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Full Paper

Hyaluronic Acid Graft Copolymers with Cleavable Arms as Potential Intravitreal Drug Delivery Vehicles¹

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Treatment of retinal diseases currently demands frequent intravitreal injections due to rapid clearance of the therapeutics. The use of high molecular weight polymers could extend the residence time in the vitreous and prolong the injection intervals. This study reports a water soluble graft copolymer as a potential vehicle for sustained intravitreal drug delivery. The copolymer features a high molecular weight hyaluronic acid (HA) backbone and poly(glycerol glycerol) (PGG) side chains attached via hydrolysable ester linkers. PGG, a polyether with 1,2-diol groups in every repeating unit available for conjugation, serves as a detachable carrier. The influence of synthesis conditions and incubation in physiological media on the molecular weight of HA is studied. The cleavage of the PGG grafts from the HA backbone is quantified and polymer-from-polymer release kinetics are determined. The biocompatibility of the materials is tested in different cell cultures.

¹ **Supporting Information** is available online from the Wiley Online Library or from the author.

1. Introduction

Posterior segment ocular diseases are a leading cause of visual impairment and blindness in the aging societies.^[1,2] Due to the specific barriers of the eye, these diseases are commonly treated by intravitreal injections every 4 - 8 weeks.^[3-5] Sustained release systems have been proposed to prolong the intravitreal injection intervals and to deliver drugs at controlled levels over prolonged periods of time. These systems usually consist of either polymer particles or solid polymer implants.^[5-8] A water soluble delivery vehicle could overcome certain limitations associated with particles or implants, such as visual disturbances, aggregation, increased ocular pressure and other adverse ocular effects.^[9-13] Furthermore, drug release and degradation rate of implants or polymer particles need to be carefully synchronized to avoid the accumulation of “ghost” devices inside the eye.^[14]

Water-soluble graft copolymers are promising carrier materials for drug and gene delivery applications.^[15,16] Especially graft copolymers with polysaccharide backbones are desirable due to their biocompatibility, biodegradability and unique bioactivity.^[17] Polysaccharides typically feature a very high molecular weight, which is beneficial for increasing the retention times of carriers in the body.^[16,18,19] Hyaluronic acid (HA) is a naturally occurring polysaccharide and a main constituent of the vitreous humor.^[20] Our approach utilizes a high molecular weight HA backbone, which is grafted with shorter poly(glyceryl glycerol) (PGG) side chains. PGG is a multi-functional polyether consisting of a polyethylene glycol (PEG) backbone and 1,2-diol moieties in every repeating unit that can be functionalized with a variety of drugs, probes and possibly targeting ligands.^[21] In addition to PGG being versatile in terms of functionalization, the multitude of hydroxyl groups in this kind of polyethers improve their biocompatibility and lower their immunogenicity compared to PEG.^[22]

The HA-PGG graft copolymer is intended as a delivery vehicle for a potential two-stage drug release (**Scheme 1**). First, the grafts, which could carry the drugs, are slowly released from the HA backbone

in the vitreous. Second, with the use of special linkages the potential drugs could be released from grafts after cell uptake.^[23] The release of drug-carrying grafts prior to the release of drugs would reduce their cytotoxic effects in the eye and minimize drug clearance through the blood-ocular barriers.^[14] Compared with nanoparticles, short polymer chains have higher diffusivity and higher permeation into the retinal layers.^[9,14]

To achieve long retention times in the vitreous, it is crucial to preserve the length of the polysaccharide backbone. Therefore we investigated the effect of synthesis and incubation in physiological conditions on the chain length of HA and developed a new esterification route. We further compared methods to quantify the release of the grafts from the HA backbone. It is of fundamental interest to establish procedures for the quantification of polymer-from-polymer release, a topic that is rarely covered in the literature. A knowledge of the release kinetics is also essential in order to predict the action time of the delivery vehicle. Hence, we evaluated commonly used release setups for the study of soluble polymeric systems. Finally, we report the biocompatibility of the graft copolymer and PGG in different cell cultures.

2. Experimental Section

2.1. Materials

Research grade sodium hyaluronate (HA, 752 kDa according to manufacturer) was obtained from Lifecore Biomedical (U.S.) and used as received. All other chemicals and solvents were purchased from Sigma Aldrich (Finland) or Fisher Chemical (U.S.) and used as received unless otherwise noted. Propargyl mesylate was prepared according to a previously reported procedure.^[24] α -Azido-poly(glyceryl glycerol) (N₃-PGG) was synthesized as reported earlier.^[21] Pyrene-labeling of PGG (N₃-PGG-pyr) is described in Supporting Information Section 4. Phosphate-buffered saline (PBS) was prepared from tablets yielding 0.01 M phosphate buffer (0.137 M sodium chloride, 0.0027 M

potassium chloride) with pH adjusted to 7.40 using 0.1 M sodium hydroxide solution (NaOH). Dialysis was conducted in regenerated cellulose tubular membranes with molecular weight cut off (MWCO) of 6 - 8 kDa (CelluSep T2, Membrane Filtration Products, U.S.) or 25 kDa (Spectra/Por, Spectrum Labs, Netherlands). The latter was used in conjunction with a QuixSep® micro dialyzer (1 mL, Membrane Filtration Products, U.S.) for release experiments, as were Slide-A-Lyzer® Mini dialysis units (100 µL, MWCO: 20 kDa, Thermo Scientific, U.S.). Deuterated solvents for NMR spectroscopy (deuterium oxide, D₂O, 99.96 % D; methanol-d₄, MeOD, 99.80 % D) were obtained from Euriso-Top (France).

2.2. Characterization

2.2.1. NMR Spectroscopy

NMR spectra were recorded on a Bruker Avance III 500 spectrometer (¹H: 500.13 MHz, ¹³C: 125.77 MHz) at 23 °C and analyzed using Bruker TopSpin 3.0 or SpinWorks 2.5.5. Chemical shifts are given in parts per million (ppm) and calibrated relative to the residual solvent signals. Degrees of substitution (DS) of HA derivatives are given in % per 100 disaccharide units.

2.2.2. Size Exclusion Chromatography (SEC)

Molecular weights were determined by SEC using a Waters 515 HPLC pump connected to a Waters 2487 UV and Waters 2410 refractive index detector. The elution rate was 0.8 mL min⁻¹ in all runs. Samples were eluted with 0.1 M aqueous sodium nitrate (NaNO₃) containing 3 vol% acetonitrile and calibrated with polyethylene oxide (PEO) standards (PSS Polymer Standards Service, Germany). The columns used were: TOSOH (Japan) Guard column PWXL, TSKgel G3000 PWXL, G5000 PWXL and G6000 PWXL. Curves were analyzed with OmniSEC 4.7.0 software and OriginPro 8.6.

2.2.3. Fluorescence Spectroscopy

Fluorescence measurements were conducted using a Horiba Jobin Yvon FluoroMax-4 spectrofluorometer and FluorEssence 3.8 software. Emission spectra were recorded in the range of 361 - 550 nm with excitation wavelength of 343 nm and excitation and emission slit widths of 3 nm.

2.3. Syntheses

2.3.1. Esterification of HA

In a typical reaction, HA (1.0 eq., 503 mg, 1.25 mmol -COO^-) was dissolved in 25 mL water under gentle stirring. The solution was cooled in an ice bath and 70 mL dimethyl sulfoxide (DMSO) were added dropwise. The reaction flask was immersed into an oil bath at 45 °C and stirred for 5 min. Thereafter, solutions of triethylamine (1.0 eq., 127 mg, 1.25 mmol) and propargyl mesylate (1.0 eq., 169 mg, 1.26 mmol) in each 2 mL DMSO were added and the respective vials were washed with each 0.5 mL DMSO, which was also added to the reaction mixture. The mixture was stirred at 45 °C for 24 h, subsequently cooled to room temperature and dialyzed (MWCO 6 – 8 kDa) against water (3 times), 0.1 M aqueous sodium chloride (NaCl, 3 times) and again water (3 times). The product was lyophilized to give 378 mg (75 %) of a fluffy white solid (DS = 17 %). ^1H NMR(500 MHz, D_2O , δ): 2.01 (s, 3H, -NH-CO-CH_3), 3.04 (s, 0.17 H, $\text{-C}\equiv\text{CH}$), 3.10-4.10 (m, 10H, sugar backbone), 4.30-4.70 (d, 2H, anomeric protons), 4.80-4.90 (q, 0.34H, $\text{-CH}_2\text{-C}\equiv\text{CH}$); IR (ATR): ν = 3367 (s), 2897 (w), 1745 (w), 1644 (s), 1613 (s), 1559 (m), 1407 (m), 1375 (m), 1317 (m), 1263 (w), 1207 (w), 1152 (s), 1076 (s), 1042 (s), 946 (w), 895 (w), 799 (w), 704 (w), 610 (w).

2.3.2. Comparison of Click Conditions

HA-propargyl ester (DS = 17 %) was subjected to different click reaction conditions in presence of sodium azide (NaN_3) as a model compound to study the degradation of the polysaccharide backbone.

A) Click reaction with copper(I): HA-propargyl ester (1.0 eq., 21 mg, 8.7 μmol propargyl) was dissolved in 2.5 mL water. NaN_3 (1.4 eq., 0.8 mg, 12.4 μmol) in 0.1 mL water was added, followed

by dropwise addition of 6.5 mL DMSO. The mixture was degassed by 3 freeze-pump-thaw cycles and backfilled with nitrogen. Copper(I) bromide (5.1 eq., 6.3 mg, 43.9 μmol) in 1 mL DMSO was added via syringe. The mixture was degassed again by 2 freeze-pump-thaw cycles and backfilled with argon. *N,N,N',N'',N''*-Pentamethyldiethylenetriamine (PMDETA, 4.8 eq., 8.7 μL , 42.0 μmol) was added via automatic pipette under argon and the reaction mixture **was** stirred for 24 h at room temperature, protected from light. The product was purified by dialysis (MWCO 6 - 8 kDa) against 0.01 mM aqueous ethylenediaminetetraacetic acid (EDTA, 3 times), 0.1 M NaCl (3 times) and water (3 times) and lyophilized. Yield: 19 mg, 92 %.

B) Click reaction with copper(II): HA-propargyl ester (1.0 eq., 20 mg, **8.5** μmol **propargyl**) was dissolved in 9 mL water. NaN_3 (1.5 eq., 0.8 mg, 12.6 μmol) in 1 mL water was added and the solution was bubbled with argon for 90 min. Copper(II) sulfate pentahydrate (0.1 eq., 0.3 mg, 1.2 μmol) in **0.2** mL water and sodium ascorbate (1.4 eq., 2.3 mg, 11.8 μmol) in **1** mL water were added via syringe. The mixture was stirred under slow argon bubbling and protected from light for 24 h at room temperature. The same purification procedure as described above was followed. Yield: 19 mg, 95 %.

2.3.3. Grafting of PGG-pyr onto HA-Propargyl Ester

HA-propargyl ester (DS = 17 %; 1.0 eq., 71 mg, 30.0 μmol propargyl) and N_3 -PGG-pyr (M_n = 9.2 kDa and PDI = 1.45 determined by SEC; 0.3 eq., 71 mg, 7.7 μmol) were dissolved in 5 mL water. The flask was cooled in an ice bath and 14 mL of DMSO were added dropwise under stirring. The resulting solution was degassed by 3 freeze-pump-thaw cycles and backfilled with nitrogen. Copper(I) bromide (2.0 eq., 9 mg, 61.3 μmol) in 1 mL DMSO was added via syringe and the mixture was degassed again by 2 freeze-pump-thaw cycles and backfilled with argon. PMDETA (0.2 eq., 1.24 μL , 5.9 μmol) was added via automatic pipette under argon. The reaction mixture was stirred for 24 h at room temperature under argon and protected from light, then dialyzed (MWCO 25 kDa) against 0.01 mM EDTA (3 times), 0.1 M NaCl (3 times) and water (3 times) and lyophilized. Yield: 75 mg, 53 % (DS = 8 %, i.e. 44 % of propargyl groups functionalized; graft copolymer contains 56 - 65 wt%

PGG-pyr). ^1H NMR(500 MHz, D_2O , δ): 2.01 (s, 3H, $-\text{NH}-\text{CO}-\text{CH}_3$), 3.10-4.20 (m, 45H, HA sugar backbone & PGG), 4.40-4.70 (d, 2H, anomeric protons), 5.30-5.60 (d, 0.32H, $-\text{CH}_2$ -triazole), 8.15-8.20 (s, 0.08 H, triazole); IR (ATR): $\nu = 3324$ (s), 2917 (m), 2876 (m), 1735 (w), 1611 (s), 1564 (m), 1453 (w), 1407 (m), 1375 (m), 1318 (m), 1076 (s), 1040 (s), 946 (w), 860 (w), 685 (w), 611 (w).

2.4. Stability of HA Backbone and Hydrolysable Linker

2.4.1. Preparation of Vitreous

Porcine eyes were procured from a local slaughter-house. Eyes were kept on an ice bath during the isolation of vitreous humor and were cleaned of extra-ocular material by dipping in 70 % ethanol. The eyes were opened by incision with a dissecting knife and the clear vitreous humor was separated gently from the neural retina. Isolated vitreous humor was homogenized on ice, centrifuged and the supernatant was sterile-filtered using a $0.22\ \mu\text{m}$ filter to remove cellular debris and microbial contamination. It was stored at $-80\ ^\circ\text{C}$ until further use. For degradation and hydrolysis studies, 10 vol% vitreous in PBS containing 1 vol% antibiotics ($100\ \text{U ml}^{-1}$ penicillin, $100\ \mu\text{g ml}^{-1}$ streptomycin, Gibco) was used, hereafter referred to as vitreous.

2.4.2. Incubation of HA-Derivatives

Separate solutions were prepared of sodium hyaluronate, HA-propargyl ester (DS = 17 %) and HA-propargyl amide (DS = 40 %, synthesis described previously)^[25] with polymer concentrations of $1\ \text{g L}^{-1}$ in either PBS or vitreous and left to dissolve in the fridge overnight. The solutions were subsequently incubated at $37\ ^\circ\text{C}$ in an oven for 36 days, after which each solution was dialyzed (MWCO 25 kDa) against water (4 times) and lyophilized. Yield: between 53-100 % (mean = 92 %). The residues were dissolved in D_2O and investigated by ^1H NMR spectroscopy, afterwards lyophilized again and used for SEC measurements.

2.4.3. NMR Hydrolysis Kinetics

HA-propargyl ester (DS = 17 %, 1 g L⁻¹) was dissolved in PBS containing 5 vol% D₂O in the fridge overnight. The pH was adjusted to 7.40 ± 0.01 using 0.1 M NaOH. 0.7 mL of this solution was transferred to a NMR tube and inserted into the NMR spectrometer at 37 °C. Proton spectra were recorded every hour using the Bruker water presaturation pulse sequence *zgpr* with pulse strength of 1 mW, measurement delay (D1) of 3 s, acquisition time of 4 s and 128 scans. All spectra were phase- and baseline-corrected, as well as referenced towards the HA acetamide peak (1.96 ppm) and automatically integrated in the same spectral regions - i.e. 1.855 - 2.068 ppm (HA acetamide), 2.789 - 2.821 ppm (released propargyl alcohol) and 3.008 - 3.043 ppm (propargyl ester), respectively - using TopSpin 3.0. The integrals were calibrated towards the HA acetamide signal. The experiment was conducted twice with independently prepared solutions.

2.5. Release experiments

2.5.1. SEC

HA-PGG-pyr was dissolved in PBS at a concentration of 1 g L⁻¹ (~11 mL) in a fridge overnight. The pH was adjusted to 7.40 ± 0.01 using 0.1 M NaOH. The solution was distributed between 11 Eppendorf tubes (1 mL per tube). The first sample (t = 0) was frozen in liquid nitrogen immediately, the other samples were placed in an oven at 37 °C. At predetermined intervals samples were withdrawn and frozen in liquid nitrogen. All samples were lyophilized and stored in the freezer until the SEC measurements. Immediately before SEC measurement a sample was thawed and dissolved in 1 mL SEC eluent containing 1 g L⁻¹ uracil as an internal standard. The sample was filtered and measured. The chromatograms were baseline corrected, referenced toward the uracil elution volume, smoothed and integrated using OriginPro 8.6 (see Supporting Information Section 7.3).

2.5.2. Dialysis

Slide-A-Lyzer® Mini dialysis units were soaked in water prior to the experiment to remove glycerol. 26.7 μL of a 1 g L^{-1} solution of PGG-pyr (or HA-PGG-pyr) in PBS were added to each dialysis unit. The units were closed with the provided caps and placed in 1.5 mL Eppendorf tubes containing 1 mL PBS and a magnetic stirring flea. The assembled device was wrapped with Parafilm® to prevent water evaporation. The tubes were fitted with a Styrofoam™ swimmer and immersed in an oil bath at 37 °C and under stirring at 375 rpm. At predetermined intervals the contained dialysis units were transferred to new Eppendorf tubes with fresh PBS and placed back. The amount of permeated PGG-pyr in the dialysate was determined by fluorescence spectroscopy by comparing the intensity at 375 nm to a PGG-pyr calibration curve (Figure S14b). The experiment was carried out in triplicate starting from the same PGG-pyr or HA-PGG-pyr stock solutions.

In the case of the QuixSep® micro-dialyzer, 800 μL of PGG-pyr in PBS (1 g L^{-1}) were dialyzed against 30 mL PBS under stirring at 400 rpm at 37 °C. At predetermined intervals the micro-dialyzer was transferred to a new beaker containing 30 mL of fresh PBS and dialysis was continued. The amount of permeated PGG-pyr was determined as described above and the experiment was repeated twice.

2.5.3. Sample and Separate Method

A stirred solution of HA-PGG-pyr in either PBS or vitreous (1 g L^{-1} , ~2 mL) was placed in an oil bath at 37 °C. At predetermined intervals 100 μL samples were withdrawn, immediately frozen in liquid nitrogen and lyophilized. 1.2 mL methanol was added to the dried material and placed on a shaker overnight. The suspension was subsequently centrifuged for 5 min at 20 238 $\times g$ to separate the insoluble fraction (containing the intact HA-PGG-pyr copolymer and HA) from the methanol solution (containing free PGG-pyr). The supernatant was carefully pipetted off and the pellet was dried in vacuum. The dry pellet was dissolved in 1 mL PBS overnight and its fluorescence intensity at 375

nm was measured and compared to the intensity determined at $t = 0$ (start sample). The experiments were carried out in triplicate with separate solutions.

2.6. Biocompatibility

Conditions for the cell culturing and cytotoxicity assay are described in Supporting Information Section 8.

3. Results and Discussion

3.1. The HA Backbone

3.1.1. Preparation of HA for Efficient Grafting

The glucuronic acid moieties of HA were functionalized with hydrolysable linkers to introduce reactive centers for grafting of PGG side chains. In addition, the modification slows down the already low turnover rate of HA in the vitreous by masking of the hyaluronidase recognition sites.^[26–29] Ester bonds serve as the hydrolysable linkage between HA and the side chains, having fast enough hydrolysis kinetics to release the grafts before the copolymer is cleared from the vitreous and regenerating native HA as the only side product upon release. We prepared propargyl esters of HA for subsequent use in azide-alkyne cycloaddition (click reaction) with azide-functional PGG (**Scheme 2**). Most of the esterification methods reported for HA involve a pre-treatment of the polysaccharide (acidification or hydrophobic salt) in order to solubilize it into polar aprotic solvents, such as DMSO.^[30,31] This additional step is time-consuming, may lead to degradation of the chains and complicates the purification of the products,^[32,33] hence we explored ways to functionalize the commercially available sodium salt of HA directly.

We found that, when HA was dissolved in water first, it was possible to add up to 3-times the volume of DMSO to the solution and to obtain a homogeneous clear mixture. In water/DMSO (1:3) the rate

of esterification was sufficiently high to obtain products in a reasonable time scale (~24 h), while at higher water contents no esters were obtained (**Table S1**). The reaction proceeded via base-catalyzed nucleophilic substitution between HA and a propargyl substrate carrying a good leaving group, such as a bromide or mesylate group. Degrees of substitution (DS) ranged from about 17 - 40 % and are comparable to or higher than those of previously reported HA-esters.^[30,34–37] Nishikubo *et al.*^[38] noticed in the esterification of poly(methacrylic acid) (PMAA) with propargyl bromide and chloride an increase in reaction rate with increasing temperature. Also, one might expect an increase in reaction rate with increasing reactant concentration according to the rate law of a bimolecular reaction: $r = k \cdot [RX] \cdot [Nu^-]$, with $[RX]$ being the concentration of propargyl substrate and $[Nu^-]$ the concentration of HA carboxylate groups. No appreciable trends concerning those factors were observed in this study. However, the type of base, employed as catalyst in the reactions, had a strong influence on the rate of esterification. While 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was the preferred base in esterification of PMAA,^[38] triethylamine (TEA) performed better in our system. Only 0.5 to 1 equivalent of TEA compared to carboxylate groups gave HA-esters within 24 h. In contrast, DBU had to be employed in 5-times excess and reactions took 96 h. As DBU is a stronger base than TEA (pK_a 24.34 compared to 18.82 in acetonitrile, respectively),^[39] it was expected that DBU would deprotonate hyaluronic acid more efficiently leading to an increased esterification rate. Although we did not study the mechanism of the esterification in detail, we suspect that DBU acts as a nucleophile in addition to its role as base,^[40,41] attacking the propargyl substrate itself. TEA, on the other hand, is a non-nucleophilic base and thus only involved in deprotonation of HA.

Excess amounts of base led to degradation of HA as shown by the decrease in molecular weight (Table S1), hence we chose the following conditions as a general procedure for the preparation of HA-propargyl esters: $[HA] = 5 \text{ g L}^{-1}$, base: TEA (1 eq.), substrate: propargyl mesylate (1 eq.), 45 °C, water/DMSO (1:3), 24 h. Mesylates were favored, as they are generally easier to prepare than alkyl

halides in cases where the substrate is not commercially available. Notably, our procedure does not require excess reactants.

The structure of the HA-propargyl ester was confirmed by 2D NMR spectroscopy, as well as by NMR diffusion measurements (**Figure S1 and S2**). The FT-IR spectra of HA-propargyl esters exhibited the characteristic ester carbonyl stretch around 1740 - 1750 cm^{-1} (**Figure S3**). Although this study was focused on HA-ester derivatives, in some of the following experiments we also included a previously reported HA-propargyl amide to get a qualitative idea of how both derivatives would compare during release tests.^[25]

3.1.2. Degradation During Synthesis

Hyaluronic acid is prone to degrade under harsh reaction conditions, such as in strongly alkaline, acidic or oxidative solutions, or when subjected to excessive heat, shear and ultrasound irradiation.^[42–44] Since our designed graft copolymer relies on its high molecular weight backbone for prolonged retention time in the eye, we aimed to keep the HA degradation as low as possible. Therefore, we determined the effect of chemical modifications on the molecular weight of the HA derivatives by SEC after each synthesis step. It was already mentioned above that the base-catalyzed esterification decreased the molecular weight of HA. The same is observed in **Figure 1a** (and **Table S2**). For grafting of side chains to the HA backbone, two click reaction procedures were evaluated using sodium azide as a model compound to avoid the introduction of side groups that would significantly alter the solution behavior of HA in SEC.

The standard aqueous click reaction protocol uses copper(II)sulfate and sodium ascorbate to create the catalytically active Cu^+ species in situ by reduction.^[25] At the same time hydroxyl radicals are formed that readily degrade HA.^[45] If the reaction is performed in organic solvent (here: water/DMSO (1:3)) with copper(I)bromide and a ligand, no redox reaction takes place and degradation is less pronounced, as shown in the SEC results (Figure 1a). Rheological measurements support these

findings. The zero-shear viscosity of HA derivatives (3 g L⁻¹ in water), which is known to be closely related to the molecular weight of the polysaccharide, decreased from 88 mPa·s of unmodified HA to 16 mPa·s in case of the esterified HA (**Figure S4**). It further decreased to 7.2 and 3.8 mPa·s for the click derivatives prepared with copper(I) and copper(II), respectively. Also, neither of the derivatives shows the shear thinning behavior of unmodified HA, which can be a result of the lower molecular weight. In order to maintain the length of HA chains, we chose the copper(I) click conditions for preparation of the HA-PGG graft copolymer.

3.1.3. Stability of HA During Release Experiments

We investigated the stability of the polysaccharide backbone under physiological conditions. Samples were incubated in either PBS or vitreous at 37 °C for 36 days to estimate the extent of non-enzymatic and enzymatic hydrolysis, respectively. Sodium hyaluronate, HA-propargyl ester and HA-propargyl amide, which was reported earlier,^[25] were used to determine whether polysaccharides with masked glucuronic acid groups were more stable towards enzymatic degradation. The incubated solutions were dialyzed (MWCO 25 kDa) to remove the buffer salts and approximately 90 % of the employed starting materials could be recovered. The high yield indicates no formation of small molecular weight degradation products that could be removed during the dialysis step. SEC studies (**Figure 1b**) further show that molecular weight and molecular weight distribution of the incubated HA sample were similar to the starting material, constituting almost no sign of degradation. The polysaccharides were stable during the studied period even in the presence of enzymes contained in the vitreous. No difference between native and modified HA could be observed (**Figure S5** and **Table S3**). Therefore we concluded that degradation of the HA backbone is negligible during our investigation and will not affect the release of side chains from the graft copolymer.

3.1.4. Cleavage of the Hydrolysable Linker

In order to release the grafts from the HA-PGG graft copolymer the ester bonds have to be cleaved. This was studied in a similar way as above, but using NMR spectroscopy. HA-propargyl ester and HA-propargyl amide were incubated in PBS or vitreous at 37 °C for 36 days and afterwards dialyzed. The NMR spectra of the derivatives before and after incubation were compared. As can be seen in **Figure 2**, the signals of the propargyl ester groups have disappeared after 36 days in both media. The hydrolysis product, i.e. small molecular weight propargyl alcohol, was removed during the dialysis step and the remaining material showed the characteristic signals of hyaluronic acid. In contrast, the amide derivative was expected to be quite stable. Accordingly, the NMR spectra showed only a slight decrease in propargyl signal intensity after incubation, which was more pronounced in the sample containing vitreous (20 % decrease in PBS versus 28 % decrease in vitreous).

The hydrolysis kinetics of HA-propargyl ester were further studied in situ in PBS (containing 5 vol% D₂O) at 37 °C using NMR spectroscopy with a water suppression pulse sequence. This approach was chosen to circumvent two problems that arise when studying ester degradation in D₂O: First, hydrolysis reactions proceed 2- to 5-times faster in D₂O as a result of isotope effects.^[46] Second, the pH changes in unbuffered systems in the course of the reaction. Hence, we followed the integrals of the decreasing HA-propargyl ester peak at 3.01 - 3.04 ppm as well as the emerging signal of the released propargyl alcohol at 2.79 -2.82 ppm over time (**Figure S6 and S7**). The HA-propargyl ester had a half-life ($t_{1/2}$) of about 3.3 h (**Table S4**), which is only half that of a reported HA-corticosteroid derivative ($t_{1/2} \sim 6.5$ h).^[47] The result was expected, considering that the steroid ester is much more hydrophobic and contracted than a propargyl derivative, hindering the attack of water molecules.^[48,49]

3.2. The HA-PGG graft copolymer

3.2.1. Grafting of PGG onto HA

Grafting of azido-functional PGG side chains ($M_n = 3.6$ kDa) to the HA-propargyl ester (DS = 25%) was achieved by click reaction using copper(I) bromide and a ligand in water/DMSO (1:3). Product

formation was confirmed by ^1H NMR spectroscopy and SEC. All propargyl groups were functionalized, as can be seen from the disappearance of the propargyl $-\text{C}\equiv\text{CH}$ signal at 2.99 ppm, as well as a shift of the propargyl $-\text{CH}_2-$ protons from 4.84 - 4.95 ppm to 5.33 - 5.54 ppm (**Figure 3**). Furthermore a new signal at 8.16 ppm appeared, which can be attributed to the formed triazole ring. The peaks of PGG and the HA sugar moieties overlap in the region between 3 - 4 ppm.

The SEC eluogram of the HA-PGG graft copolymer showed an increase in molecular weight compared to the HA-propargyl ester (**Figure 4**). The change is less pronounced than could be expected from simple addition of the molecular weights of the components. This is due to the very flexible nature of PGG and its low intrinsic viscosity as well as the branched structure of the copolymer, which leads to an underestimation of its molecular weight by SEC.^[21] A bimodal distribution was observed after purification by dialysis (MWCO 25 kDa), suggesting that the method was not effective to remove uncoupled PGG (**Figure 4** and **S11**). It could be removed almost completely by extraction of the lyophilized solid material with methanol. Meanwhile, the HA-PGG graft copolymer remained insoluble and could be collected by centrifugation (**Figure S12 & S13**). The small low molecular weight shoulder still present in the resulting chromatogram could be a result of ester hydrolysis during the SEC measurement, which takes about 1 hour

3.2.2. Release Studies

In order to study the release of PGG grafts from the HA-PGG copolymer, two major challenges have to be overcome. First, the macromolecules, which feature very similar chemical structures (plethora of hydroxyl groups) and solubility behavior, have to be quantitatively separated. Second, the amount of free PGG needs to be quantified. The latter problem was solved by employing a pyrene-labeled PGG (PGG-pyr) for fluorescence detection (see Supporting Information Section 5), where pyrene also acts as a hydrophobic model compound. Thus, the graft copolymer employed in the release experiments was prepared from PGG-pyr and the same HA-propargyl ester used previously in the

NMR kinetics study. Further, all experiments were carried out at the same concentration of HA-PGG-pyr (1 g L^{-1}), as the solution viscosity has been shown to influence the ester hydrolysis rate.^[47] Under these conditions, the HA chains were below their critical overlap concentration and electrostatically completely shielded due to the presence of buffer salts (137 mM NaCl).^[50] We therefore assumed no interactions between free PGG-pyr and HA that would complicate the release study.

We first used SEC to separate and detect the polymers simultaneously. Due to the low refractive index increment of both HA and PGG the experiment consumed a large amount of copolymer (tens of milligrams). The shear forces acting on the molecules when passing through the chromatography columns facilitated breaking of possible aggregates, thus SEC should give a true picture of the amount of released grafts. The chromatograms (see Supporting Information Section 7.1) after different hydrolysis times produced the release curve depicted in **Figure 5d**. The profile followed the pseudo-first order kinetics of an ester hydrolysis, but proceeded slower than the release of low molecular weight propargyl alcohol from the same HA derivative. The grafts were released over the course of one week with a rate constant of $(2.95 \pm 0.27) \cdot 10^{-2} \text{ h}^{-1}$ and $t_{1/2}$ of about 23 h. This result indicated a 7-times longer half-life of the HA-PGG-pyr copolymer compared with the HA-propargyl ester. To verify the result and to decrease the required amount of sample we continued to explore other methods as well.

There are two kinds of in vitro setups commonly used for drug release studies, namely dialysis and the sample and separate method.^[51] In dialysis, small molecular weight drugs or peptides are separated from a drug depot, such as a polymeric prodrug, liposome or microsphere, with the help of a membrane that is only permeable for the released compound.^[52,53] The release rate determined by this method depends on several factors. These comprise the actual release of compound from the drug depot, the diffusion rate across the dialysis membrane and possible binding of the drug/peptide to the depot and/or membrane.^[54] The sample and separate method relies on the separation of the released

compound from the drug depot by means of filtration or centrifugation.^[55,56] Therefore, drug binding to the delivery vehicle might also distort the measured release rate.

A variation of the sample and separate method, our methanol-extraction procedure, was not suited to quantitatively remove PGG-pyr from the samples (Figure 5c and Supporting Information Section 7.2). To overcome previously encountered problems with dialysis and to miniaturize the experimental setup in order to save precious HA-PGG-pyr graft copolymer, we tested two dialysis devices of different scales (**Table 1** and Figure 5a). The QuixSep® micro-dialyzer (1 mL) and the Slide-A-Lyzer® Mini dialysis units (100 µL) consisted of a cylindrical sample reservoir with a membrane attached to the base area. In both setups the membrane surface-area-to-sample-volume (SA:V) ratio was higher than in conventional dialysis and the dialysate compartment was constantly stirred to disturb water-layers at the membrane surface.^[51–53] The sample-to-dialysate volume ratio was kept constant in order to stay above the fluorescence detection limit of PGG-pyr, which is 0.1 µg mL⁻¹ in the dialysate corresponding to 0.4 % released PGG-pyr (**Figure S17**). The MWCO of the membranes was comparable, being 25 and 20 kDa, respectively, which was the largest cutoff available for the Slide-A-Lyzer® units. Free PGG-pyr of approximately 9.2 kDa was dialyzed against PBS at 37 °C to study its permeability across the dialysis membrane.

Figure 5a shows that free PGG-pyr could completely escape from the Slide-A-Lyzer® device within 48 h, while only 40 % of PGG-pyr chains were released from the QuixSep® setup during this time. The higher dialysis efficiency of the Slide-A-Lyzer® was likely due to the larger membrane SA:V ratio in our experiment. We continued with this setup to investigate the release of PGG-pyr from the graft copolymer (Figure 5b). The grafts were released over the course of one week and fitting the profile to the integrated ester hydrolysis rate law, we obtained a rate constant of $(2.10 \pm 0.20) \cdot 10^{-2} \text{ h}^{-1}$ and $t_{1/2}$ of about 33 h. Notably, the release of PGG-pyr measured by SEC was faster than in the dialysis experiment. This can be explained by the fact that the rate observed in dialysis contains the hydrolysis step and the diffusion across the membrane, which is slow for polymers. In order to

separate these two processes we used the model of consecutive reactions to fit the results. We considered the hydrolytic release of PGG-pyr inside the dialysis device as the first, and diffusion of PGG-pyr across the membrane as the second reaction step (see Supporting Information Section 7.3). This approach led to a hydrolysis rate constant that very well matched the result obtained from SEC (**Table 2**).

Unsaturated neighboring groups, such as propargyl and triazole groups, can have an electron-withdrawing effect that enhances the hydrolysis rate, but inductive effects die out rapidly over distance.^[57] As the changing substituents are located in the alkyl component that is further away from the reaction center (the carbonyl carbon), the effect would be even less pronounced and does not satisfactorily explain the 7-times slower hydrolysis rate of the graft copolymer. The slow release of grafts compared to small molecular weight propargyl alcohol is likely due to steric crowding in the graft copolymer, which shields the ester bonds from attack by water. Hence, a graft copolymer with drugs attached to the polymeric side chains could enable longer lasting release than a prodrug with drug molecules attached to the backbone. Furthermore, once verified by a different method, dialysis can be used to study polymer-from-polymer release.

With the developed methods, we are in the future able to determine the release rate constants of ester- and amide-linked HA-PGG graft copolymers in vitreous liquid. Those values will be essential in modeling the pharmacokinetics and pharmacodynamics of the delivery platform and to find the most suitable derivative for the intended application.

3.3. Biocompatibility

The biocompatibility of a HA-PGG graft copolymer and PGG towards human retinal pigment epithelial cells (ARPE-19) and human umbilical vein endothelial cells (HUVEC) was evaluated using the MTT cytotoxicity assay. The metabolic activity of cells was measured after 5 h of incubation with the polymers at different concentrations and 20 h in growth medium. In living cells, NAD(P)H-

dependent cellular oxidoreductase enzyme reduces the tetrazolium dye MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble formazan, which can be detected spectrophotometrically (see Supporting Information Section 8).^[58] Epithelial (ARPE-19) and endothelial (HUVEC) cells were chosen due to their relevance in ocular drug development. The cytotoxicity of HA-PGG and PGG was compared to polyvinyl alcohol (PVA), which is non-toxic and FDA-approved for ocular use, and branched polyethylene imine (PEI), which is cytotoxic.

Figure 6 shows that HA-PGG and PGG exhibit similar levels of biocompatibility to PVA for concentrations up to 0.1 mg mL^{-1} . At higher concentrations both polymers cause a decrease in cell viability that is more pronounced for PGG than HA-PGG. The polymers are much less toxic than PEI and their slight cytotoxic effect may be attributed to residual impurities from the synthesis, such as copper ions originating from the click grafting. Those may be further reduced by filtration over an ion exchange resin. The solvents used for the coupling and extraction, DMSO and methanol, respectively, are generally regarded as having low toxicity.^[12] A similar behavior was observed in monkey kidney fibroblast (CV-1) and human ovarian adenocarcinoma (SKOV-3) cells (**Figure S20**), indicating that HA-PGG and PGG are equally well tolerated by healthy and cancer cells and might be useful for other biomedical applications as well.

We believe that the results are promising, considering that the concentration of HA-PGG graft copolymer in the vitreous could be around 0.125 mg mL^{-1} after intravitreal injection. The volume of the human vitreous is approximately 4 mL and the maximum volume for intravitreal injection is 50 μL .^[12,14] Assuming a concentration of 10 mg mL^{-1} for HA-PGG graft copolymer, which is not very viscous yet, one injection could yield 500 μg of administered graft copolymer. With these encouraging results we will continue to build on our design strategy by functionalization of the PGG grafts with drugs and targeting moieties. Selected degradable linkages that are stable in the vitreous shall enable the controlled drug release from the grafts after uptake by the target cells.

4. Conclusion

We set out to prepare ester-linked HA-PGG graft copolymers of high molecular weight that could slowly release their grafts over time intended as vehicles for controlled intravitreal drug delivery. Our results suggested that the HA chain length was very sensitive to the employed synthetic conditions and thus procedures were optimized to keep degradation at a minimum. In contrast, incubation in PBS and vitreous at 37 °C had no significant effect on the molecular weight of HA, which supports our proposition that HA copolymers can achieve long retention times in the vitreous. The release studies of HA-propargyl ester and HA-PGG-pyr indicated that polymeric side chains were cleaved at significantly slower rate than small molecular linkers, which we attributed to steric crowding at the ester bond hindering the attack of water molecules. These results encourage our two-stage release design, in which drugs bound to polymer grafts will be released over longer periods than drugs bound directly to the polymer backbone, as in classic prodrugs.

We tested different setups for the quantification of polymer-from-polymer release and found that dialysis, which is commonly used to study release of small drugs, can also be used for polymeric systems. However, it is important to validate the measured rate constants with a second method (in our case SEC) and, if necessary, to build a model taking into account all underlying processes and interactions of the components within the system.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Appendix

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Keywords: hyaluronic acid, poly(glyceryl glycerol), drug delivery vehicle, polymer-from-polymer release, vitreous

- [1] A. Urtti, *Adv. Drug Deliv. Rev.* **2006**, 58, 1131–1135.
- [2] E. M. del Amo, A. Urtti, *Drug Discov. Today* **2008**, 13, 135–143.
- [3] M. W. Stewart, *Mayo Clin. Proc.* **2012**, 87, 77–88.
- [4] D. H. Nguyen, J. Luo, K. Zhang, M. Zhang, *Discov. Med.* **2013**, 15, 343–348.
- [5] V. Delplace, S. Payne, M. Shoichet, *J. Controlled Release* **2015**, 219, 652–668.
- [6] J.-E. Chang-Lin, M. Attar, A. A. Acheampong, M. R. Robinson, S. M. Whitcup, B. D. Kuppermann, D. Welty, *Invest. Ophthalmol. Vis. Sci.* **2011**, 52, 80–86.
- [7] J. A. Haller, F. Bandello, R. Belfort Jr, M. S. Blumenkranz, M. Gillies, J. Heier, A. Loewenstein, Y. H. Yoon, J. Jiao, X.-Y. Li, et al., *Ophthalmology* **2011**, 118, 2453–2460.
- [8] V. A. N. Huu, J. Luo, J. Zhu, J. Zhu, S. Patel, A. Boone, E. Mahmoud, C. McFearin, J. Olejniczak, C. de Gracia Lux, et al., *J. Controlled Release* **2015**, 200, 71–77.
- [9] J. Park, P. M. Bungay, R. J. Lutz, J. J. Augsburger, R. W. Millard, A. Sinha Roy, R. K. Banerjee, *J. Controlled Release* **2005**, 105, 279–295.
- [10] Q. Xu, N. J. Boylan, J. S. Suk, Y.-Y. Wang, E. A. Nance, J.-C. Yang, P. J. McDonnell, R. A. Cone, E. J. Duh, J. Hanes, *J. Controlled Release* **2013**, 167, 76–84.
- [11] V. Andrés-Guerrero, M. Zong, E. Ramsay, B. Rojas, S. Sarkhel, B. Gallego, R. de Hoz, A. I. Ramírez, J. J. Salazar, A. Triviño, et al., *J. Controlled Release* **2015**, 211, 105–117.
- [12] Y. Hamdi, F. Lallemand, S. Benita, *J. Drug Deliv. Sci. Technol.* **2015**, 30, Part B, 331–341.
- [13] S. M. Whitcup, M. R. Robinson, *Ann. N. Y. Acad. Sci.* **2015**, 1358, 1–12.
- [14] E. M. del Amo, A.-K. Rimpelä, E. Heikkinen, O. K. Kari, E. Ramsay, T. Lajunen, M. Schmitt, L. Pelkonen, M. Bhattacharya, D. Richardson, et al., *Prog. Retin. Eye Res.* **2017**, 57, 134–185.
- [15] H. Petersen, P. M. Fechner, A. L. Martin, K. Kunath, S. Stolnik, C. J. Roberts, D. Fischer, M. C. Davies, T. Kissel, *Bioconjug. Chem.* **2002**, 13, 845–854.
- [16] T. Etrych, P. Chytil, T. Mrkvan, M. Šírová, B. Říhová, K. Ulbrich, *J. Controlled Release* **2008**, 132, 184–192.
- [17] Y. Hu, Y. Li, F.-J. Xu, *Acc. Chem. Res.* **2017**, 50, 281–292.
- [18] E. M. del Amo, K.-S. Vellonen, H. Kidron, A. Urtti, *Eur. J. Pharm. Biopharm.* **2015**, 95, Part B, 215–226.
- [19] W. Shatz, P. E. Hass, M. Mathieu, H. S. Kim, K. Leach, M. Zhou, Y. Crawford, A. Shen, K. Wang, D. P. Chang, et al., *Mol. Pharm.* **2016**, 13, 2996–3003.
- [20] J. Necas, L. Bartosikova, J. Kolar, *Vet. Med. (Praha)* **2008**, 53, 397–411.
- [21] T. Borke, A. Korpi, F. Pooch, H. Tenhu, S. Hietala, *J. Polym. Sci. Part Polym. Chem.* **2017**, 55, 1822–1830.
- [22] M. Imran ul-haq, B. F. L. Lai, R. Chapanian, J. N. Kizhakkedathu, *Biomaterials* **2012**, 33, 9135–9147.
- [23] X.-M. Liu, L. Quan, J. Tian, F. C. Laquer, P. Ciborowski, D. Wang, *Biomacromolecules* **2010**, 11, 2621–2628.
- [24] M. Rivara, M. K. Patel, L. Amori, V. Zuliani, *Bioorg. Med. Chem. Lett.* **2012**, 22, 6401–6404.
- [25] T. Borke, F. M. Winnik, H. Tenhu, S. Hietala, *Carbohydr. Polym.* **2015**, 116, 42–50.

- [26] U. B. G. Laurent, J. R. E. Fraser, *Exp. Eye Res.* **1983**, 36, 493–503.
- [27] J. Yeom, S. H. Bhang, B.-S. Kim, M. S. Seo, E. J. Hwang, I. H. Cho, J. K. Park, S. K. Hahn, *Bioconjug. Chem.* **2010**, 21, 240–247.
- [28] C. Schanté, G. Zuber, C. Herlin, T. F. Vandamme, *Carbohydr. Polym.* **2011**, 86, 747–752.
- [29] C. E. Schanté, G. Zuber, C. Herlin, T. F. Vandamme, *Carbohydr. Polym.* **2012**, 87, 2211–2216.
- [30] C. Huin-Amargier, P. Marchal, E. Payan, P. Netter, E. Dellacherie, *J. Biomed. Mater. Res. A* **2006**, 76A, 416–424.
- [31] Q. Li, Y. Bao, H. Wang, F. Du, Q. Li, B. Jin, R. Bai, *Polym. Chem.* **2013**, 4, 2891–2897.
- [32] B. P. Purcell, I. L. Kim, V. Chuo, T. Guenin, S. M. Dorsey, J. A. Burdick, *Biomater. Sci.* **2014**, 2, 693–702.
- [33] L. Pravata, C. Braud, M. Boustta, A. El Ghzaoui, K. Tømmerraas, F. Guillaumie, K. Schwach-Abdellaoui, M. Vert, *Biomacromolecules* **2008**, 9, 340–348.
- [34] S. Sahoo, C. Chung, S. Khetan, J. A. Burdick, *Biomacromolecules* **2008**, 9, 1088–1092.
- [35] S. Manju, K. Sreenivasan, *J. Colloid Interface Sci.* **2011**, 359, 318–325.
- [36] J. Li, P. Huang, L. Chang, X. Long, A. Dong, J. Liu, L. Chu, F. Hu, J. Liu, L. Deng, *Macromol. Res.* **2013**, 21, 1331–1337.
- [37] A. Takahashi, Y. Suzuki, T. Suhara, K. Omichi, A. Shimizu, K. Hasegawa, N. Kokudo, S. Ohta, T. Ito, *Biomacromolecules* **2013**, 14, 3581–3588.
- [38] T. Nishikubo, A. Kameyama, Y. Yamada, Y. Yoshida, *J. Polym. Sci. Part Polym. Chem.* **1996**, 34, 3531–3537.
- [39] I. Kaljurand, A. Kütt, L. Sooväli, T. Rodima, V. Mäemets, I. Leito, I. A. Koppel, *J. Org. Chem.* **2005**, 70, 1019–1028.
- [40] W.-C. Shieh, S. Dell, O. Repič, *J. Org. Chem.* **2002**, 67, 2188–2191.
- [41] M. Baidya, H. Mayr, *Chem. Commun.* **2008**, 1792.
- [42] E. Dřimalová, V. Velebný, V. Sasinková, Z. Hromádková, A. Ebringerová, *Carbohydr. Polym.* **2005**, 61, 420–426.
- [43] R. Stern, G. Kogan, M. J. Jedrzejewski, L. Šoltés, *Biotechnol. Adv.* **2007**, 25, 537–557.
- [44] A. Maleki, A.-L. Kjøniksen, B. Nyström, *Macromol. Symp.* **2008**, 274, 131–140.
- [45] L. Šoltés, K. Valachová, R. Mendichi, G. Kogan, J. Arnhold, P. Gemeiner, *Carbohydr. Res.* **2007**, 342, 1071–1077.
- [46] D. Lundberg, K. Holmberg, *J. Surfactants Deterg.* **2004**, 7, 239–246.
- [47] E. Payan, J. Y. Jouzeau, F. Lapique, K. Bordji, G. Simon, P. Gillet, M. O'Regan, P. Netter, *J. Controlled Release* **1995**, 34, 145–153.
- [48] A. J. M. D'Souza, E. M. Topp, *J. Pharm. Sci.* **2004**, 93, 1962–1979.
- [49] A. Taglienti, P. Sequi, M. Valentini, *Carbohydr. Res.* **2009**, 344, 245–249.
- [50] H. G. Garg, C. A. Hales, *Chemistry and Biology of Hyaluronan*, Elsevier, Amsterdam; Boston, **2004**, pp. 7-9.
- [51] S. S. D'Souza, P. P. DeLuca, *AAPS PharmSciTech* **2005**, 6, E323–E328.
- [52] T. G. Park, W. Lu, G. Crotts, *J. Controlled Release* **1995**, 33, 211–222.
- [53] J. W. Kostanski, P. P. DeLuca, *AAPS PharmSciTech* **2000**, 1, 30–40.
- [54] S. Modi, B. D. Anderson, *Mol. Pharm.* **2013**, 10, 3076–3089.
- [55] S. J. Wallace, J. Li, R. L. Nation, B. J. Boyd, *Drug Deliv. Transl. Res.* **2012**, 2, 284–292.
- [56] S. D'Souza, *Adv. Pharm.* **2014**, 2014, e304757.
- [57] *The Chemistry of Carboxylic Acids and Esters*, Interscience-Publishers, London ; New York, **1969**, pp. 514-520.
- [58] M. B. Hansen, S. E. Nielsen, K. Berg, *J. Immunol. Methods* **1989**, 119, 203–210.
- [59] G. Ebner, A. Hofinger, L. Brecker, T. Rosenau, *Cellulose* **2008**, 15, 763–767.
- [60] A. Laukkanen, F. M. Winnik, H. Tenhu, *Macromolecules* **2005**, 38, 2439–2448.
- [61] M. L. Phillips, R. L. White, *J. Chromatogr. Sci.* **1997**, 35, 75–81.

[62] P. W. Atkins, J. De Paula, *Physikalische Chemie*, Wiley-VCH, Weinheim, **2012**, p. 898.

Scheme 1. Mechanism of proposed two-stage drug release from HA-PGG graft copolymers: 1) HA-PGG, administered by intravitreal injection, slowly releases drug-carrying grafts via hydrolysis of the linker. 2) PGG-drug conjugates prevent fast clearance of drugs and are able to diffuse into retinal layers to release their cargo after cell uptake.

Scheme 2. Synthetic approach for the esterification of HA and subsequent click grafting of poly(glyceryl glycerol) (PGG). Note: The scheme only shows the structure of the modified HA repeating units.

a) Degradation during synthesis

b) Degradation during incubation

Figure 1. SEC eluograms of HA and HA derivatives after (a) chemical modification and (b) incubation in either PBS or vitreous at 37 °C for 36 days.

Figure 2. ^1H NMR spectra of HA-propargyl ester (left) and amide (right) starting materials (top) and after incubation in PBS (middle) or vitreous (bottom) at 37 °C for 36 days. The characteristic propargyl signals have vanished in the case of the ester derivative, while they are only slightly decreased for the amide derivative. **N*-methylmorpholinium impurity originating from synthesis of the amide as detailed in ref.^[25]. ** Signal originating from the vitreous.

Figure 3. ^1H NMR spectra of HA-propargyl ester (bottom) and HA-PGG after click reaction (top). Insets show enlarged regions, illustrating the appearance of the triazole signal after click reaction (left), shifting of the propargyl $-\text{CH}_2-$ signal (middle) as well as the disappearance of the propargyl $-\text{C}\equiv\text{CH}$ signal (right).

Figure 4. SEC traces of HA-propargyl ester (black) and HA-PGG graft copolymer purified by dialysis (MWCO 25 kDa; blue) or extraction (red). The inset shows a close-up of the HA ester and HA-PGG peaks, respectively.

Figure 5. Results of different release experiments, conducted in PBS at 37 °C unless otherwise noted. a) Dialysis of PGG-pyr using QuixSep® (squares, n = 2) or Slide-A-Lyzer® (circles, n = 3) devices to evaluate the permeability of the polymer through the membrane. b) Release of PGG-pyr from the graft copolymer (triangles, n = 3) studied by dialysis with the Slide-A-Lyzer setup (permeation of free PGG-pyr is shown for comparison). c) Residual PGG-pyr in extracted samples of the HA-PGG-pyr graft copolymer after different hydrolysis times (n = 3) in PBS (squares) or vitreous (circles). d) Release of PGG-pyr from the graft copolymer studied by SEC (squares) and dialysis (triangles), as well as release of propargyl alcohol from the HA-ester determined by NMR (circles). If applicable, data is reported as mean $\pm$ s.d. Release profiles were fitted with the integrated ester hydrolysis rate law, as shown in b) and d).

Figure 6. Viability of a) human retinal pigment epithelium (ARPE-19), b) human umbilical vein endothelial cells (HUVEC) after incubation with HA-PGG (squares) or PGG (circles) determined by MTT cytotoxicity assay. Polyvinyl alcohol (PVA, triangles) and polyethylene imine (PEI, diamonds) were measured for comparison. Experiments were conducted in triplicate (except for HUVEC) and data is presented as mean $\pm$ s.d.

Table 1. Characteristics of the dialysis devices.

	MWCO [kDa]	Sample [μ L]	Dialysate [mL]	Volume Ratio	Membrane surface area [cm ²]	SA:V ^{a)} [cm ² mL ⁻¹]
QuixSep®	25	800.0	30	37.5	1.77	2.21
Slide-A-Lyzer®	20	26.7	1	37.5	0.28	10.59

^{a)}Surface area-to-sample volume ratio

Table 2. Summary of hydrolysis and diffusion rate constants (k_{hydr} and k_{diff} , respectively) and half-lives of the HA derivatives in PBS at 37 °C.

	HA-PGG-pyr (SEC)	HA-PGG-pyr (Dialysis)	HA-propargyl ester (NMR)
k_{hydr} (10^{-2} h^{-1})	2.95 ± 0.27	2.97 ± 0.38	17.63 ± 1.00
k_{diff} (10^{-2} h^{-1})	-	10.50 ± 3.31	-
$t_{1/2}$ (h)	23.51 ± 2.19	23.34 ± 3.04	3.93 ± 0.22
adj. R^2 ^{a)}	0.986	0.997	0.970

^{a)}Coefficient of determination adjusted by the degrees of freedom of the fit model

Design and synthesis of a water soluble delivery vehicle for prolonged retention times in the vitreous is reported. Synthesis conditions are optimized to maintain the high molecular weight of the hyaluronic acid graft copolymer. Kinetics of graft cleavage from the backbone are determined and show sustained release compared to small molecules. Biocompatibility is demonstrated in cell cultures.

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Hyaluronic Acid Graft Copolymers with Cleavable Arms as Potential Intravitreal Drug Delivery Vehicles