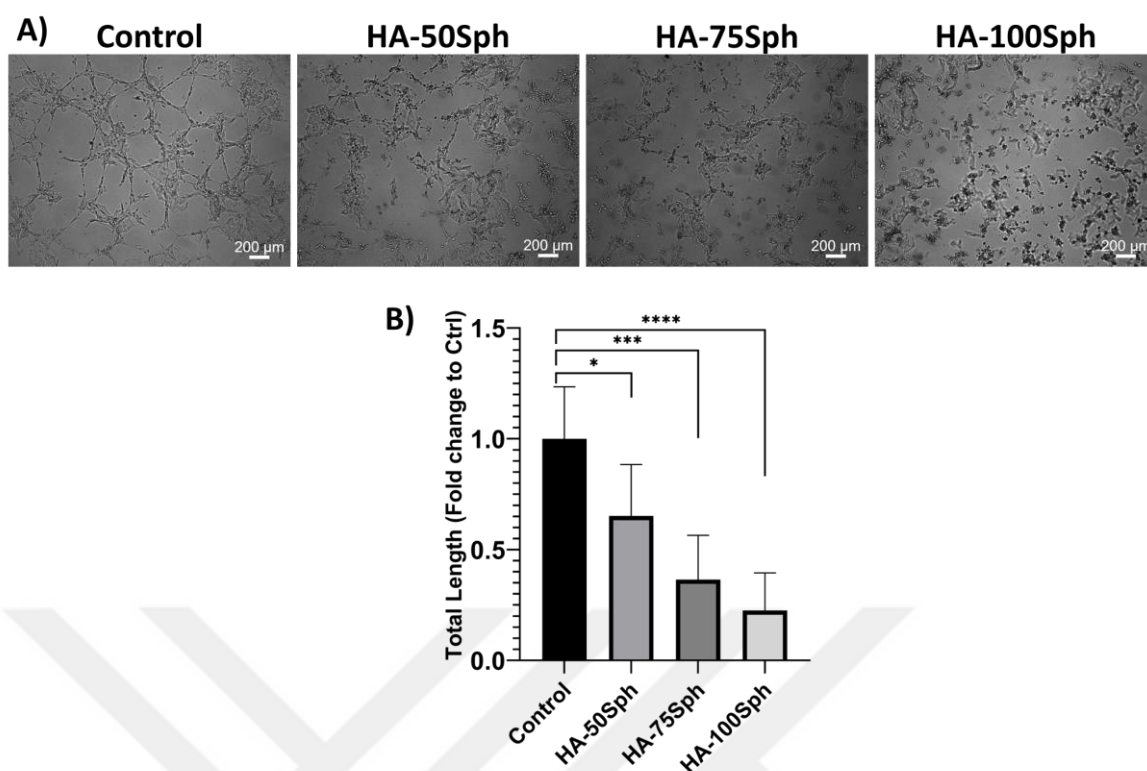


Figure 17. A) Representative brightfield microscope images of tube formation assay with HA-Sph polymersomes (150 µg/mL). **B)** Change in the total length of tubes formed by HUVECs after 5 hr. compared to HA-Sph polymersomes. Data normalized to control groups of each replicate. Each experiment was conducted in triplicates, and all results are presented as mean ± SD. (p < 0.05 is represented as *, p < 0.01 as **, p < 0.001 as ***, and p < 0.0001 as ****)

4.6. *Ex vivo* whole porcine eye penetration study

An *ex vivo* study was undertaken using whole porcine eyes to evaluate the ocular penetration of HA-50Sph polymersomes, chosen for their advantageous smaller size in facilitating ocular delivery [2]. Employing the whole porcine eye model, the experiment focused on assessing the ability of these polymersomes to reach the retina, an important step in ocular drug delivery. The corneal epithelium serves as the primary barrier hindering drug absorption [149]. The confocal images showed that Rhod B intensities were higher at corneal epithelial layers, as shown in Figure 18. Notably, the confocal microscope images of retinal tissue sections from the porcine eyes exhibited the successful penetration of HA-50Sph polymersomes into the retina, which indicate their potential to traverse ocular barriers and reach to the posterior eye segment, as seen in Figure 18. However, when comparing the Rhod B intensity of loaded HA-50Sph polymersomes at the corneal tissues, it was clearly observed that a relatively less HA-

50Sph polymersomes reached to the retina. Additionally, a prior study suggested that polymersomes of approximately 100 nm in size could remain in the vitreous humor for up to 92 days following intravitreal injections into the rabbit eyes [9]. This previous study also revealed that clearance rate of polymersomes in vitreous is 1,7–8,7 $\mu\text{L/h}$ which is significantly less than polymeric micelles with a clearance rate of 3,6–5,4 $\mu\text{L/h}$ [9].

Moreover, fluorescence spectra of vitreous humor of the porcine eyes were measured, the presence of polymersome at the posterior segment was confirmed (data not shown). These results suggested the significant potential of HA-50Sph polymersomes as effective carriers for targeted ocular drug delivery to the posterior segment of the eye.

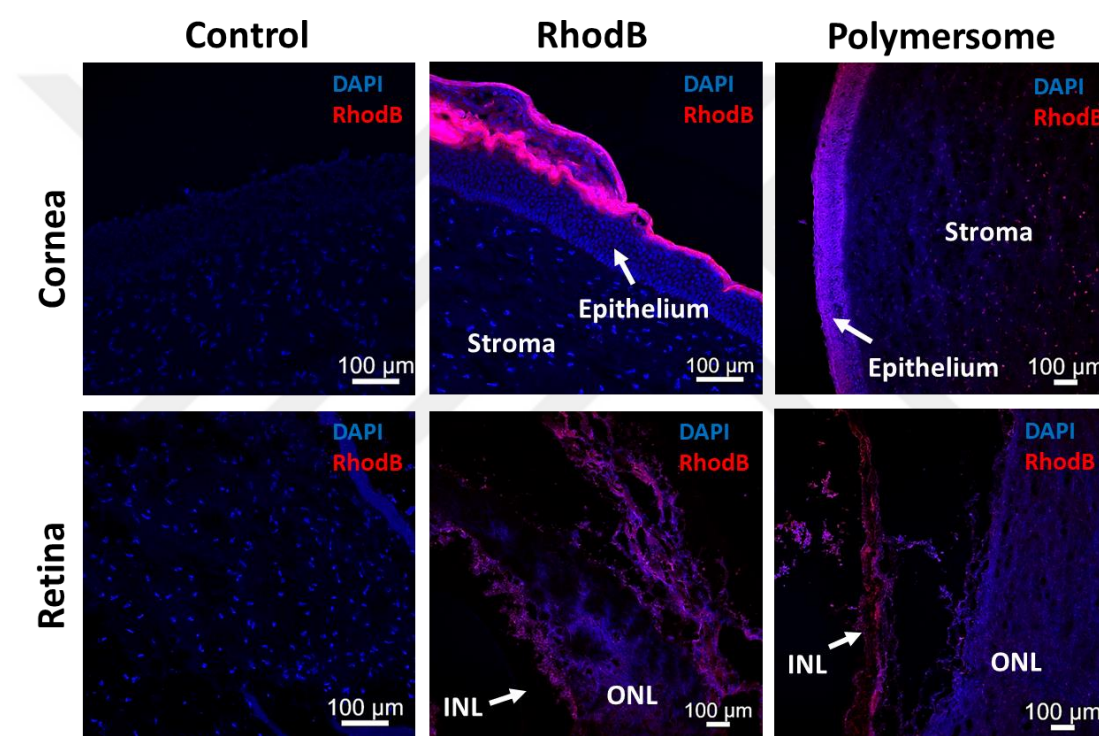


Figure 18. Representative images of ex vivo whole porcine eye permeation experiment (Blue for DAPI and red for Rhod B) (n=4). Corneal and retinal sections of porcine eyes were stained with DAPI and visualized under fluorescent microscopy. For control and RhodB groups, PBS and rhodamine B solution were dropped onto the eyes, respectively. For Polymersome group, RhodB encapsulated HA-50Sph polymersome solution was used as the eye drop. (Scale bars: 100 μm)

5. Conclusion

In the current study, we developed and characterized amphiphilic polymers made from hyaluronic acid grafted with sphingosine (HA-Sph), and prepared self-assembled

polymersomes. Our aim was to facilitate the targeted delivery of sphingosine to the retina for treating ocular neovascularization-based pathologies. Our findings exhibit a significant inhibition of HUVEC proliferation by HA-Sph polymersomes while no adverse effects observed on ARPE-19 cells. The efficacy in hindering angiogenesis was further confirmed by tube formation assays, showing a substantial reduction in total tube length across all HA-Sph polymersomes compared to the control. Additionally, the ocular penetration activity of the HA-50Sph polymersomes were verified through an *ex vivo* ocular penetration study.

It is imperative to acknowledge the existing gaps in our comprehension of Sph metabolism and its impacts on ocular tissues, especially the potential dose-dependent toxicity which linked with clearance rates, necessitates further investigations. Assessing sphingosine toxicity and HA-Sph polymersome clearance rates within ocular tissues is essential for safety of polymersomes. The potential of sphingolipids for the treatment of ocular diseases has been investigated in limited studies. In this regard, this study has the potential to be at the forefront of the treatment of ocular neovascularization diseases. The polymersomes demonstrated superior ocular penetration capabilities. The polymersomes showcased remarkable ocular penetration abilities, highlighting their suitability for topical administration. Additionally, due to the advantages of polymersomes for the encapsulation of various therapeutic agents, these HA-Sph polymersomes could evolve into more complex drug delivery systems, promising to protect the retina and effectively treat ocular diseases.

Optimization of Dual Drug Incorporated Hyaluronan-Sphingosine-Cholesterol Polymersomes for Treatment of Ocular Neovascularization: Design of Experiments Approach

1. Introduction

Abnormal and excessive angiogenesis is a central feature of many pathologies that occur in the posterior segment of the eye, including age-related macular degeneration (AMD), proliferative diabetic retinopathy, and retinopathy of prematurity. Wet AMD, which affects only 10-15% of AMD patients, is responsible for almost all vision loss associated with the disease [14]. It is characterized by the invasion of the retina by leaky choroidal neovascularization, which leads to rapid vision loss. High expression of vascular endothelial growth factor (VEGF), a key regulator of angiogenesis, is the main cause of these neovascularization-based diseases [167]. Treatment options for these diseases have improved significantly following the development of anti-VEGF intravitreal injections including pegaptanib (Macugen®), ranibizumab (Lucentis®), aflibercept (Eylea®), and brolucizumab (Beovu®) [7]. These treatments require injecting the drug into the eye, either once or twice a month, for a long period of time [126]. Therefore, adverse effect such as frequent intravitreal injections, which are a burdensome and inconvenient procedure for patients, poor membrane permeability, low bioavailability, high immunogenicity, and high costs of these drug may decrease the benefits for patients [8, 127, 168].

These disadvantages underscore the need for new approaches to the delivery of anti-VEGF drugs that can provide sustained treatment with less patient burden. In this regard, anti-VEGF peptides have advantages such as low cost, low immunogenicity, high tissue permeability, and high reproducibility [168]. Recently, Zhang *et al.* discovered a potential anti-VEGF peptide, called HRH (HRHTKQRHTALH), which is an anti-angiogenic peptide by utilizing Ph.D.-12 phage display library against VEGFR-Fc fusion protein [127]. The study suggests that HRH peptide is a mimotope for binding site of VEGFR-1, which competitively binds to VEGFR-1 thus, inhibits VEGF-A and VEGF-B [127]. Later, Chen *et al.* prepared self-assembled nanofibers from the amphiphilic HRH peptide derivative, which was enhanced the *in vivo* bioactivity of HRH peptide compared

to free HRH peptide for the treatment of corneal neovascularization [169]. Recent study was formulated conjugated linoleic acid coated and paclitaxel loaded superparamagnetic iron oxide nanoparticles (SPION), and decorated the surface of the SPIONs with HRH peptide for inhibition of angiogenesis in tumor environment [170]. HRH decorated SPIONs significantly reduced VEGF-A levels of human dermal microvascular endothelial cells *in vitro* [170].

Sphingolipids comprise approximately 11-13 mole% of the total lipids in rat and bovine retinas, highlighting their significant presence in ocular tissues [21]. Despite their abundance, the precise roles of individual sphingolipids as signaling molecules in ocular tissues remain to be fully elucidated [23]. Sphingosine (Sph) is phosphorylated by sphingosine kinases to generate sphingosine-1-phosphate (S1P), which exhibits contrasting biological effects compared to Sph [22]. Recently, Zhang *et al.* developed S1P-loaded mesoporous silica nanoparticles (MSNs) encapsulated within an aldehyde hyaluronic acid (AHA)/N,O-carboxymethyl chitosan (NOCC) nanocomposite hydrogel [52]. Consequently, S1P effectively promoted HUVEC proliferation and enhanced *in vivo* vascularization of bone defects [52]. In our previous study, we demonstrated the anti-angiogenic property of sphingosine and further evaluated the properties of HA-Sph polymersomes as potential therapeutics for the treatment of ocular neovascularization (Figure 13 and 17). According to this study, HA-Sph polymersomes decreased the proliferation of HUVECs and inhibited the tube formation. However, these HA-Sph polymersomes showed dose-dependent cytotoxicity to corneal endothelial cells (Figure 15).

Polymersomes, also known as polymeric vesicles, have emerged as versatile drug delivery systems, demonstrating the ability to encapsulate a diverse range of small and large, hydrophilic and hydrophobic molecules [55]. Polymersomes can be tailored through various modifications of their size, surface potential, and composition, enabling controlled drug release, targeted delivery to specific sites, enhanced bioavailability in the vitreous humor, and protection of sensitive drugs from degradation [50, 97, 98]. Polymersomes could allow dual drug delivery of various drug including hydrophilic and hydrophobic small drugs, proteins, and antibodies [50, 94]. Additionally, Junnuthula *et al.* explored the *in vivo* ocular pharmacokinetics of polymersomes, and polymeric micelles assembled from distinct polymers [98]. Intravitreal injection of polymersomes

(~100 nm) and micelles (~30 nm) into rabbit eyes revealed prolonged residence in the vitreous humor, with half-lives ranging from 11,4 to 32,7 days for polymersomes and 4,3 to 9,5 days for micelles [98]. Intriguingly, polymersomes exhibited substantial retention in the vitreous for up to 92 days post-injection and accumulated in the optic nerve head for 111 days [98].

However, the fabrication of polymersomes is a complex process, influenced by numerous factors such as polymer type, solvent choice, temperature, and pH [13]. To optimize polymersome synthesis, systematic testing of factor combinations and their effects on desired outcomes is crucial. The design of experiments (DoE) method provides a structured approach to identify key factors, determine their ideal levels, and assess their interactions [171]. Among various DoE designs, Box-Behnken design (BBD) is particularly suitable for modeling quadratic relationships with three levels for each factor [172]. BBD's ability to yield robust estimates of primary effects and interactions while requiring fewer experimental runs compared to full factorial designs makes it ideal for optimizing nonlinear or quadratic processes or products [171, 172].

2. Aim of the Study

In this study, we synthesized amphiphilic polymers, including sphingosine-grafted hyaluronan with variable grafting density (HA-50Sph and HA-100Sph) and cholesterol-grafted hyaluronan (HA-Chol), for the purpose of fabricating self-assembled blend polymersomes as a novel treatment approach for ocular neovascularization. We utilized a three-level BBD approach with response surface method (RSM) predictions to determine the optimum parameters for preparing the polymersomes, including the HA-Sph/HA-Chol polymer ratio, total polymer to HRH peptide mass ratio, and preparation temperatures. We extensively characterized the HRH-encapsulated blend polymersomes and compared the experimental results with the predictive results. We also evaluated the drug release kinetics of the polymersomes with and without enzymatic degradation, as well as the synergistic therapeutic efficiency of the blend polymersomes using an *in vitro* proliferation assay on HUVECs.

3. Materials and Methods

3.1. Materials

Hyaluronan (<10 kDa) was purchased from Creative PEGWorks (North Carolina, USA). Sphingosine (d18:1) was purchased from Cayman Chemical (Michigan, USA). N-Ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), N,N-dimethylformamide (DMF), cholesterol, ethanol, hyaluronidase, and pyrene were purchased from Sigma Aldrich (Missouri, USA). Diethyl ether (99,5%) was purchased from Isolab (Eschau, Germany). FAM-tagged HRH peptide (HRHTKQRHTALH) synthesized and purchased from GenScript Biotech (Rijswijk, Netherlands). Cell counting kit-8 (CCK-8) was purchased from Abcam (Cambridge, UK). Endopan-3 medium with supplement kit, fetal bovine serum (FBS), L-glutamine (200 mM), and penicillin-streptomycin were purchased from PAN-Biotech (Aidenbach, Germany).

3.2. Synthesis and characterization of HA-Sph and HA-Chol amphiphilic polymers

Sphingosine-grafted hyaluronic acid (HA-Sph) polymers were synthesized using the EDC/NHS coupling reaction [30]. To produce these polymers, 2 mg hyaluronic acid is dissolved in acidified milliQ-grade (MQ) water (pH 3.0-3.5) and EDC and NHS with 10-times unit moles of HA was added to the HA solution (Table 4). The solution was mildly stirred for 30 minutes, at room temperature (RT) to activate the carboxylic groups of the HA. Next, appropriate amounts of sphingosine in DMF (1 mg/mL), which equals to 50- and 100-unit mole% of HA, were added dropwise to the solution. The final water-to-DMF ratio of the solution is set to 1:1. The EDC/NHS coupling reaction continued for 24 hours at RT with constant stirring. The reaction is then quenched with MQ water and dialyzed with 3500 Da dialysis membranes against ethanol/water (1:1 v/v) ratio for at least 20 hours, and the dialysis media is changed several times. Afterwards, the HA-Sph solutions were dialyzed against dH₂O for at least 24 h several media changes. Finally, amphiphilic HA-Sph solutions are freeze-dried and stored at -20°C. The corresponding samples, based on Sph amounts, are entitled as HA-100Sph, and HA-50Sph, respectively.

Synthesis of cholesterol-grafted hyaluronic acid (HA-Chol) amphiphilic polymers were conducted similar to HA-Sph polymers. However, only EDC with 10-times molar excess to unit mole of HA was used. After 30 min stirring of solution, cholesterol with 75-unit mole% to HA was added to the HA solution. Reaction finished after 24 h and solution

was dialyzed to separate by-products and organic solvent (DMF). Products were freeze-dried and stored in the freezer until use.

HA-Sph and HA-Chol polymers were characterized by ^1H NMR and FTIR for confirming the grafting of Sph and Chol, as well as degree of substitutions on the HA chains. FTIR spectrums of polymers were collected between $600 - 4000\text{ cm}^{-1}$ by ATR-FTIR (Shimadzu IRAffinity-1S, Japan). Samples that dissolved in D_2O were measured by ^1H NMR. Measurements were conducted using Varian VNMRs 600 spectrometer operating at 400 MHz for ^1H resonance frequency and 5 mm NMR sample tubes [146]. Data acquisition and processing were conducted using Vnmrj 4.2 software and Bruker TopSpin 4.1.4 software, respectively.

Table 4. Amounts used for the synthesis of amphiphilic HA polymers and calculated degree of substitutions

HA-Sph	Unit moles of HA (μmol)	Moles of Sph (μmol)	Moles of EDC and NHS (μmol)	% Sph to the unit moles of HA	Degree of Substitutions (%)
HA-50Sph	2,5	1,25	25	50%	15,445
HA-100Sph	2,5	2,5	25	100%	25,235
HA-Chol	1,875	1,875	25 ^a	75%	4,555

a: Only EDC was used.

3.3. Box-Behnken design and response surface method for predictions of polymersome formulations

Three-factor Box-Behnken design with a categorical factor was utilized to study the effects of HA-Sph/HA-Chol polymer ratio, polymer/drug ratio, preparation temperature, and type of HA-Sph polymer on the response variables of size (nm), PDI, zeta potential, and EE% (encapsulation efficiency) of the peptide loaded polymersomes, as seen in Table 5. The design consisted of 30 base and total runs with six center points. The Minitab 19.2020.1 software (Minitab, LLC) was used to generate and analyze the design. According to BBD, 30 experimental observations were made by preparing peptide encapsulated polymersomes according to BBD with 100 $\mu\text{g/mL}$ and final volume of 500 μL . Then, the response surface method was used for generation of plots and contour plots

to visualize the effects of the factors and their interactions on the response. Response optimization was conducted for determining optimal factors for preparation of HRH peptide loaded HA-50Sph-Chol and HA-100Sph-Chol polymersomes that had maximum EE% and minimum size, PDI, and zeta potential responses (Table 6). Results of all observations and model fitting found at supporting information.

Table 5. Independent variables with levels for BBD optimization

Continuous factors	Variable	Low (-1)	Intermediate (0)	High (1)
A	HA-Sph/HA-Chol polymer ratio	2:1	1:1	1:2
B	Polymer/peptide ratio	4	6	8
C	Preparation temperature	25	50	75
Categorical factor				
D	Type of HA-Sph polymer	HA-50Sph	HA-100Sph	

Table 6. Response surface method optimization parameters

Response	Goal	Lower	Target	Upper	Weight
EE%	Maximum	50	99,000		1,0
Zeta (mV)	Minimum		-35,480	-20,0	0,1
PDI	Minimum		0,108	0,3	0,1
Size (nm)	Minimum		117,300	200,0	1,0

3.4. Preparation of HRH peptide encapsulated HA-Sph-Chol polymersomes

HA-Sph and HA-Chol amphiphilic polymers were blended and self-assembled into HA-Sph-Chol polymersomes via solvent switch (evaporation) method [96]. Amphiphilic polymers were completely dispersed in diethyl ether (100 µg/mL) with vortexing and 30 min sonication in sonic bath. The appropriate amount of polymers were dispersions were mixed according to predictions of BBD results and final ether volume was set to 9 mL. HRH peptide dissolved in MQ water and added dropwise to the polymer dispersions during vigorous stirring. Final ether-to-water ratio was adjusted to 9:1 (v/v). Diethyl ether was completely evaporated with constant vigorous stirring and HRH encapsulated

polymersomes (HRH@HA-50Sph-Chol and HRH@HA-100Sph-Chol) self-assembled in the aqueous phase. Finally, polymersomes were filtered by 0,45 μm cellulose acetate (CA) filter.

3.5. CAC determination of designed HA-Sph-Chol blends

Critical aggregation concentrations (CAC) of amphiphilic polymers were determined by serial dilution of polymersomes with pyrene as fluorescent probe. Amphiphilic polymers were dispersed in diethyl ether via vortexing and 30 min sonication. Appropriate amount of pyrene stock solution (in acetone with 10^{-3} M) was added to amphiphilic polymer dispersions. Polymer dispersions vigorously stirred and MQ water was added dropwise onto the polymer dispersions to obtain a final polymer solution of 0,25 mg/mL with 5×10^{-6} M pyrene. Ether evaporated with constant vigorous stirring. Serial dilutions of polymer solution were prepared and fluorescence spectrums of pyrene containing polymer dilutions were collected for each amphiphilic polymer and polymer blend with $\lambda_{\text{Ex}} = 333$ nm and $\lambda_{\text{Em}} = 350\text{-}500$ nm wavelength by fluorescence spectrofluorophotometer (RF-6000, Shimadzu).

3.6. Characterization of HA-Sph-Chol blend polymersomes

All solutions were filtered through 0,45 μm CA filter to eliminate any dust or aggregates. Hydrodynamic sizes and zeta potentials of polymersomes were measured by DLS (Zetasizer Pro, Malvern Panalytical). Measurements were taken in disposable DLS (ZEN0040) and zeta cuvettes (DTS1070) at 25°C in triplicates.

The morphologies of polymersomes were observed by SEM micrographs. 4 μL samples were deposited onto glass slides and allowed to dry at room temperature for SEM imaging. Afterward, the samples were coated with a layer of Au/Pd using a Sputter Coater 108 (Cressington) for 120 seconds at a current of 40 mA. The SEM micrographs were captured using a field emission scanning electron microscope (Supra 35VP FE-SEM, Zeiss) equipped with secondary electrons (SE) and in-lens detectors. Micrographs captured with in-lens detector and mixed signal of SE and in-lens detector (mixing ratio of 0.6), at an accelerating voltage of 3-5 kV.

3.7. HRH peptide encapsulation and release from polymersomes

After preparation of peptide loaded HA-50Sph-Chol, HA-100Sph-Chol, HA-50Sph, and HA-100Sph polymersomes, fluorescence intensities of HRH- FAM (HRH-6-carboxyfluorescein) peptide-loaded polymersomes were measured before and after filtering with 0.45 µm CA filter. Encapsulation efficiencies (EE%) and drug loading capacities (LC%) were calculated according to Eq. 1 and Eq. 2, respectively.

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Mass of encapsulated HRH peptide}}{\text{Mass of feeded HRH peptide}} \times 100 \quad (\text{Eq. 1})$$

$$\text{Loading capacity (\%)} = \frac{\text{Mass of encapsulated HRH peptide}}{\text{Mass of HRH encapsulated polymersomes}} \times 100 \quad (\text{Eq. 2})$$

FAM-HRH peptide-loaded polymersomes were put inside dialysis membranes (MWCO of 50 kDa) and dialyzed against 2 mL PBS (pH 7.4) at 37°C. Dialysis media was collected at each time points of 1, 2, 4, 10, 24, 48, 72, 96, and 168 hr., and fresh PBS was put against peptide-loaded polymersome solutions. Fluorescence intensities of $\lambda_{\text{Emission}} = 525$ nm of released HRH-FAM were measured at each time point with fluorescence spectrophotometer (RF-6000, Shimadzu). Cumulative drug releases from polymersomes were calculated using Eq. 3.

$$\text{Cumulative Release (\%)} = \frac{\text{Mass of released HRH peptides}}{\text{Mass of encapsulated HRH peptides}} \quad (\text{Eq. 3})$$

Drug release profiles of polymersomes were investigated by fitting them on pharmacokinetics model of the Korsmeyer Peppas kinetics model, as given in the Eq. 4 [1].

$$M_t/M_\infty = Kt^n \quad (\text{Eq. 4})$$

Additionally, drug release rates of polymersomes under enzymatic degradation with hyaluronidase were investigated. In this regard, hyaluronidase (Hyase) with 20 U/mL was added to HA-50Sph-Chol and HA-100Sph-Chol polymersome solutions on 0th, 1st, 2nd, 3rd, and 4th days of drug release. All of the measurements were done at least three times and results given as mean ± SE.

3.8. Cell culture

Human umbilical vein endothelial cells (HUVEC; CRL-1730, ATCC) were cultured in Endopan-3 media containing growth supplement kit, 10% FBS, 1% L-glutamine, and 1%

penicillin/streptomycin. HUVECs were incubated at 37°C, 5% CO₂, with media changes every 3-4 days until cells reached 70-80% confluency.

3.9. *In vitro* proliferation assay on HUVECs

The concentration-dependent effect of blend polymersomes on cell proliferation of human umbilical vein endothelial cells (HUVEC; CRL-1730, ATCC) was evaluated using the Cell Counting Kit-8 (CCK-8) assay. HUVECs were seeded into 96-well plates with 10000 cells/well seeding density and incubated overnight. Then, old media is discarded, and cells were incubated with blank (HA-50Sph-Chol), and HRH loaded polymersomes (HRH@HA-50Sph-Chol), which dissolved in fresh media at various concentrations (50 µg/mL, 100 µg/mL, 200 µg/mL, and 300 µg/mL) for 4 days. After incubation for four days at 37°C and 5% CO₂, the media was replaced with fresh media containing CCK-8. Absorbance at 460 nm was measured using a microplate reader, and cell viability was calculated using Eq. (5). Experiment was done in duplicate.

$$Cell\ Viability\ (\%) = \frac{OD_{sample}}{OD_{Control}} \times 100 \quad (Eq. 5)$$

4. Results and Discussion

4.1. Synthesis and characterization of HA-Sph and HA-Chol amphiphilic polymers

EDC/NHS coupling system is commonly used for coupling of carboxylic groups to amine groups [151, 173]. The synthesis of amphiphilic HA-Sph polymers involved a common EDC/NHS coupling reaction between carboxyl (COO⁻) groups of HA and amine (-NH₂) groups of sphingosines (Figure 19-A) [151]. Additionally, EDC generated O-acylisourea ester intermediate could react with various nucleophilic groups such as hydroxyl and amino groups [173]. When the NHS is incorporated in this reaction, O-acylisourea by-products are converted into more stable NHS-esters, which selectively react with amino groups. A previous study suggested that without using NHS, EDC activated carboxylic acids could predominantly react with hydroxyl groups [173]. In this regard, HA-Chol polymers were synthesized via esterification of carboxylic groups of HA and hydroxyl groups of cholesterol by using EDC as coupling reagent without any catalysts.

In the FTIR spectrums, the sharp peak around 1033 cm^{-1} in all samples corresponded to the C-O-C hemiacetal system of saccharide units in HA chain (Figure 19-B). One of the peaks in region II (around 1600 cm^{-1}) is related to the symmetric vibration of COO^- groups of in HA, which was divided into two separate peaks in amphiphilic HA-Sph and HA-Chol polymers, due to coupling reactions. In the region (ii) of HA-Sph polymers, the peaks around 1640 cm^{-1} and 1560 cm^{-1} are related to C=O stretch and in-plane N-H bending of secondary amide groups, respectively. Additionally, there is an N-H wagging peak at 667 cm^{-1} in the region (i) for the HA-Sph amphiphilic polymers. At region (iii), two sharp peaks were observed for HA-Sph and HA-Chol polymers which indicate the C-H stretch of methylene ($-\text{CH}_2-$) and methyl ($-\text{CH}_3$) of the Sph and Chol parts. At the region (iv) for HA-Chol, the peak at 1365 cm^{-1} was corresponding to C-H rocking. At the region (v), another sharp peak was observed at around 800 cm^{-1} which indicates the aromatic C-H out-of-plane bending of cholesterol. Lastly, peak observed at 668 cm^{-1} at the region (i) in HA-Chol sample was corresponding to C-H bending. Accordingly, FTIR spectrum indicates successful synthesis of HA-Sph and HA-Chol polymers.

The chemical structures amphiphilic polymers and grafting amounts were further investigated by ^1H NMR spectroscopy, as seen in Figure 19-C. The glycosidic protons of sugar units in HA were observed at chemical shifts between 3,0-4,1 ppm, and the N-acetyl ($-\text{NHCOCH}_3$) group's methyl protons were observed at 1,9 ppm for all samples [131, 151, 174]. Two additional peaks were observed in the NMR spectrum of sphingosine-conjugated samples (HA-Sph) that indicated methylene ($-\text{CH}_2-$) protons (1,165 ppm) and terminal methyl ($-\text{CH}_3$) protons (0,95 ppm) of sphingosine molecules [131, 139]. These additional peaks confirmed the successful grafting of sphingosines to the HA chain. In the case of HA-Chol polymer, methylene protons at the tail of Chol were observed between 1,01 – 1,18 ppm [175]. Similar to terminal methyl of Sph, terminal methyl groups of Chol were shown at 0,96 ppm [175]. Several protons of benzene rings were observed as small peaks between 1,2 ppm to 2,6 ppm [176]. Additionally, solvent peak of DMF was observed around 2,8 ppm for all amphiphilic polymers [177].

The degree of substitution (DS%) of amphiphilic polymers was defined as the number of sphingosine molecules grafted onto 100 sugar units of the HA main polymer chain [131]. DS% of HA-Sph polymers were calculated from the ratio of integrals of terminal methyl of Sph to N-acetyl methyl of HA. On the other hand, there are two terminal methyl in

cholesterol with similar chemical shifts. Thus, DS% of HA-Chol was calculated from half of the ratio of integrals of terminal methyl groups to methyl of N-acetyl group of HA. Accordingly, DS% of HA-50Sph, HA-100Sph, HA-Chol polymers were calculated as 15,45%, 25,24%, and 4,56%, respectively.



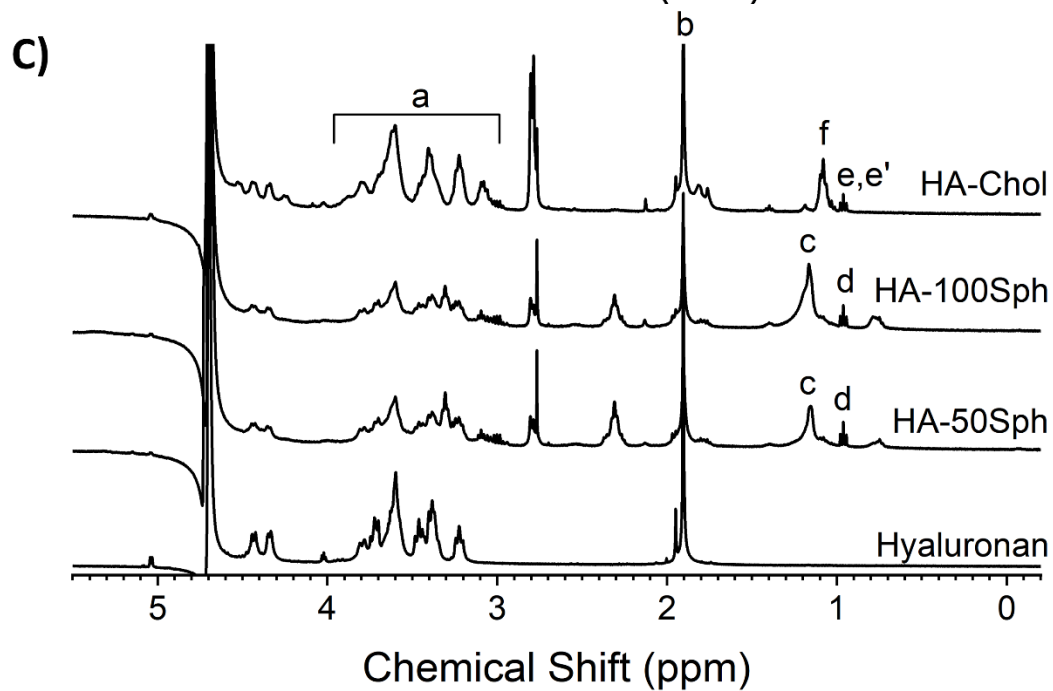
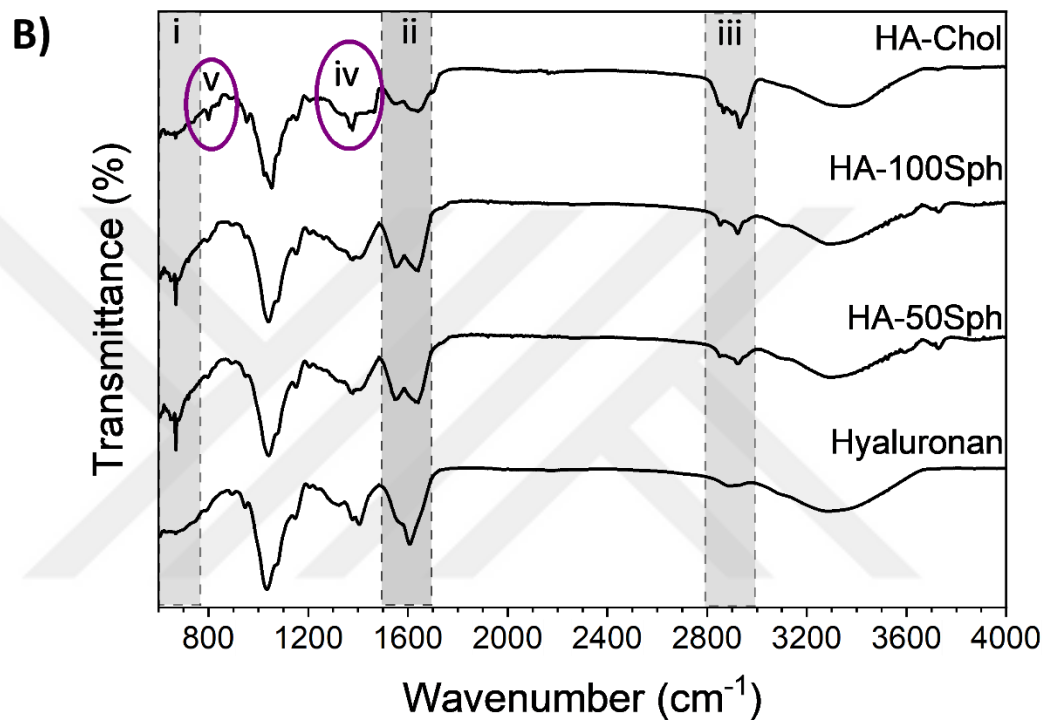
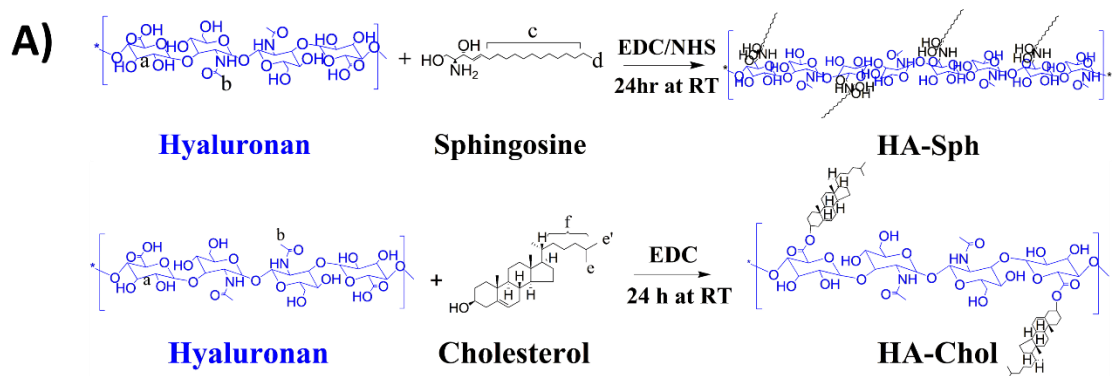


Figure 19. A) Reaction equilibrium for HA-Sph and HA-Chol amphiphilic polymer synthesis and chemical structures of hyaluronic acid, d-sphingosine, cholesterol, HA-Sph, and HA-Chol **B)** FTIR spectrums of HA-Chol, HA-100Sph, HA-50Sph, and 10kDa HA samples. **C)** ^1H NMR spectrums of HA-Chol, HA-100Sph, HA-50Sph, and 10kDa HA samples. Here, a, b, c, and d show corresponding peaks at 3,0 – 4,1 ppm, 1,9 ppm, 1,165 ppm, and 0,95 ppm. For HA-Chol sample, f indicates peaks between 1,01 – 1,18 ppm and peaks at e and e' correspond to 0,96 ppm.

4.2.Box-Behnken design (BBD) for predictions of polymersome formulations

HA-Sph and HA-Chol amphiphilic polymers were blended at various ratios for preparation of HRH peptide loaded polymersomes via solvent switch method, according to design parameters of BBD method. Experimental observations of variable parameters including HA-Sph to HA-Chol polymer ratio, polymer-to-drug ratio, and preparation temperature were introduced to Box-Behnken design (Table 7). The effects of variable parameters on blend polymersomes properties were represented as contour plots for each experimental response including PDI, size, zeta potential, and EE%, as seen in Figure 19. The optimum preparation temperature was determined to be 25°C.

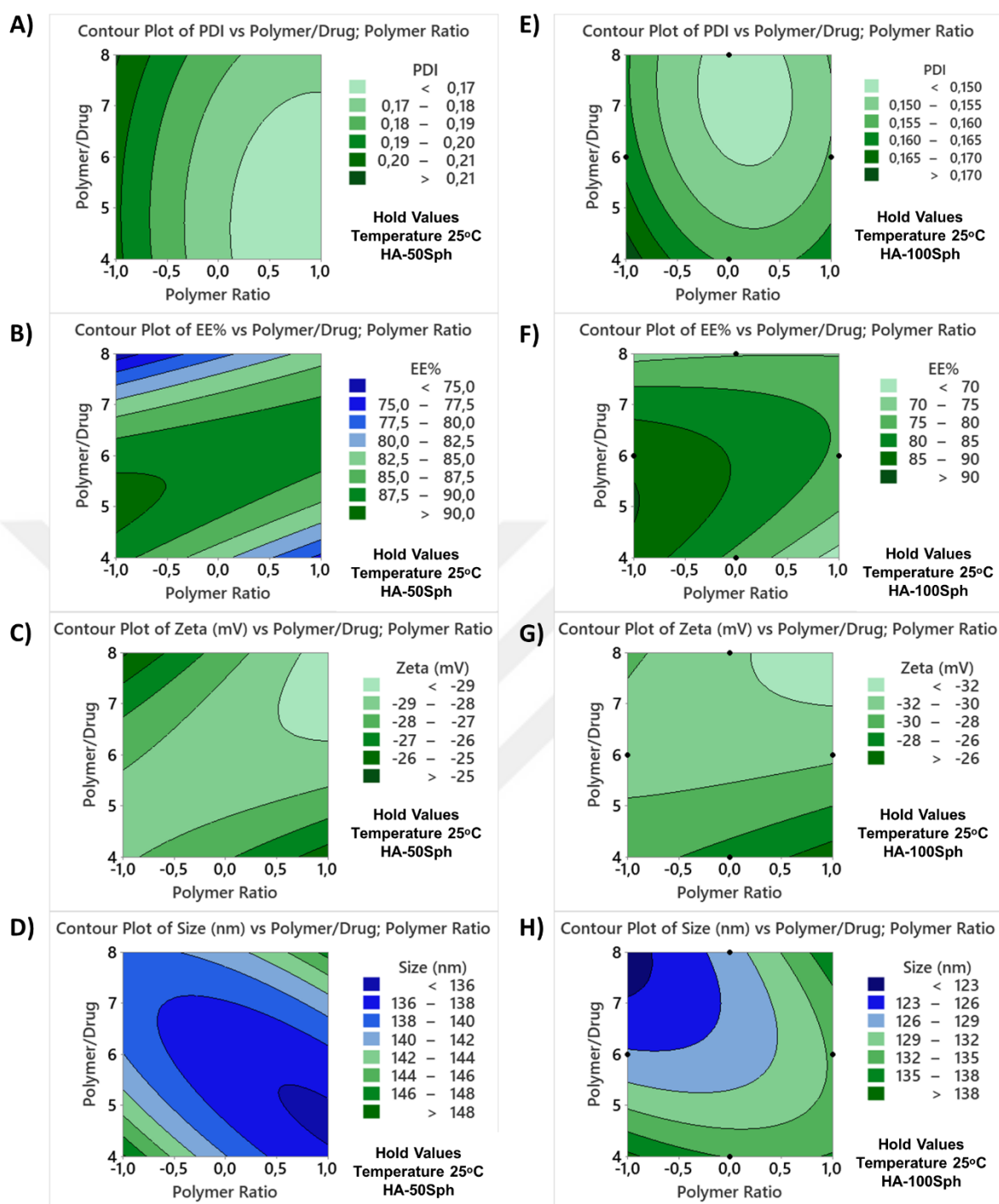


Figure 20. Contour plots of dependent variables including PDI, encapsulation efficiency (EE%), zeta, and size which are depending on polymer/drug ratio and amphiphilic polymer ratio as a result of the Box-Behnken method. **A-D)** Contour plots for HA-50Sph-Chol blend polymersome at constant temperature of 25°C. **E-H)** Contour plots for HA-100Sph-Chol blend polymersome at constant temperature of 25°C.

Table 7. Three-factor three-level Box-Behnken design with experimental observations and fitted responses

	Experimental	Fitted
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Polym er Ratio	Tempe rature (°C)	Polymer/ Drug	HA- Sph	Size (nm)	PDI	Zeta (mV)	EE%	FIT (Size)	FIT (PDI)	FIT (Zeta)	FIT (EE%)
0	25	4	50	140,10	0,15	-23,71	69,05	140,08	0,17	-27,12	83,28
1	25	6	50	151,90	0,19	-29,18	89,13	137,53	0,16	-28,76	88,24
-1	25	6	50	152,00	0,20	-30,68	86,78	140,03	0,20	-27,78	88,97
0	25	8	50	140,50	0,19	-27,43	81,10	141,15	0,18	-27,48	79,17
0	25	4	100	127,70	0,18	-29,16	83,04	134,81	0,16	-27,18	78,55
-1	25	6	100	117,30	0,18	-29,37	93,05	126,40	0,16	-30,41	88,60
1	25	6	100	129,90	0,13	-30,99	74,43	132,42	0,16	-30,37	79,26
0	25	8	100	120,70	0,14	-30,24	84,06	127,68	0,15	-31,66	74,56
1	50	4	50	139,00	0,13	-30,80	70,83	159,07	0,15	-26,49	57,33
-1	50	4	50	168,10	0,22	-27,50	81,91	165,64	0,20	-29,23	64,30
0	50	6	50	150,40	0,17	-31,85	74,29	156,15	0,16	-30,75	69,85
0	50	6	50	155,40	0,15	-28,03	66,98	156,15	0,16	-30,75	69,85
0	50	6	50	150,50	0,16	-29,27	85,69	156,15	0,16	-30,75	69,85
-1	50	8	50	141,30	0,18	-29,73	34,44	156,07	0,19	-28,12	54,21
1	50	8	50	161,50	0,14	-31,81	67,25	168,39	0,14	-32,63	68,81
1	50	4	100	176,60	0,18	-20,06	40,39	161,30	0,15	-23,99	40,20
-1	50	4	100	161,10	0,14	-29,06	45,66	159,35	0,17	-27,75	55,78
0	50	6	100	165,60	0,13	-30,55	45,89	150,02	0,13	-30,82	57,08
0	50	6	100	147,40	0,15	-32,24	53,48	150,02	0,13	-30,82	57,08
0	50	6	100	149,20	0,12	-32,76	54,46	150,02	0,13	-30,82	57,08
1	50	8	100	165,10	0,13	-35,48	45,87	162,42	0,12	-34,24	51,80
-1	50	8	100	161,10	0,14	-28,76	51,90	141,57	0,14	-30,76	45,82
0	75	4	50	172,90	0,19	-30,03	55,96	161,83	0,18	-28,27	85,10
-1	75	6	50	148,50	0,21	-30,72	103,84	155,20	0,20	-31,19	91,05
1	75	6	50	169,00	0,15	-30,36	95,03	163,45	0,14	-31,97	99,41
0	75	8	50	176,30	0,13	-33,13	97,73	160,50	0,15	-32,95	90,60
0	75	4	100	159,60	0,17	-23,94	81,89	163,03	0,17	-24,23	64,19
1	75	6	100	156,40	0,11	-29,24	76,38	164,82	0,13	-29,48	74,25

-1	75	6	100	142,90	0,16	-29,14	65,67	148,05	0,16	-29,72	74,51
0	75	8	100	144,80	0,14	-34,27	72,43	153,51	0,11	-33,02	69,81

All the responses obtained from individual formulations of polymersomes were fitted to response surface method (RSM) and the models for HA-50Sph-Chol and HA-100Sph-Chol polymersomes were formulated (Figure 21). The models are then used to predict optimum parameters for HRH loaded polymersomes which have maximum EE% and minimum size, zeta potential, and PDI, as given in Table 5. Additionally, composite desirability is a method to optimize multiple responses in response surface methods such as BBD. Composite desirability could be between 0 as undesirable and 1 as desirable [178]. The composite desirability of the model's predictions was 0,864 for HA-50Sph-Chol and 0,899 for HA-100Sph-Chol polymersomes, which showed that parameters may give highly favorable experimental results. The polymersomes were prepared and characterized, according to modelled parameters. The results demonstrated that the predicted and experimental responses were highly comparable with each other (Table 8). Especially, HA-100Sph-Chol polymersomes have highly comparable predicted and experimental results for size, zeta potential, and EE%. On the other hand, results of some experimental responses slightly vary from predicted results. For instance, the EE% value of HA-50Sph-Chol polymersomes was calculated as 79,8% however, predicted value was 89,18%. Nonetheless, all experimental results were in the 95% confidence interval (CI) of the predictive model.

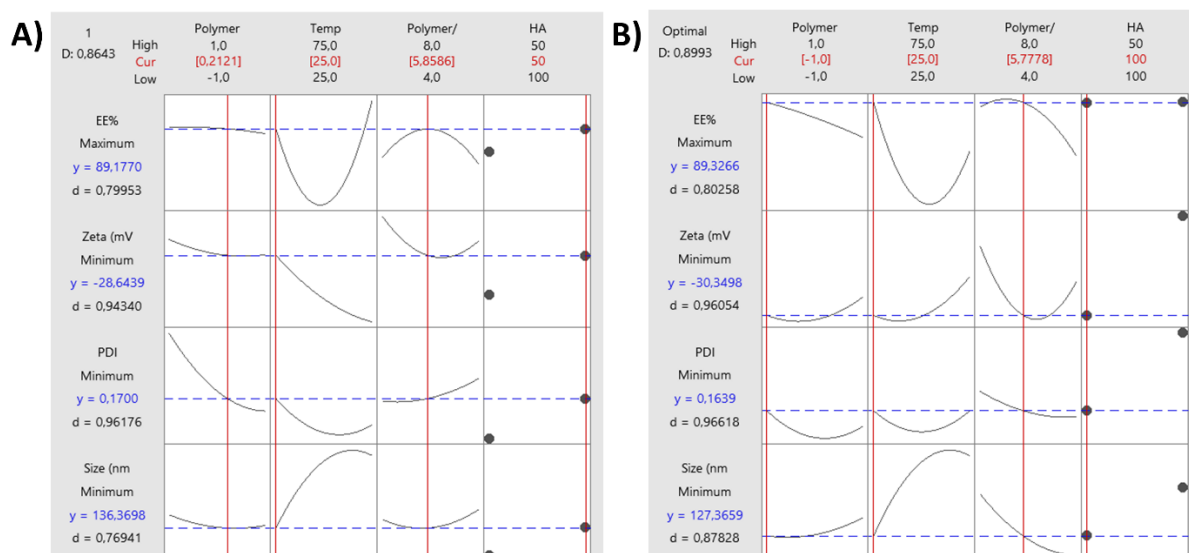


Figure 21. RSM response optimization graphs. For optimum parameters (given as red lines), maximum or minimum points of each response (given as blue dash) determined. **A)** HA-50Sph-Chol, **B)** HA-100Sph-Chol (Graphs were obtained from Minitab software)

Table 8. Results of HA-Sph-Chol blend polymersomes prepared according to solution of response surface method (n=4). (All data presented here are in the 95% confidence interval (CI) of the prediction of BBD design.)

	HA-Sph	Polymer Ratio (HA-Sph/HA-Chol)	Temperature (°C)	Polymer/Drug Ratio	Size (nm)	Zeta (mV)	PDI	EE%	LC%	Composite Desirability
Optimized responses	HA-50Sph-Chol	1:1,21	25	5,86	136,4±7,2	-28,64 ±1,37	0,170±0,012	89,18±7,9	-	0,864
	HA-100Sph-Chol	2:1	25	5,78	127,4±9,7	-30,35±1,83	0,164±0,017	89,33±10,6	-	0,899
Experimental results	HA-50Sph-Chol	1:1,21	25	5,86	141,3±12,2	-25,8±6,2	0,140±0,044	79,8±6,8	14,28	-
	HA-100Sph-Chol	2:1	25	5,78	127,9±21,1	-29,54±4,6	0,210±0,054	88,9±8,7	17,84	-

4.3. CAC determination of designed HA-Sph-Chol blends

One of the defining characteristics of amphiphilic polymers is the critical aggregation concentration (CAC) [35]. The CAC is the specific concentration, at which amphiphilic polymers begin to self-assemble and form aggregates such as polymersomes [93]. Pyrene exhibits five distinct fluorescence peaks between 374 nm and 410 nm with variable intensities corresponding to polarity of the molecular environment, in its monomeric

state. The CACs of HA-50Sph, HA-100Sph, HA-Chol, HA-50Sph-Chol, and HA-100Sph-Chol were calculated from the intersection points of two linear regression lines of I_{374}/I_{384} ratio and were found to be 22,04 $\mu\text{g/mL}$, 13,01 $\mu\text{g/mL}$, 22,0 $\mu\text{g/mL}$, 19,04 $\mu\text{g/mL}$, and 15,18 $\mu\text{g/mL}$, respectively (Figure 22-A and C). In the HA-Sph amphiphilic polymers, the CAC values are inversely proportional to the degree of substitution (DS%) of the polymers. As the DS% increases, the CAC decreases [35]. According to Pedro *et al.*, the hydrophobicity of the polymer chains increases with an increase in DS%, resulting in stronger self-aggregation due to more compact hydrophobic interactions [155]. In our CAC data suggests, HA-Sph and HA-Chol polymers combined in a highly compatible manner, as demonstrated by intermediate CAC values of HA-50Sph-Chol and HA-100Sph-Chol blend polymersomes.

According to Xu *et al.*, the intensity ratio of the excimer peak to the peak at 374 nanometers (I_{Ex}/I_{374}) increases rapidly after the CAC of amphiphilic molecules is reached, when bilayer or vesicular structures form [156]. In our study, the I_{Ex}/I_{374} peak ratios of pyrene increased significantly after passing the CAC, in all of the samples. Especially, HA-50Sph, HA-50Sph-Chol, and HA-100Sph-Chol samples demonstrated significant increase of I_{Ex}/I_{374} , due to pyrenes in the bilayers of these polymersomes are closer to each other (Figure 22-B). This indicates bilayers of HA-50Sph, HA-50Sph-Chol, and HA-100Sph-Chol polymersomes are thinner compared to HA-100Sph and HA-Chol polymersomes.

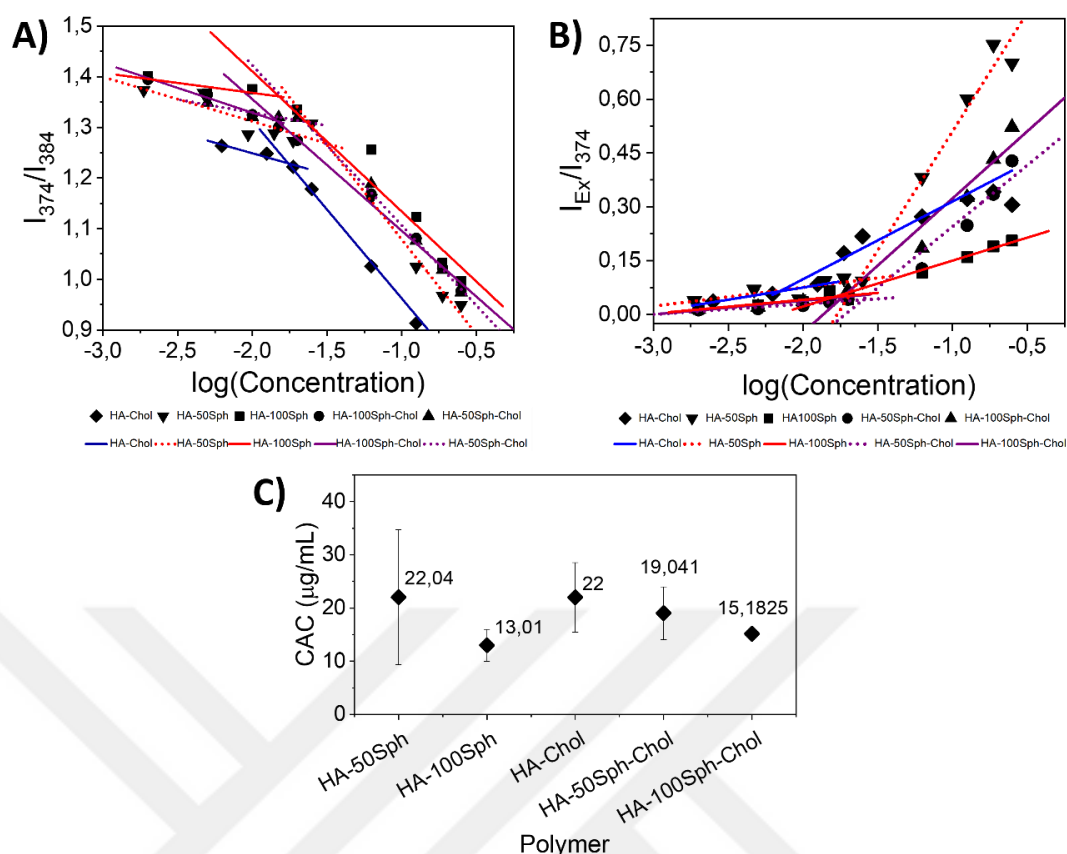


Figure 22. Determination of critical aggregation concentrations (CAC) of amphiphilic polymers via fluorescence probe method with pyrene, **A)** ratios of pyrene peak intensities of 374 nm and 384 nm versus $\log(\text{Concentration})$ **B)** the ratios of pyrene peak intensities of excimer peak (~470 nm) to 374 nm versus $\log(\text{Concentration})$. **C)** Corresponding CAC values of HA-50Sph, HA-100Sph, HA-Chol, HA-50Sph-Chol, and HA-100Sph-Chol amphiphiles.

4.4. Characterization of HA-Sph-Chol blend polymersomes

Blend polymersomes of HA-50Sph-Chol and HA-100Sph-Chol that are encapsulated with HRH peptide prepared with solvent switch method according to BBD parameters, as seen in Table 6. The polymersomes are polymersomes had sizes of 141,3 nm and 127,9 nm, and PDI values of 0,140 and 0,210 for HA-50Sph-Chol and HA-100Sph-Chol polymersomes, respectively. Compared to each other, HA-100Sph-Chol polymersomes have smaller sizes, however, the size distribution of HA-50Sph-Chol polymersomes are more homogeneous. In the literature, nanocarrier size <200 nm is considered favorable for ocular drug delivery applications, and PDI values <0,3 is indicating homogeneous particle size distribution [2, 108]. Additionally, nanocarriers with zeta potentials of < -20 mV and > +20 mV are considered to have high colloidal stabilities [2]. In our study, peptide loaded blend polymersomes have zeta potentials of -25,8 mV and -29,54 mV for

HA-50Sph-Chol and HA-100Sph-Chol, respectively. Vitreous humor consists of a highly negatively charged network with pore sizes around 550 nm, which is made from collagen and hyaluronic acid. Therefore, negative or neutral charged nanocarriers with sizes <550 nm could cross the vitreous [179]. Accordingly, both blend polymersomes could be favorable nanocarriers for dual drug delivery to posterior segment of the eye.

The HRH loaded HA-50Sph and HA-100Sph polymersomes had sizes, PDI values, and zeta potentials of $181,8 \pm 1,2$ nm, 0,28, and $-29,7 \pm 1,2$ mV and $174,5 \pm 6,0$ nm, 0,18, and $-24,8 \pm 0,4$ mV, respectively. When comparing to HA-50Sph polymersomes, HA-50Sph-Chol polymersomes are demonstrating lower size and PDI values however, zeta potential was slightly increased. Similarly, size of HA-100Sph-Chol polymersomes are reduced, meanwhile the homogeneity of polymersomes slightly decreased compared to HA-100Sph polymersome. The incorporation of HA-Chol polymers into the formulation might enhance the compactness of polymersomes.

In addition, SEM micrographs of blend polymersomes revealed the nanoparticles with sizes <200 nm (Figure 23). A highly homogeneous size distribution of polymersomes is further confirmed by SEM micrographs. Moreover, the edges of the nanoparticles in the SEM micrographs of in-lens detector are brighter than the center of polymersome. This phenomenon might indicate the collapsed and flattened aqueous core of the polymersomes [11].

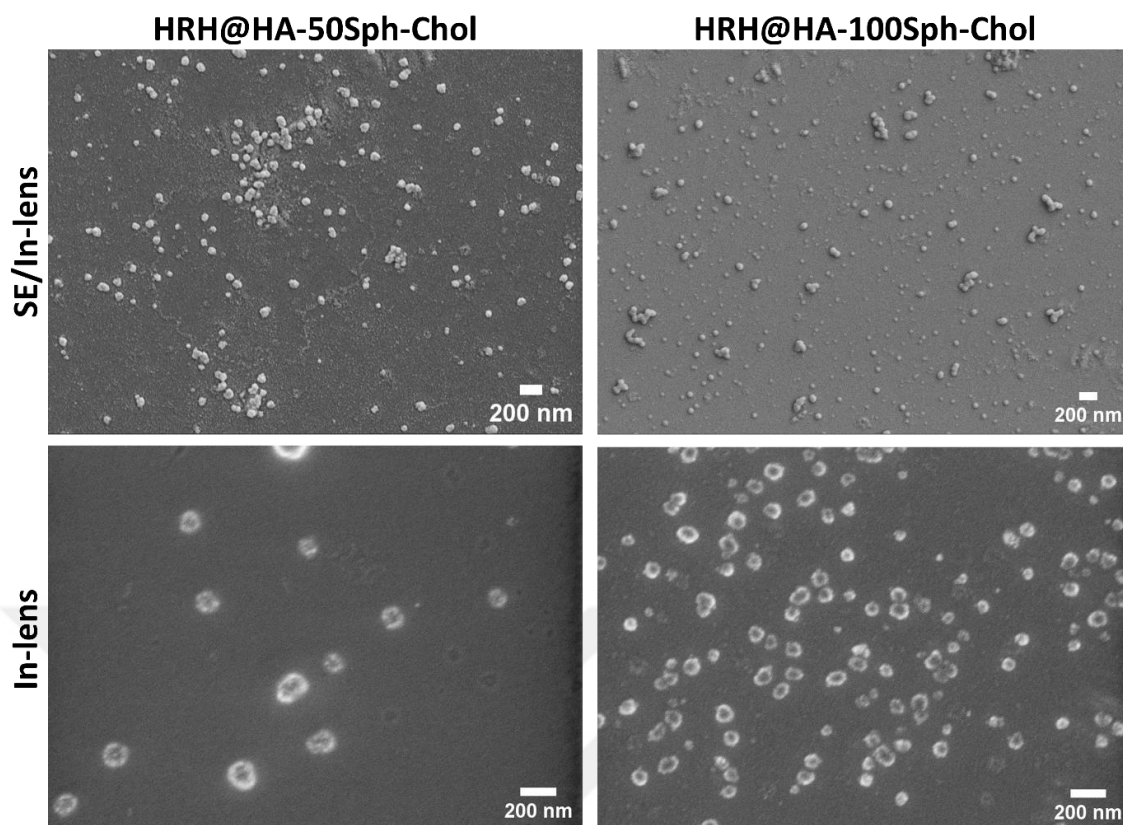


Figure 23. SEM micrographs of HRH peptide encapsulated blend polymersomes. SE/In-lens micrographs were taken by mixing signal of two detector with ratio of 0,6 with 50000x magnification. Micrographs in the below row are taken by in-lens detector with 100000x magnification. (Scale bars = 200 nm)

4.5. HRH peptide encapsulation and release from polymersomes

Polymersomes designed by BBD method showed high encapsulation efficiency of HRH peptides. RSM method's predicted EE% values of $89,18 \pm 7,9\%$ and $89,33 \pm 10,6\%$ meanwhile EE% of experimental results calculated as $79,8 \pm 6,8\%$ and $88,9 \pm 8,7\%$ for HA-50Sph-Chol and HA-100Sph-Chol polymersomes, respectively. When compared to predicted and experimental results, HA-50Sph-Chol showed a high difference in EE% despite the fact, the difference is not significant. Moreover, HRH encapsulated HA-50Sph and HA-100Sph polymersomes were prepared with EE% of $74,8 \pm 8,1\%$ and $83,9 \pm 3,6\%$, respectively. The blend polymersomes demonstrated higher EE% compared to their HA-Sph polymersome counterparts. The loading capacities of HA-50Sph-Chol and HA-100Sph-Chol were calculated as 14,28% and 17,84%, respectively.

Release kinetics of HRH peptide encapsulated blend polymersomes investigated and compared with release kinetics of the polymersome made from HA-50Sph and HA-100Sph. All polymersomes demonstrated sustained drug release up to a week, compared to free HRH peptide in solution that released completely in 4 days. HA-50Sph-Chol, HA-100Sph-Chol, HA-50Sph, and HA-100Sph were released $35,2 \pm 6,5\%$, $33,4 \pm 4,6\%$, $52,8 \pm 8,2\%$, and $44,3 \pm 6,2\%$, respectively (Figure 24). These results suggest that blend polymersomes have prolonged peptide release, which could be beneficial for long-term drug release. Additionally, the release behavior of polymersomes under enzymatic degradation was investigated by incorporating 20 U/mL hyaluronidase in the polymersomes solution daily until the 4th day of drug release. HA-50Sph-Chol, HA-100Sph-Chol, and HA-50Sph polymersomes demonstrated enhanced release rate with enzymatical degradation, and $70,5 \pm 8,0\%$, $64,4 \pm 7,9\%$ and $98,7 \pm 6,1\%$ of the encapsulated peptides released at 7 days. Amounts of peptides released from polymersomes were doubled with enzymatical degradation. Compared to blend polymersomes, HA-50Sph polymersomes completely released the peptides in 7 days.

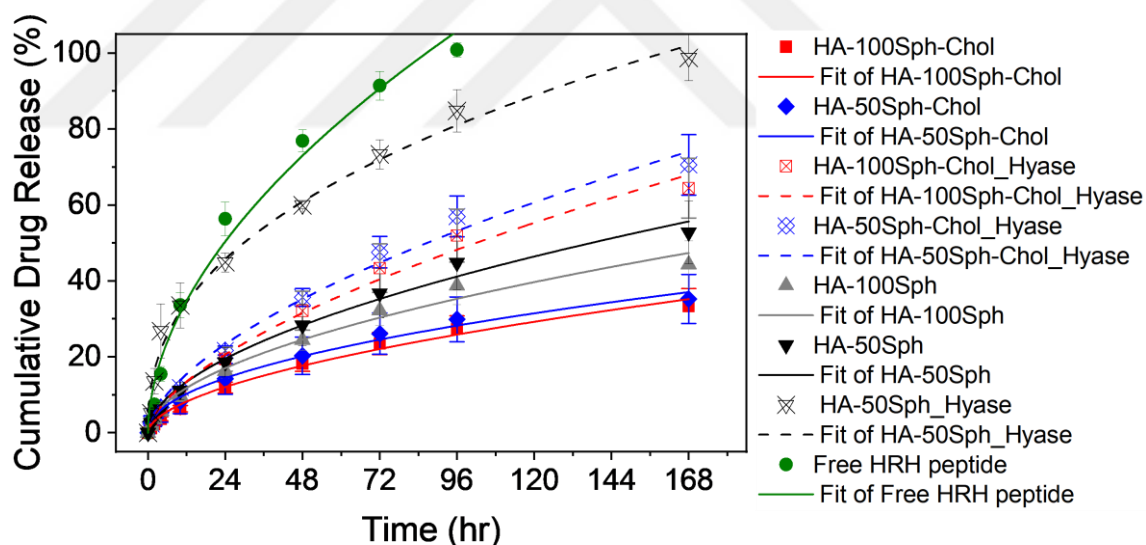


Figure 24. Release of HRH peptide from polymersomes with and without the influence of daily 20 U/mL hyaluronidase (Hyase). Lines corresponding to fitting of Korsmeyer-Peppas pharmacokinetics model. (n=3-4; mean $\pm$ SE)

The drug release profiles of peptide-loaded polymersomes were found to conform to the Korsmeyer-Peppas kinetic model, and the values of the kinetic constants K (h^{-1}), which represents the rate constant, and n , which is the coefficient for the release mechanism, as well as the coefficient of determination (R^2), are presented in Table 9 [1, 180]. As anticipated, the highest kinetic constants were observed in the enzymatically degraded

polymersomes, specifically HA-50Sph with a value of 12,32 h⁻¹. The values of K for the hyaluronidase-degraded HA-50Sph-Chol and HA-100Sph-Chol were 3,53 h⁻¹ and 2,93 h⁻¹, respectively. In contrast, the blended polymersomes without enzymatic degradation exhibited the lowest release rates of 3,06 h⁻¹ and 2,09 h⁻¹ for HA-50Sph-Chol and HA-100Sph-Chol, respectively. The coefficient (K) of release mechanism indicates whether drug release occurs by Fickian diffusion ($n < 0,43$) or non-Fickian or anomalous transport ($0,43 < n < 0,85$) for spherical polymeric particles[181]. In this case, the polymersomes displayed non-Fickian transport, which is characterized by drug release via swelling and/or erosion of the polymer matrix, in addition to Fickian diffusion [180, 181].

Table 9. Korsmeyer-Peppas pharmacokinetic model analysis and fitting values of the drug release of polymersomes

Sample	R ²	K (h ⁻¹) (± SE)	n (± SE)
HA-50Sph-Chol	0,993	3,06±0,33	0,49±0,023
HA-100Sph-Chol	0,991	2,09±0,29	0,55±0,030
HA-50Sph-Chol_Hyase	0,992	3,53±0,51	0,59±0,031
HA-100Sph-Chol_Hyase	0,990	2,93±0,50	0,61±0,037
HA-50Sph	0,991	3,51±0,47	0,54±0,029
HA-100Sph	0,988	3,20±0,50	0,53±0,034
HA-50Sph_Hyase	0,989	12,32±1,31	0,41±0,024

4.6. *In vitro* proliferation assay on HUVECs

In the current study, an anti-angiogenic peptide, HRH peptide was encapsulated into blend polymersomes for further enhancing the treatment efficiency of drug formulation and prolonged the therapeutic effect by sustained release of HRH peptides. To minimize the potential toxicity of Sph against ocular tissues, blend polymersome with lowest Sph amount (HA-50Sph-Chol) was chosen for the *in vitro* proliferation assay.

HUVEC proliferation was inhibited by both blank polymersomes (HA-50Sph-Chol), and HRH loaded blend polymersomes (HRH@HA-50Sph-Chol) with increased polymersome concentration, as seen in Figure 25. Polymer concentration at 300 µg/mL, cell viability was measured as 84,6% and 71,0% for blank and peptide loaded polymersomes, respectively. The concentration of HRH peptide is approximately 21,6

μM , at this polymer concentration. In the previous study of Chen *et al.*, the bioactivity of HRH peptide against corneal neovascularization was enhanced through preparation of nanofibrous structure of amphiphilic HRH peptide (NapFFHRH) [169]. In the study, they indicated that NapFFHRH significantly inhibited human retinal microvascular endothelial cell (HRMEC) and HUVEC proliferation at concentrations of $50\ \mu\text{M}$ [169]. Compared to effective concentrations, in our study, HRH@HA-50Sph-Chol polymersomes demonstrated higher anti-proliferative effect against endothelial cells. Therefore, in this concentration, HRH@HA-50Sph-Chol demonstrated significant inhibition of cell proliferation compared to blank polymersomes.

Moreover, our previous study demonstrated the high potential of sphingosine grafted hyaluronan polymersomes for inhibiting HUVEC proliferation and reducing the tube formation (Figure 13 and 17). In current study, around $50\ \mu\text{M}$ Sph was incorporated to HRH@HA-50Sph-Chol, when polymer concentration is $300\ \mu\text{g/mL}$. In previous study, effective HA-50Sph polymersome was contained Sph around $55\ \mu\text{M}$, and HUVEC viability in this concentration was around 78,9%, which is comparable to blank HA-50Sph-Chol polymersomes in the current study. Meanwhile, HRH encapsulation further inhibited the HUVEC proliferation. Consequently, HRH peptide encapsulation and Sph grafting synergistically improved the anti-angiogenic efficiency of blend polymersomes.

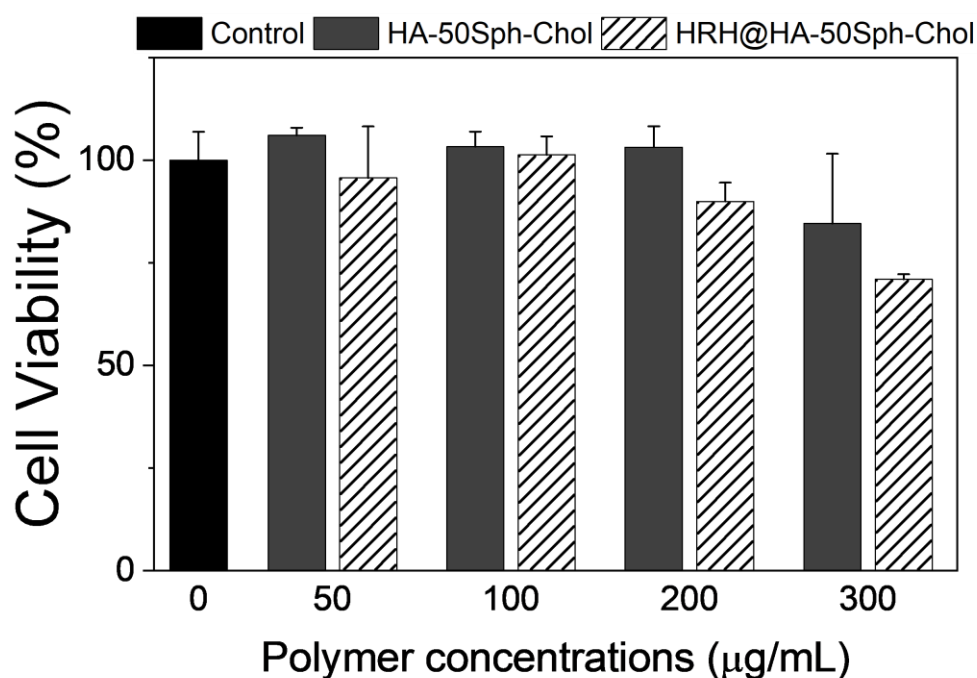


Figure 25. Effect of blank (HA-50Sph-Chol) and HRH peptide encapsulated (HRH@HA-50Sph-Chol) blend polymersomes on HUVEC proliferation *in vitro* for 4 days. HRH peptide amount in HRH@HA-50Sph-Chol was approximately 3,6 μM , 7,2 μM , 14,4 μM , and 21,6 μM for 50 $\mu\text{g/mL}$, 100 $\mu\text{g/mL}$, 200 $\mu\text{g/mL}$, and 300 $\mu\text{g/mL}$ polymer concentration, respectively. (n=2; mean $\pm$ SD)

5. Conclusion

As a conclusion, our findings indicate that the HA-50Sph-Chol blend polymersomes possess superior anti-angiogenic efficiency due to effect of HRH peptide and sphingosine and could serve as an alternative ocular drug delivery carrier for the treatment of ocular neovascularization. The design parameters for these polymersomes were optimized using a three-factor, three-level Box-Behnken design approach with response surface method. The optimized blend polymersomes demonstrated maximum encapsulation efficiency for the HRH peptide and minimal size, PDI, and zeta potential, making them more effective as ocular drug carriers. The sustained release of the HRH peptide from these polymersomes was observed for up to 7 days, and enzymatic degradation of the blend polymersomes doubled the amount of released peptides. The biological activity of the HRH peptide encapsulated in the HA-50Sph-Chol polymersomes was confirmed through *in vitro* experiments, which showed a synergistic anti-angiogenic effect of the dual drug loading. However, further studies are needed to fully understand the potential of these peptide-loaded polymersomes as a novel treatment option for ocular neovascularization-based diseases, including wet AMD.

Future Perspectives

The results revealed that sphingosine is highly anti-angiogenic and significantly reduced HUVEC proliferation *in vitro*. Additionally, the promising potential of HA-50Sph polymersomes for topical ocular drugs are demonstrated by an *ex vivo* whole porcine eye ocular penetration study. However, further investigations are needed for confirming the efficiency of HA-Sph polymersomes as novel therapeutics for ocular neovascularization. In this regard, comparing the effects of HA-Sph polymersomes against commercially available anti-VEGF antibodies such as ranibizumab must be examined to determine the therapeutic efficiency. Furthermore, the investigation for proliferation inhibition effect of HA-Sph polymersomes at elevated VEGF conditions should be evaluated. Our developed HA-Sph polymersomes behave differently in ARPE-19 and HUVEC cells. Therefore, cellular uptake of HA-Sph polymersomes to HUVEC and ARPE-19 cells should be evaluated for better understanding the bioactivity of polymersomes. The safety profile of the developed polymersomes is another subject of concern. Accordingly, further evaluations are needed for determination of working mechanism of HA-Sph polymersomes against ocular neovascularization.

At the second part of the thesis, the synergistic anti-angiogenic effect of HRH peptide loaded polymersomes demonstrated by higher inhibition of proliferation of HUVECs. Similarly, investigations are required for understanding the mechanism and potential of the HA-Sph-Chol polymersomes as dual drug loaded ocular carriers. Especially, bioactivity of polymersomes against ARPE-19 cells and cellular uptake of the HRH-loaded polymersomes should be evaluated. Drug loaded polymersomes production in the large scale is currently a problem due to limitations of solvent switch method. The cost of organic solvent and time-consuming production process are also the limitations that should be overcome. Production method of polymersomes should be further modified and developed for large scale production of polymersome and reducing the cost of production. However, compared to cost of commercially available anti-VEGF antibodies that are over 1000\$ per doses, the overall cost of sphingosine, hyaluronan, cholesterol, and HRH peptide, is less expensive.

To this date, various strategies have been explored in ocular drug delivery, yet achieving optimal treatment efficacy remains a persistent challenge. The applications of polymersomes in ocular drug delivery has been relatively limited and only a few studies

are available. Moreover, the metabolism and anti-angiogenic mechanism of sphingosine for the treatment of ocular diseases has not been investigated. In this regard, this study has the potential to be at the forefront of the treatment of ocular neovascularization diseases.



APPENDIX

Publications

Yetisgin, A.A., Durak, S., Kutlu, O., & Cetinel, S., "Hyaluronan-Sphingosine Polymersomes for Treatment of Ocular Neovascularization: Synthesis and Evaluation", under revisions, *Molecular Bioscience*, Wiley.

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Proceedings

E-MRS Spring 2023 Meeting (40th Anniversary), “*Development of Topical Drug Formulations for the Treatment of Ocular Neovascularization*”. Strasbourg, France

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