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Research paper

Thermosensitive hybrid hydrogels for the controlled release of bioactive vancomycin in the treatment of orthopaedic implant infections

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ABSTRACT

The purpose of this work was the development of antibacterial delivery systems for vancomycin, with potential application in the prevention or treatment of orthopedic implant infections. Previous studies have shown tandem thermal gelling and Michael addition cross-linking of hydrogels based on methacrylate, acrylate or vinylsulfone triblock copolymers of PEG-p(HPMAm-lac$_{1-2}$) and thiolated hyaluronic acid. In this work we exploited these α-β unsaturated derivatives of PEG-p(HPMAm-lac$_{1-2}$) triblock copolymers and used them in combination with thiolated hyaluronic acid as controlled delivery systems for vancomycin. It was found that the antibiotic was sustainably released from the hydrogel networks for at least 5 days with release kinetics depending on diffusion and dissociation of the positively charged vancomycin from the negatively charged hyaluronic acid. The release of vancomycin could be tailored mainly by HA-SH solid content and degree of thiolation. The developed hydrogels were demonstrate efficacious in preserving the structural and functional integrity of the encapsulated drug by physical immobilization within the gel network and ionic interaction with hyaluronic acid, thereby preventing vancomycin deamidation processes. Furthermore, the antimicrobial activity of vancomycin loaded hydrogels was assessed, demonstrating retention of inhibitory activity towards *Staphylococcus aureus* during formulation and release, with slightly increased activity of vancomycin encapsulated in hydrogels of higher HA-SH content as compared to controls.

1. Introduction

Implantable medical devices employed in healthcare practice are often susceptible to microbial contamination, despite considerable research and development efforts [1]. Bacteria can readily colonize surfaces of synthetic materials, such as those used for the fabrication of hip and knee implants, causing device failure, significant morbidity in terms of pain and loss of function and need for surgery revision [2]. Limitations in the treatment of implant-associated infections are especially related to the formation of a bacterial biofilm at the implant surface, consisting of adherent bacteria encased in a polymeric complex and protecting microorganisms from antibiotic activity and from immune cells phagocytosis [3,4]. Thus, there is a strong need to mitigate bacterial colonization of implants in an effort to maximize the success rate of biomaterials based technologies. Established treatments to prevent bacterial colonization of implanted biomaterials rely on the use of antibacterial surface coatings, such as bone cements, nanoparticles and hydrogels for the controlled and local release of antibiotics [5–12]. These coatings are designed to offer (1) efficacy toward early bacterial colonization in the first hours after surgery through surface properties modification (i.e. hydrophilicity) [13,14]; (2) safety, as local and controlled release of therapeutic doses of active antibiotic may avoid induction of antibiotic resistance and possible risks for long-term effects on bone healing; (3) versatility, through loading of specific and/or multiple antibacterial agents; (4) ease of handling; and (5) reduced costs for large-scale application.

In the evolving panorama of biomaterials based coatings for antibiotic release, porous materials such as cements, cancellous bone [15], collagen sponges and poly(methyl methacrylate) biomaterials have been proposed [16,17]. These eluting systems proved efficacious in

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keeping the implant surface sterile while eradicating the short-term bacterial contamination of surrounding tissues [18–20]. More advanced and biodegradable systems for the controlled supplementation of antibiotics in bone infections are based on poly(lactic-*co*-glycolic acid) microparticles [21,22] or implants [23–26]. Other approaches comprise surface tethering of antibiotics [27]. In this paper we focus our attention on the use of biodegradable thermosensitive hydrogels as potential antimicrobial delivery systems with potential application in implant associated infections. Thermosensitive hydrogels are cross-linked networks of amphiphilic polymers displaying lower critical solution temperature (LCST) in aqueous medium. Below their LCST they are water-soluble, while undergoing phase separation and gel network formation above their LCST [28]. Hydrogels attracted long-lasting interest as biomaterials as they may act as controlled release systems, being particularly suited for protein and peptide release [29–31], have been successfully applied in tissue engineering applications [32–34], are often regarded as biocompatible materials [35,36] and their high moldability make them suitable to surface coating [37–39]. In our previous studies, a novel hydrogel system based on (meth)acrylated triblock copolymers consisting of a central PEG chain flanked at both sides by copolymers of poly(hydroxypropyl methacrylamide lactate)(p(HPMAm-lac$_{1,2}$)) have been developed for the controlled release of protein drugs and for cartilage tissue engineering applications [31,40]. When combined with thiolated hyaluronic acid, used as a cross-linker, these polymers formed *in situ* biodegradable networks dually cross-linked by thermal gelation and Michael addition [41]. The combination of thermosensitivity and in-situ Michael addition cross-linking offers significant advantages: (1) the delivery system can be administered *in vivo* by minimally invasive methods, as the system is liquid at room temperature, therefore injectable; (2) immediately after injection, the thermosensitive system jellifies assuring stability of the biomaterial at the injection site, and preventing premature polymer dissolution in the body fluids; (3) in time, when Michael additions occurs, the hydrogel is chemically stabilized so that degradation time and release profiles are prolonged and sustained. The choice of using thiolated hyaluronic acid as chemical cross-linker is motivated by its well-known biocompatibility and biological relevance in the field of orthopaedics. Furthermore, thiol mediated Michael addition is a biocompatible reaction, that occurs through mild processes and requires no exogenous, cytotoxic initiators [42].

In the present work, the previously developed hydrogel technology was used as releasing matrix for vancomycin, an amphoteric glycopeptide antibiotic, first reported in 1956 [43], active against gram-positive bacteria [44]. In recent years, vancomycin has received renewed attention as a therapeutic agent in bone and joint infections, including osteomyelitis and septic arthritis, due to the emergence of methicillin-resistant bacteria [45]. Gram-positive pathogens are the dominant bacteria in orthopaedics, with *Staphylococcus aureus* and the coagulase-negative *staphylococci* especially occurring [46,47].

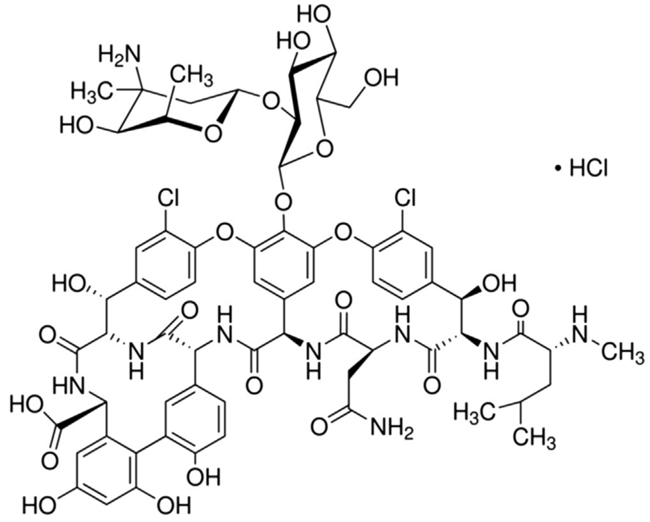
Limitations associated with the long-term release of vancomycin, however, reside in the poor stability of the drug, that is susceptible to asparagine deamidation and aspartate isomerization [48–50], leading to rapid decay of its antimicrobial activity.

The present paper focuses on the controlled release of vancomycin from injectable thermosensitive hydrogels, with particular attention on strategies to tailor the release kinetics and to preserve the structural and functional integrity of the drug through *in vitro* studies.

2. Materials and methods

2.1. Materials

Unless indicated otherwise, chemicals were obtained from Sigma-Aldrich and were used as received. Research grade sodium hyaluronate produced from microbial fermentation and hydrolyzed to a molecular weight of 33,000 Da, was supplied by Lifecore Biomedical, LLC. Hydroxyl propyl methacrylamide monolactate (HPMAm-lac$_1$) and dilactate (HPMAm-lac$_2$) were synthesized according to a previously reported method [51]. The synthesis of p(HPMAm-lac$_{1\text{-}2}$)-PEG triblock copolymers was described previously [52]. 3,3′-Dithiobis(propanoic dihydrazide) (DTP) was synthesized by the method described by Vercruysse et al. [53] Vancomycin hydrochloride (trade name Vancotex 500 mg) was kindly donated by PharmaTex Italia – Milano (Scheme 1). For bacteria growth inhibition tests, *S. aureus* (Gram-positive bacterium) ATCC 25923 was obtained from PBPI International. *S. aureus* was grown using Tryptone Soya Broth (TSB) obtained from OXOID Microbiology Products as well as Mueller Hinton Agar and Antimicrobial Susceptibility Test Discs.



Scheme 1. Chemical structure of the vancomycin hydrochloride (molecular weight 1450 Da).

2.2. Synthesis of thermosensitive methacrylated triblock copolymer

ABA triblock copolymer of PEG 10 kDa as hydrophilic B-block and p(HPMAm-lac) as thermosensitive outer A-blocks with a feed ratio HPMAm-monolactate/HPMAm-dilactate of 50/50 was synthesized by free-radical polymerization using (PEG-ABCPA)$_n$ as macroinitiator. Subsequently, methacrylic side groups at 30% of the available OH groups were introduced according to the procedure described by Censi et al. [41]. The chemical structure of metacrylated triblock copolymer is depicted in Scheme 2.

Before Methacrylation. ^{1}H NMR, $CDCl_3$, δ in ppm: 7.0 (1H, $-\mathrm{N}\mathit{H}\mathrm{CH_2CHCH_3}$), 5.5 (2H, $-\mathrm{NHCH_2C}\mathit{H}\mathrm{(CH_3)O}$ and $-\mathrm{COC}\mathit{H}\mathrm{(CH_3)O}$), 4.5 (1H, $-\mathrm{COC}\mathit{H}\mathrm{(CH_3)OH}$), 3.8–3.3 (909H, $-\mathrm{OC}\mathit{H_2}\mathrm{C}\mathit{H_2}$ PEG protons), 3.1–0.6 (main chain protons).

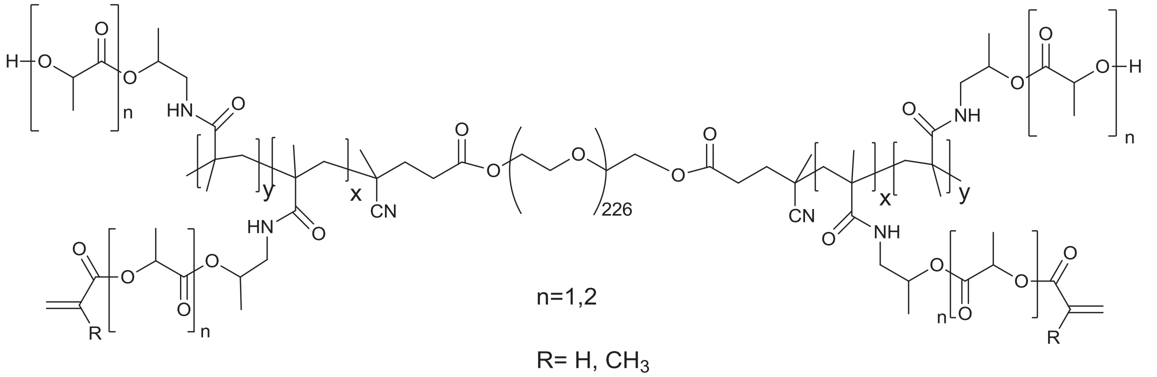
After Methacrylation. ^{1}H NMR, DMSO-d_6, δ in ppm: 7.2 (1H, $-\mathrm{N}\mathit{H}\mathrm{CH_2CHCH_3}$), 6.1–5.7 (2H, $\mathrm{CH_2CCH_3}$), 5.5–5.3 (1H, $-\mathrm{O}\mathit{H}\mathrm{CHCH_3}$), 4.7 (2H, $-\mathrm{NHCH_2C}\mathit{H}\mathrm{(CH_3)O}$ and $-\mathrm{COC}\mathit{H}\mathrm{(CH_3)O}$), 4.25 (1H, $-\mathrm{COC}\mathit{H}\mathrm{(CH_3)OH}$), 3.6–3.3 (909H, $-\mathrm{OC}\mathit{H_2}\mathrm{C}\mathit{H_2}$ PEG protons), 3.2–0.6 (main chain protons).

The degree of methacrylation, defined as the percentage of OH groups derivatized by methacrylate moieties, was determined by ^{1}H NMR.

2.3. Synthesis of thermosensitive acrylated triblock copolymer

The OH side groups of HPMAm-lac$_{1\text{-}2}$ were partially acrylated slightly modifying the procedure already described by Censi et al. [41].

Triblock copolymer (1 g) was dissolved in 100 mL of dry DCM under N_2 atmosphere. Triethylamine (0.060 mL) and a spatula tip of 4-Methoxyphenol were added. Acryloyl chloride was added dropwise in



Scheme 2. Chemical structure of ABA triblock copolymer with PEG 10,000 as middle B-block and poly(HPMAm-lac$_{1-2}$) as A block, partially modified with acrylic moieties (R = H) and methacrylic moieties (R = CH_3) respectively.

30 min at 0 °C, in a chloride/OH molar ratio of 0.15. The reaction mixture was subsequently stirred for 24 h at room temperature. Afterward, the polymers were diluted with THF and dialyzed (membrane with a cutoff of 12–14 kDa) against THF at 4 °C and deionized water for 2 days and ultimately isolated as a white powder by freeze-drying. The chemical structure of acrylated triblock copolymer is depicted in Scheme 2.

Before Acrylation. ^{1}H NMR, $CDCl_3$, δ in ppm: 7.1 (1H, $-NHCH_2CHCH_3$), 5.2 (2H, $-NHCH_2CH(CH_3)O$ and $-COCH(CH_3)O$), 4.4 (1H, $-COCH(CH_3)OH$), 3.8–3.3 (909H, $-OCH_2CH_2$ PEG protons), 3.2–0.5 (main chain protons).

After acrylation. ^{1}H NMR, DMSO-d_6, δ in ppm: 7.2 (1H, $-NHCH_2CHCH_3$), 6.6–6.7 (2H, CH_2CH), 5.4–5.2 (1H, $-OHCHCH_3$), 4.7 (2H, $-NHCH_2CH(CH_3)O$ and $-COCH(CH_3)O$), 4.2 (1H, $-COCH(CH_3)OH$), 3.7–3.4 (909H, $-OCH_2CH_2$ PEG protons), 3.2–0.6 (main chain protons).

The degree of acrylation, defined as the percentage of OH groups derivatized by acrylate moieties, was determined by ^{1}H NMR.

2.4. Synthesis of vinyl sulfonated triblock copolymer

According to the procedure described by *Lutolf and Hubbell* [54], part of the terminal OH groups were modified with vinyl sulfonate units (Scheme 3).

Triblock copolymer (1 g) was dissolved in dry DCM under nitrogen atmosphere and in presence of molecular sieves. Dry NaH (0.046 g) was added and after 10 min, time necessary for NaH to react, divinyl sulfone (DVS, 0.058 mL) was supplemented to the reaction. The molar ratio used between OH groups and DVS was 1:1. The reaction was stirred for 3 days and then neutralized with concentrated acetic acid (equimolar to NaH, 0.102 mL). The solvent was evaporated and the product precipitated in ice-cold diethyl ether. The vinyl sulfonated triblock copolymer was obtained as a white solid with a yield of 47%.

Before Vinyl Sulfonation. ^{1}H NMR, $CDCl_3$, δ in ppm: 6.7 (1H, $-NHCH_2CHCH_3$), 5.0 (2H, $-NHCH_2CH(CH_3)O$ and $-COCH(CH_3)O$), 4.3 (1H, $-COCH(CH_3)OH$), 3.6–3.4 (909H, $-OCH_2CH_2$ PEG protons), 3.0–0.8 (main chain protons).

After Vinyl Sulfonation. ^{1}H NMR, DMSO-d_6, δ in ppm: 7.4 (1H, $-NHCH_2CHCH_3$), 6.9 (1H, CH_2CHSO_2), 6.4–6.2 (2H, CH_2CHSO_2), 5.44 (1H, $-OHCHCH_3$), 5.1–4.8 (2H, $-NHCH_2CH(CH_3)O$ and $-COCH(CH_3)O$), 4.2 (1H, $-COCH(CH_3)OH$), 3.6–3.3 (909H, $-OCH_2CH_2$ PEG protons), 3.05–0.78 (main chain protons).

The degree of vinyl sulfonation, defined as the percentage of OH groups derivatized by acrylate moieties, was determined by ^{1}H NMR.

2.5. Synthesis of thiolated hyaluronic acid (HA-SH)

Hyaluronic acid was derivatized with thiol groups, to a varying extent, slightly modifying the procedure described by Shu et al. [55]. The reaction pathway is described in Scheme 4. The extent of thiol derivatization, also called degree of substitution (DS), is defined as the number of 3-3′-dithiobis propanoic hydrazide (DTP) residues per 100 disaccharide units. As a typical reaction procedure, to obtain a DS of 25%, 1.0 g of sodium hyaluronate (Mn 33 kDa) was dissolved in 100 mL sterile water and 240 mg of DTP was added while stirring. The pH was adjusted to 4.75 with HCl 2 M and, subsequently, 194 mg of 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) was added while keeping the pH at 4.75. The solution was stirred at room temperature for 48 h and the reaction was stopped by increasing the pH to 7 using 5 M NaOH. Then, a 20-fold excess of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was added as reducing agent. The reaction mixture was stirred for additional 24 h at 4 °C and subsequently purified by dialysis (MWCO 12–14 kDa) against dilute HCl (pH 3.5) containing 100 mM NaCl and finally against water at 4 °C. The final product was obtained as a white powder after lyophilization. The synthetic route for thiolated hyaluronic acid is depicted in Scheme 3. The DS was determined by ^{1}H NMR [41] and Ellman's method [56]. Thiolated hyaluronic acid of different DS is indicated as HA-SH_n′, where n′ indicates the DS.

^{1}H NMR, D_2O δ in ppm: 4.6–3.2 protons of hyaluronic acid, 2.7 (CH_2SH), 2.5 (CH_2CH_2SH), 1.8 ($NHCOCH_3$).

2.6. ^{1}H NMR spectroscopy

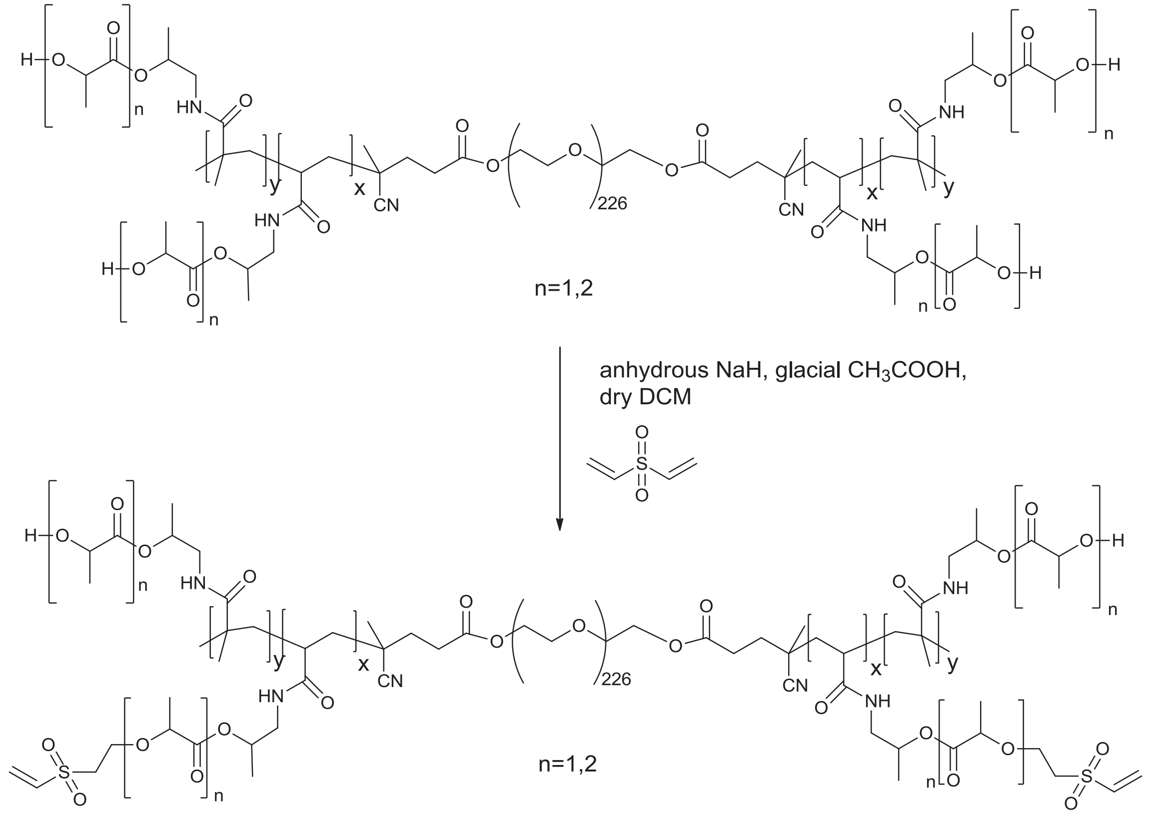
NMR spectra were recorded with a Varian Mercury Plus 400 NMR spectrometer. The polymers were dissolved in $CDCl_3$, DMSO-d_6 and D_2O. Chemical shifts were referred to the solvent peak.

2.7. Gel permeation chromatography (GPC)

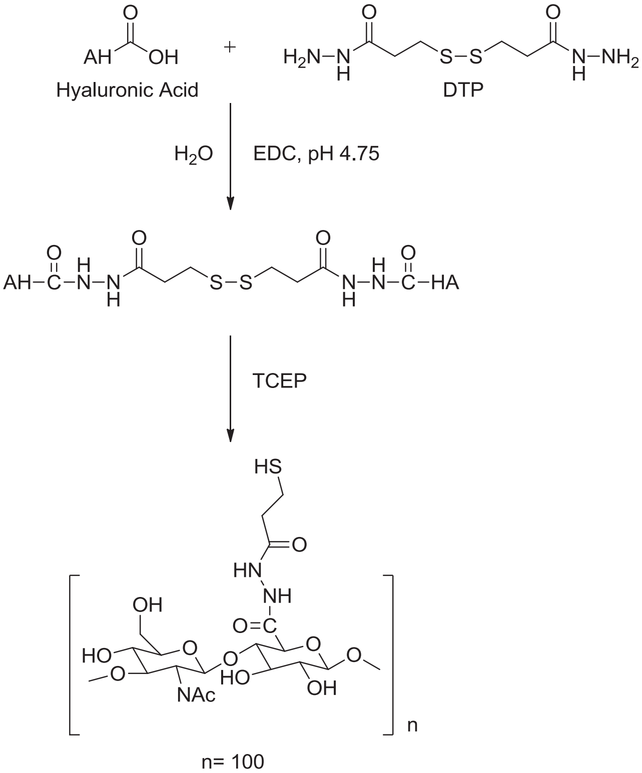
The weight average molecular weight (Mw), the number average molecular weight (Mn) and the Polydispersity Index (PDI) were determined by GPC using a TSKgel G4000HHR column (TOSOH BIOSCIENCE), 7.8 mm ID × 30.0 cm L, pore size 5 µm. Polystyrenes (range molecular weights 580–377.400) were used as calibration standards. The eluent was THF, the elution rate was 1.0 mL/min and the column temperature was 35 °C. The samples were dissolved in THF at a concentration of approximately 5 mg/ml.

2.8. Cloud point (CP)

The cloud point of the polymer was determined by light scattering using a Zetasizer Nano-S90 of Malvern Instruments. The temperature gradient range between 5 °C and 40 °C, 1 °C/min. The samples are dissolved in Ammonium Acetate buffer 120 mM pH 5.0 with the concentration of 3–5 mg/ml. Light scattering measurements were performed at a fixed scattering angle of 90° during temperature ramps from 5 to 40 °C, at a heating rate of 1 °C/min. The CP was determined as the



Scheme 3. Synthetic pathway of vinyl sulfonated triblock copolymer.



Scheme 4. Synthesis route of thiolated hyaluronic acid.

onset of increasing light scattering intensity.

2.9. Preparation of the placebo and vancomycin loaded hydrogels

Gels of a volume of 100 µL were prepared in cylindrically shaped glass vials (diameter of 5 mm) as follows. Triblock copolymers were dissolved in PBS buffer pH 7.4 (150 mM, containing 0.02% w/vol NaN_3 to prevent bacterial contamination). The samples were gently mixed using a needle and stored at 4 °C to allow the complete dissolution of the polymer. Separately, HA-SH at different degree of thiolation was dissolved in the same buffer at room temperature and mixed using a needle. Upon complete dissolution, the HA-SH solutions were mixed with the methacrylated/acrylated and vinyl sulfonated triblock copolymer solutions at room temperature and incubated at 37 °C overnight. The final concentrations of the triblock copolymers were 15 and 10% w/w and the concentration of HA-SH was determined in order to obtain a ratio between methacrylate/acrylate/vinylsulfone groups and thiol groups of 1.

Vancomycin loaded hydrogels were formulated as follows. Triblock copolymers were dissolved in PBS buffer pH 7.4; 20 µL of peptide solution (5 mg/mL) was added before the addition of HA-SH solutions. The peptide concentration was of 0.1% w/w. Upon mixing the solutions of triblock copolymers and thiolated hyaluronic acid, the gels were incubated at 37 °C.

2.10. Vancomycin release studies

Gels were prepared as described above and incubated overnight at 37 °C before starting the release studies. 0.9 mL of PBS buffer pH 7.4 or borate buffer pH 8.5 was added on top of the hydrogels and the vials incubated at 37 °C. Next, 0.15 mL of solutions were taken and replaced by an equal amount of fresh buffer. The concentration of vancomycin was determined by using high performance liquid chromatography (HPLC).

2.11. Vancomycin degradation studies

Vancomycin at the concentration of 0.1 mg/ml was dissolved in PBS pH 7.4 and incubated at 37 °C. In parallel, vancomycin loaded hydrogels, prepared as described above, were incubated at 37 °C in sealed vials to prevent water evaporation. At determined time intervals, aliquots of aged vancomycin solution were withdrawn and analyzed by HPLC as detailed below and, at the same time points, hydrogels were exposed to 500 µL of PBS buffer at pH 7.4 for 2 h to allow a certain extent of vancomycin release. The release medium was analyzed by HPLC and compared to vancomycin solutions in PBS. The extent of vancomycin degradation was evaluated considering the relative area under the chromatographic peak of degradation products as compared to that of native vancomycin.

2.12. High performance liquid chromatography (HPLC)

Analyses were performed on a HPLC-DAD, Agilent 1100 Series, using a Lichrospehere RP 18, 5 µm, 125 × 4.6 mm, column set at the temperature of 30 °C. Isocratic elution of a 25:75 mixture of ammonium formate 0.06 M buffer pH 7.7 and methanol at a flow rate of 0.8 mL/min was applied to run 20 µL volume samples. Detection was performed at the wavelength of 230 nm for a runtime of 15 min. Retention time of Vancomycin is of 6.8 min.

2.13. Bacterial growth inhibition

To study the inhibition growth by vancomycin loaded hydrogels, a methicillin-susceptible *S. aureus* strain (*S. aureus* ATCC 25923) was tested using a modified Kirby-Bauer and microdilution assay. *S. aureus* was grown aerobically at 37 °C for 18 h using Tryptone Soya Broth (OXOID) as the growth medium reaching the exponential growth phase at the concentration of 10^8 CFU/ml.

In modified Kirby-Bauer assay, agar plates were formulated from Mueller Hinton-agar (OXOID) and were evenly coated with *S. aureus* in its exponential growth phase at the concentration of 10^8 CFU/mL. Here, immediately after coating, disks of vancomycin loaded hydrogels (vancomycin concentration of 0.1 mg/ml) were applied to Mueller Hinton-agar plates coated with *S. aureus* and a vancomycin susceptibility disc was loaded with a vancomycin solution in PBS pH 7.4 at the same concentration as the hydrogel disk, and taken as positive control. Placebo hydrogel were also assayed as negative control. After 18 h of incubation at 37 °C, agar plates were digitally imaged and zones of inhibition surrounding the test samples were measured with a precision measuring magnifier.

In microdilution assay, vancomycin release samples obtained from release experiments on hydrogels after 7 days of exposure thereof to release medium release samples were tested. For comparison, a standard solution of vancomycin (0.1 mg/ml) stored at 37 °C for 7 days was also tested. Using a calibrated disposable pipette release sample and pure vancomycin solution were added in triplicate to a 96 well clear bottom plate and serially diluted with an equal volume of cation-adjusted Mueller-Hinton broth (CaMHB).

Subsequently, *S. aureus* in its exponential growth phase was added to diluted samples at a final concentration of 10^{-5} CFU/ml. In addition, controls of 0.01M PBS dilutions in media containing no vancomycin exposed to *S. aureus,* as positive control and not exposed to *S. aureus,* as negative control, were included. The plate was incubated at 37 °C for 16–18 h. After the incubation optical density at 600 nm was read using a microplate spectrophotometer (Biotek µQuant) to determine the MIC values. The Minimum Inhibitory Concentrations (MICs) were defined as the lowest concentration of the compound able to inhibit the growth of the microorganisms.

The bacteria density was normalized as follow:

$$\text{Normalized } S.\ aureus \text{ density} = (\mathrm{OD}_{600,\text{sample}} - \mathrm{OD}_{600,\text{negative control}})/(\mathrm{OD}_{600,\text{positive control}} - \mathrm{OD}_{600,\text{negative control}})$$

3. Results and discussion

3.1. Synthesis and preparation of vancomycin loaded hydrogels with different gelation mechanisms

A thermosensitive triblock copolymer TC was synthesized by radical polymerization using a PEG macroinitiator (PEG MW 10,000 Da) with a yield of 66% and an Mn of 36 kDa, as determined by ^{1}H NMR. GPC analysis, using polystyrene standards, revealed a Mn value of 12.1 kDa with a PDI of 1.59, typical of free radical polymerization procedures [31,57]. TC showed a ratio between HPMAm-lac$_1$ and HPMAm-lac$_2$ close to the feed ratio of 50%, as calculated according to ^{1}H NMR. Such ratio resulted in a thermosensitive polymer with a cloud point, determined by light scattering, of 31 °C, in accordance with previously observed values [31,40]. TC was subsequently functionalized, to a varying extent, with methacrylate, acrylate and vinyl sulfone moieties to yield hydrogels with different chemical cross-linking reactions. The methacrylate (TC_Met) and acrylate (TC_Acr) functionalized triblock copolymers, synthesized according to previously established methods [31,41], displayed 32% and 33% of their free hydroxyl groups on lactate side chains modified with cross-linkable moieties, respectively. Similarly to (meth)acrylate derivatives, newly synthesized vinyl sulfone bearing thermosensitive polymers based on triblocks of PEG and p(HPMAm-lac$_{1\text{-}2}$) (VinylSulf_TC) showed a DS of 30%. The comparison between ^{1}H NMR spectra in DMSO-d_6 of TC and VinylSulf_TC, as seen in Fig. 1, clearly showed the appearance of new peaks between 6 and 7 ppm upon vinyl sulfonation reaction. The mentioned peaks are assigned to the protons of the vinyl group and demonstrated the successful derivatization of TC. From the obtained DS, calculated according to ^{1}H NMR, it was observed that the conversion of DVS during vinyl sulfonation reaction was approximately 50%. The partial modification of the triblock copolymer with vinyl sulfone moieties led to a decrease in the CP, which dropped to from 31 to 11, 14 and 17 °C, for TC_Met, TC_Acr and TC_VinylSulf, respectively, due to an increase in the polymer hydrophobicity. The dependence of CP on hydrophobicity was observed earlier [40].

GPC analyses revealed that Mw's and PDI's were, within the experimental error, constant upon partial modification of the free hydroxyl groups with methacrylate, acrylate and vinyl sulfone moieties, indicating that no premature polymerization of vinyl sulfone residues had occurred during derivatization reaction, workup and lyophilization/precipitation procedures. The yields of the derivatization reactions were found between 82 and 98% for TC_Met and TC_Acr, respectively, while a lower yield of 47% was obtained for TC_Sulf. This observed difference was attributed to the purification method used for the synthesized polymers, concluding that dialysis/lyophilization are more efficient than precipitation procedures. Table 1 overviews the main characteristics of the synthesized polymers.

Thiolated hyaluronic acid of a molecular weight of 33 kDa was used as a cross-linker and synthetized with a yield of approximately 80% and DS values ranging of 23 and 72%. As shown in Table 2, there was good agreement between DS values calculated according to ^{1}H NMR and determined by Ellman's method, indicating no premature formation of inter- and intra-chain disulfide bonds.

Hydrogels were formulated by combining TC_Met, TC_Acr and TC_VinylSulf at the concentrations of 10 and 15% w/w with HA-SH_23 and 72 in aqueous medium at physiological pH and body temperature, using a (met)acrylate and vinyl sulfone to thiol ratio of 1. The prepared stoichiometric mixtures led to the rapid formation of a viscoelastic network by immediate thermal gelation upon temperature increase to

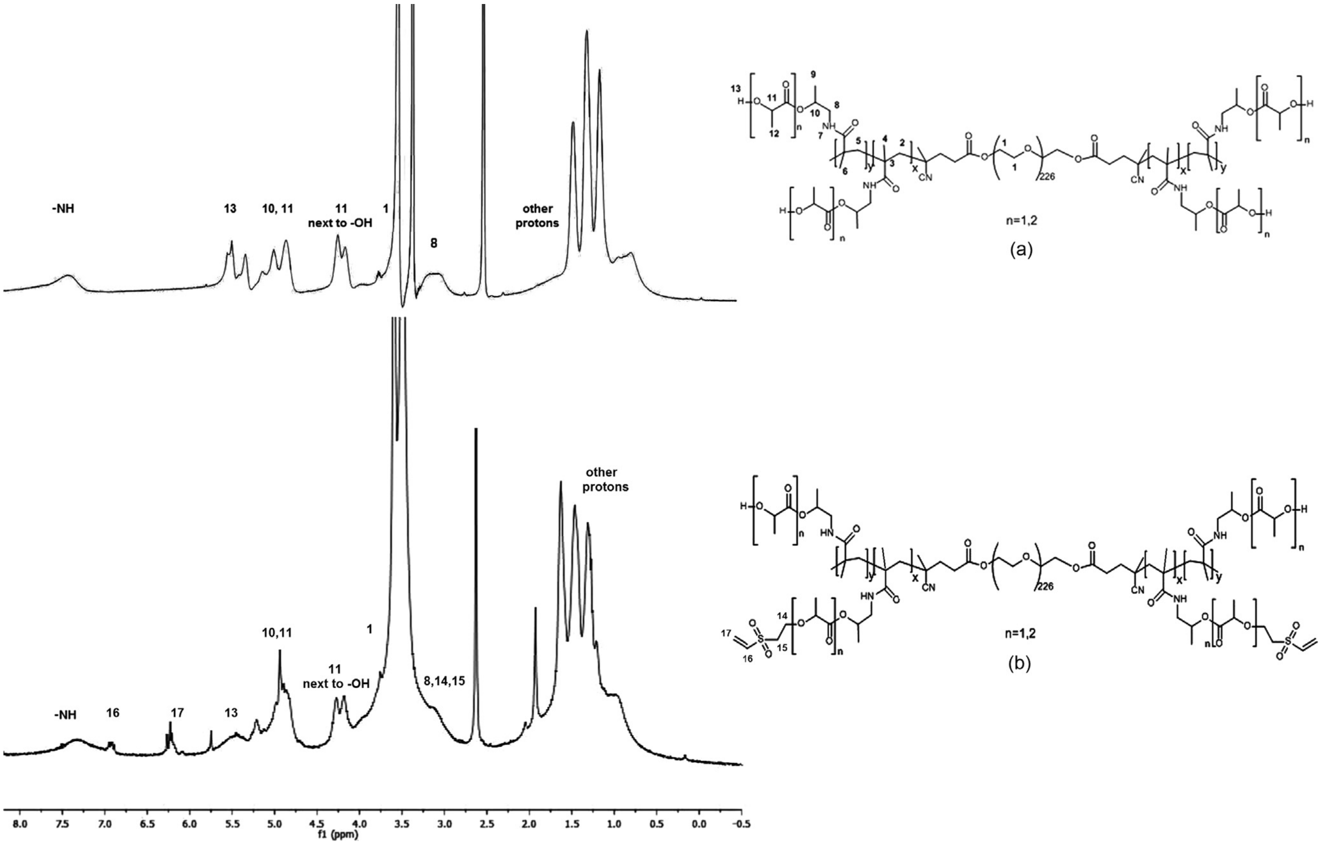


Fig. 1. ^{1}H NMR in DMSO-d_6 of (a) unmodified triblock copolymer and (b) vinyl sulfone bearing triblock copolymer.

Table 1
Overview of the main characteristics of the synthesized triblock copolymers (TC = unmodified triblock copolymer; TC_MET = methacrylated triblock copolymer; TC_ACR = acrylated triblock copolymer; TC_VINYLSULF = vinyl sulfonated triblock copolymer), as determined by ^{1}H NMR, GPC and light scattering.

	Mn[†] (kDa)	Mn[‡] (kDa)	Mw[‡] (kDa)	PDI[‡]	Cloud point[*] (°C)	Obtained DS[†] (%)	Yield (%)
TC	36	12.1	19.3	1.6	31	0	66
TC_Met	32	14	20	1.4	11	32	98
TC_Acr	37	11	14	1.3	14	33	82
TC_VinylSulf	35	10.1	16.6	1.6	17	30	43

[†] Based on ^{1}H NMR.
[‡] Based on GPC using PEG standards.
[*] Based on light scattering.

37 °C, due to the thermosensitive character of the synthetic polymers. A progressive formation of chemical cross-links via spontaneous Michael addition between thiol and metacrylate/acrylate/vinyl sulfone groups occurred in time, thereby stabilizing the network structure. As reported previously, TC_VinylSulf displayed the fastest cross-linking kinetics when combined with HA-SH, with conversion of vinyl sulfone moieties in a timeframe ranging from 9 to 60 min, depending on gel formulation [41]. TC_Acr and TC_Meth hydrogels showed a lower reactivity towards Michael addition in the presence of thiol groups [41], however full conversion of acrylate and methacrylate groups was observed for both polymers after 24 h cross-linking at 37 °C (data not shown). Vancomycin was easily loaded in the hydrogel networks by mixing an aqueous glycopeptide solution and a triblock copolymer solution prior to adding HA-SH solution and heating. It was observed that the presence of vancomycin in the hydrogel formulations did not affect the gelation time nor the mechanical stability of hydrogels.

3.2. Vancomycin release from hydrogel networks composed of TC_Met/Acr/VinylSulf and HA-SH

The suitability of hydrogels composed of TC_Met/Acr/VinylSulf at varying concentrations and HA-SH at varying degree of thiolation (as indicated in Table 3) as antibacterial system for potential orthopaedic applications was investigated. To this end, *in vitro* peptide release from Michael type cross-linked hydrogels was studied, in phosphate buffer at pH7.4, using vancomycin as an antibiotic drug. Hydrogels were prepared according to the described procedure and incubated at 37 °C for 24 and 1 h, for TC_Met/Acr and TC_VinylSulf hydrogels, respectively. The different incubation time depended on the reactivity of the three

Table 2
Overview of the main characteristics of the synthesized thiolated hyaluronic acid, as determined by ^{1}H NMR and Ellman's method.

	DTP Feed ratio (%)	Obtained DS[‡] (%)	Obtained DS[*] (%)	Conversion %	Yield (%)
HA-SH_23	35	23	25.3 ± 2.2	33	81
HA-SH_72	80	72	70.5 ± 3.3	46	79

[‡] Based on ^{1}H NMR.
[*] Based on Ellman's method.

Table 3
Solid composition of the prepared hydrogel formulations.

TC_Met/Acr/VinylSulf (% w/w)	HA-SH_72/HA-SH_23 (% w/w)	Vancomycin (% w/w)
10	3.9/12.7	0.1
15	5.9/18	0.1

studied α-β unsaturated groups toward Michael addition. It was indeed shown previously that, in order to allow complete chemical cross-linking, 24 h are necessary for TC_Met/Acr based hydrogels, while 1 h for TC_VinylSulf hydrogels [58].

A continuous release of vancomycin was observed for all hydrogel formulations in 100 h (approximately 5 days) (Fig. 2) with release kinetics differing according to gel composition. In Fig. 2a–d it can be observed that, independently from the type of modifying group on TC, formulations having same triblock copolymer content and same degree

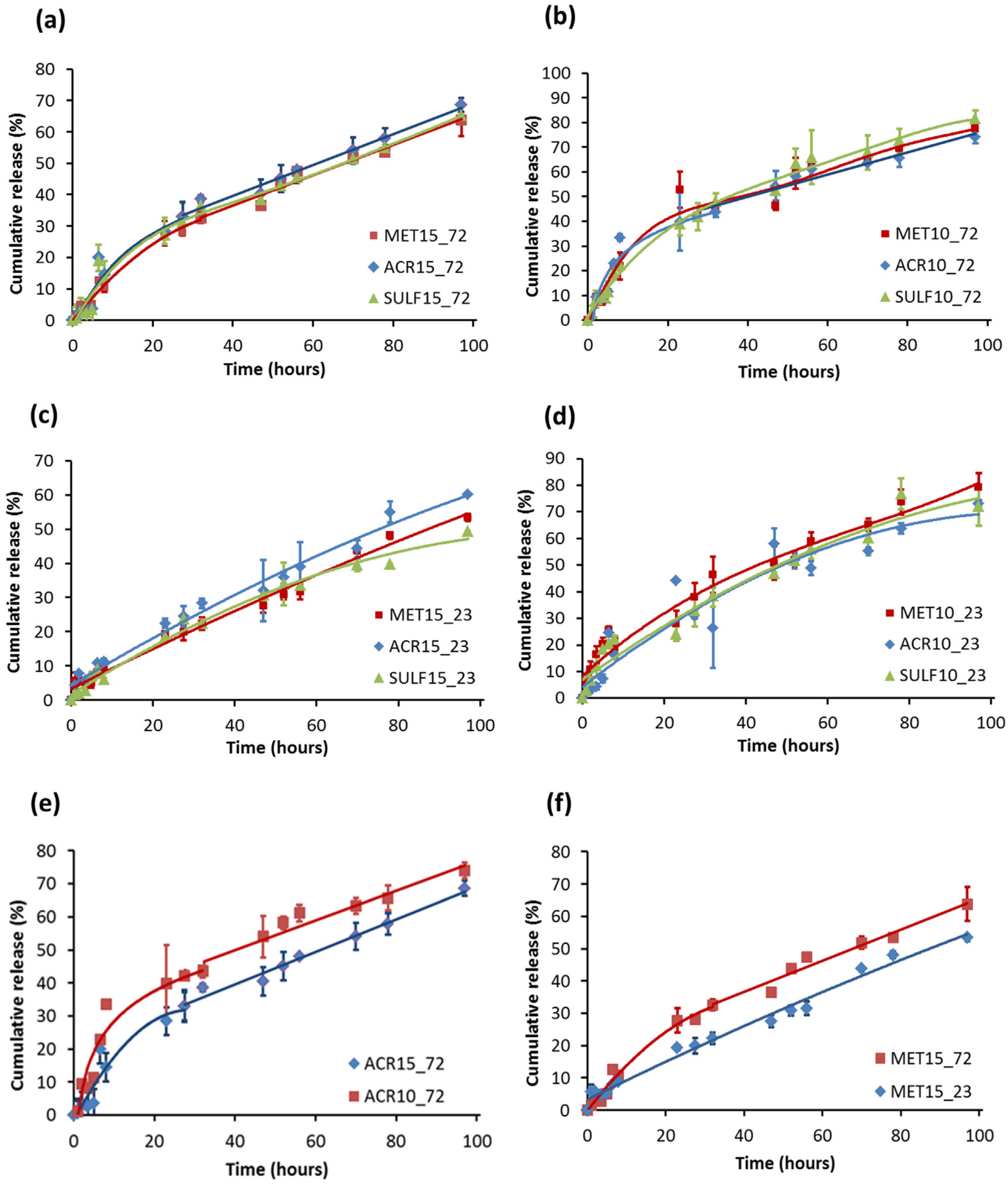


Fig. 2. Cumulative vancomycin release from hydrogel networks composed of TC_Met/Acr/VinylSulf at different triblock copolymer concentration and HA-SH at different degree of thiolation. Studies were conducted in phosphate buffer at pH 7.4. (a) Comparison between hydrogels consisting of 15% w/w TC_Met (MET15_72) or TC_Acr (ACR15_72) or TC_VinylSulf (SULF15_72) and HA-SH_72; (b) Comparison between hydrogels consisting of 10% w/w TC_Met (MET10_72) or TC_Acr (ACR10_72) or TC_VinylSulf (SULF10_72) and HA-SH_72; (c) Comparison between hydrogels consisting of 15% w/w TC_Met (MET15_23) or TC_Acr (ACR15_23) or TC_VinylSulf (SULF15_23) and HA-SH_23; (d) Comparison between hydrogels consisting of 10% w/w TC_Met (MET10_23) or TC_Acr (ACR10_23) or TC_VinylSulf (SULF10_23) and HA-SH_23; (e) Comparison between hydrogels consisting of 10 (ACR10_72) and 15% w/w (ACR15_72) TC_Acr and HA-SH_72; (f) Comparison between hydrogels consisting of 15% w/w TC_Met and HA-SH_23 (MET15_23) or HA-SH_72 (MET15_72).

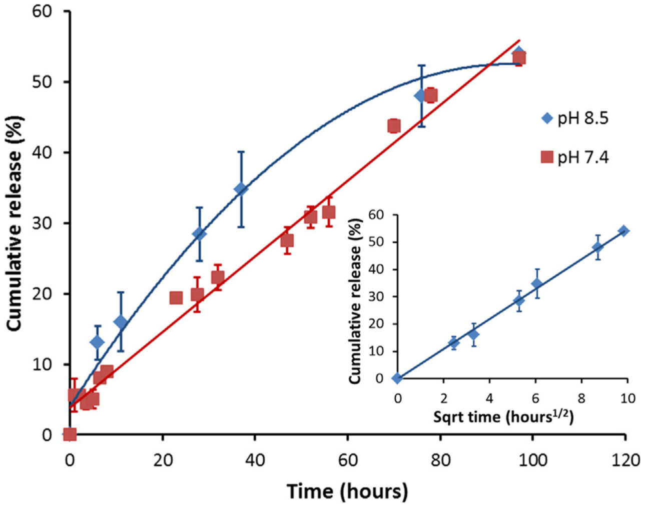


Fig. 3. Effect of pH on vancomycin release profiles from hydrogels composed of 15% w/w TC_Met and 18% HA-SH_23. The insert shows the vancomycin cumulative release in borate buffer at pH 8.5 as a function of the square root of time.

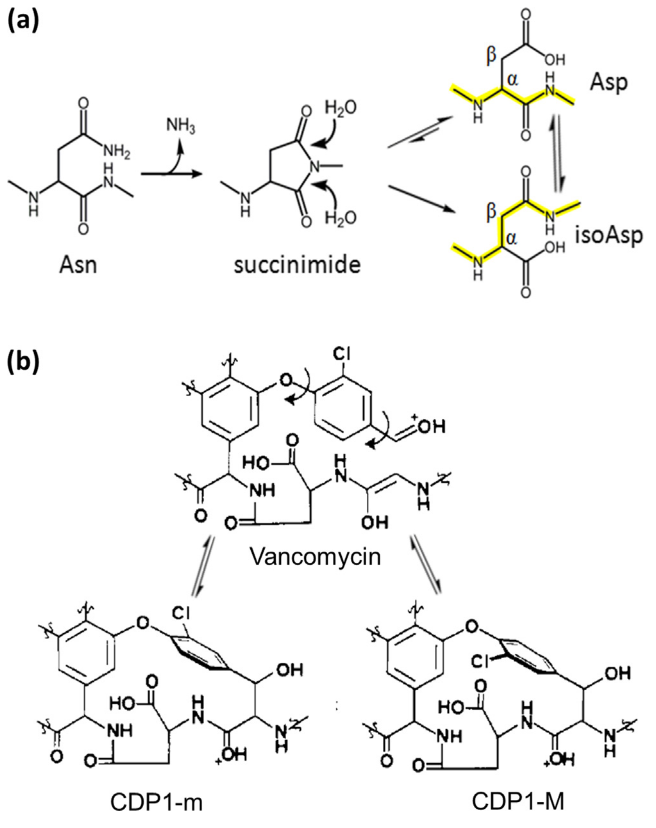


Fig. 4. Degradation pathway of vancomycin. (a) Deamidation of asparagine residues at pH > 5 and (b) rearrangement into zwitterionic rotamers CDP1-m and CDP1-M.

of HA thiolation released vancomycin with very similar kinetics and release mechanism, indicating that, although the reactivity towards Michael addition of methacrylate, acrylate and vinyl sulfone groups is extremely different, as demonstrated by the gel formation kinetics, there was no different retention of the encapsulated peptide. It can be therefore concluded that Michael type cross-linked hydrogels based on TC_Met/Acr/VinylSulf and HA-SH did not cross-react with the encapsulated vancomycin during chemical gelation. Conversely, comparing the release behavior of hydrogels consisting of different initial

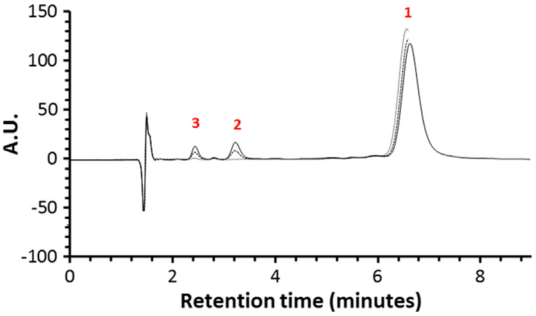


Fig. 5. Superimposed chromatograms of vancomycin solutions in PBS at pH 7.4 upon release from hydrogels at different timepoints (t = 0 is represented by the solid grey line, t = 3 days by the dotted line and t = 4 days by the solid black line). Peak 1 is native vancomycin, peak 2 is CDP1-M and peak 3 is CPD1-m.

TC content (10 and 15% w/w), we can observe that hydrogels of 10% w/w TC concentration displayed faster release kinetics as compared to 15% w/w hydrogels, as shown in Fig. 4e, where an example of release profiles for TC_Acr based hydrogels is presented. Differently from previously observed release kinetics from PEG-p(HPMAm-lac$_{1\text{-}2}$) hydrogels, relying mainly on diffusion [40,41], hydrogels showed a biphasic release with an initial phase up to 28 h showing a cumulative release of approximately 43 and 32% for 10 and 15% w/w polymer content, respectively. The following release phase up to 100 h, showed zero order release kinetics for both formulations with very similar kinetic constants (0.46 and 0.49 for 10 and 15% w/w polymer content, respectively). The observed release behavior, combined with hydrogel degradation data, showing complete dissolution of the Michael addition networks in more than 60 days (data not shown) would suggest that vancomycin was released by a combined mechanism based on diffusion and ionic dissociation from the hydrogel network. We hypothesized, indeed, that vancomycin, possessing an average net charge of +0.67 at the pH of 7.4 [59], ionically interacted with the negatively charged HA-SH and was released by diffusion through the network pores only upon dissociation from the network. The differences in the release profiles of Fig. 2a may be therefore also attributed to the different HA-SH_72 content (5.9 and 3.9% w/w for 15 and 10% w/w hydrogels, respectively) that resulted in different extent of ionic interaction with vancomycin. In Fig. 2f, hydrogels containing the same TC_Met initial content (15% w/w) but HA-SH of different DS (23 and 72%) and concentration (5.9 and 18% w/w of HA-SH_72 and HA-SH_23, respectively) also showed different release kinetics, with a similar biphasic release for 15% w/w TC_Met/HA-SH_72 hydrogels and a zero order release kinetic for 15% w/w TC_Met/HA-SH_23 hydrogels, that displayed the highest HA-SH content of 18% w/w and the highest availability of negatively ionized carboxyl groups, due to the least extent of modification.

The native peptide drug was only partially recovered during release experiments, with cumulative release values varying approximately from 50 to 80%. This observation can be ascribed to the degradation of the released vancomycin in the release medium (phosphate buffer pH 7.4), as also demonstrated in Fig. 6a.

To the scope of further elucidating the release mechanism of vancomycin from TC_Met/Acr/VinylSulf and HA-SH networks, vancomycin release studies from hydrogels consisting of 15% w/w TC_Met and 18% w/w HA-SH_23 were performed in borate buffer at the pH of 8.5, at which vancomycin does not possess net charge, and compared to release data at pH 7.4. It turned out that, upon a small burst release of approximately 4% observed for both pHs tested, vancomycin was released at higher rate at the pH of 8.5 compared to 7.4 (Fig. 3), although network degradation at the higher pH was negligible for the timeframe of the release experiment and comparable to that at pH 7.4 (data not shown). The release mechanism at pH 8.5 was investigated by fitting the release curve to the Rigter-Peppas equation:

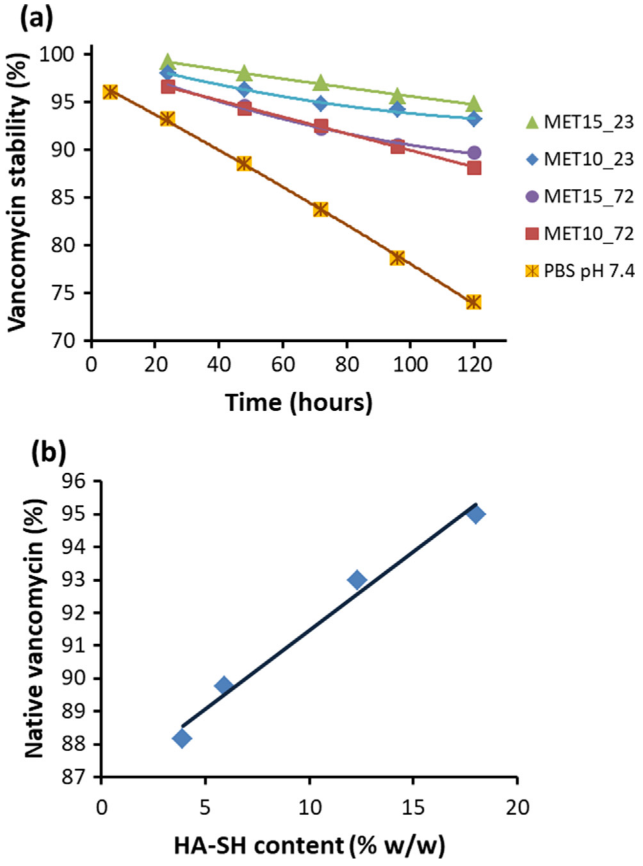


Fig. 6. Stability studies of vancomycin. (a) Concentration decay of the glycopeptide in time during storage at 37 °C within hydrogel formulations containing 10 and 15% w/w TC_Met/HA-SH_23 (MET10_23 and MET15_23, respectively) and 10 and 15% w/w TC_Met/HA-SH_72 (MET10_72 and MET15_72, respectively) as compared to vancomycin solution in phosphate buffer (PBS) pH7.4. (b) Residual native vancomycin after 5 days into hydrogel networks as a function of HA-SH concentration.

$$\frac{Mt}{M\infty} = kt^n$$

where $\frac{Mt}{M\infty}$ represent the fractional release of the loaded drug, k is a kinetic constant, t is the release time and n is the diffusional exponent that is closely related to the release mechanism of the drug. If n = 0.5 the release is governed by Fickian diffusion; if n = 1 molecules are normally released by surface erosion and if n has a value between 0.5 and 1, both mechanisms play a role.

The experimental release curve at pH 8.5 fitted to n-value of 0.5 as the cumulative release scaled linearly with square root of time, as shown in the insert of Fig. 3. This implies that the release was diffusion-controlled, no contribution of polymer-drug interaction exists at pH 8.5 and the hydrogel mesh size is bigger than the hydrodynamic diameter of vancomycin. Therefore, it can be concluded that at pH7.4, the ionic interaction between vancomycin and HA-SH actually contributed to determine the release mechanism.

3.3. Vancomycin stability studies

It was previously shown that vancomycin is a glycopeptide susceptible to degradation at physiological conditions, undergoing deamidation of its asparagine residue and rearrangement into two rotamers (CDP1-m and CDP1-M), as represented in Fig. 4 [48,49,60,61]. The two deamidated forms are both biologically inactive, limiting the shelf-life of the drug, its safety and efficacy upon administration. We investigated the stability of vancomycin when formulated into hydrogels of 10 and 15% w/w TC_Met and HA-SH_23/72, comparing the degree of drug degradation in time with that in PBS pH 7.4.

Vancomycin degradation was studied by HPLC, that revealed the presence of three different species (Fig. 5) assigned as native vancomycin (peak 1 in Fig. 5), CDP1-m (minor) (peak 2 in Fig. 5) and CDP1-M (major) (peak 3 in Fig. 5) [62]. From Fig. 5, it can be noted that the vancomycin degradation products increased in time. However, the extent of degradation was found sensibly different in vancomycin solutions as compared to hydrogel formulations and among hydrogel formulations, as shown in Fig. 6a. All vancomycin degradation profiles followed first order kinetics, with 74% of residual native vancomycin after 5 days in PBS 7.4 and recovery of 88 to approximately 95% of non-deamidated drug after the same time period in jellified networks. Differences in the degradation rate of vancomycin were observed for different hydrogel formulations, particularly, those containing higher initial solid content and HA-SH of lower degree of thiolation displayed the best ability to preserve the structural integrity of the encapsulated drug. Conversely, no differences in the capacity of the hydrogel to stabilize the protein structure were observed for hydrogels varying in type of cross-linkable moiety (methacrylate, acrylate or vinyl sulfone) (Fig. 1SI, Supporting Information). Fig. 6b shows the linear dependence of residual native vancomycin after 5 days storage in hydrogel formulations and HA-SH concentration within hydrogel formulations. By calculating the first order kinetic constants of the degradation products CDP1-m and CDP1-M, we found out that they are also linearly dependent on HA-SH concentrations, as depicted in Fig. 7. However, the same figure shows that the stabilizing effect of HA-SH was more pronounced on CDP1-M, whose kinetic constants showed a 3.5-fold decrease as HA-SH concentration increased from 3.9 to 18. Conversely, the formation of CDP1-m was negligibly affected by the HA-SH content. It derives that the stability of vancomycin is highly enhanced by the encapsulation into tridimensional gel-networks and that the hydrogel solid content and the HA-SH concentration play pivotal role in this context. These experimental observations find explanation in earlier published theories, claiming that the immobilization of proteins and peptides prone to deamidation into viscous environments stabilizes their structure [48,63].

3.4. Vancomycin biological activity upon release from hydrogels

The choice to test the antibacterial activity of vancomycin released from hydrogels against *S. aureus* was due to the common occurrence of this bacterium and its resistant strains in hospital acquired infections [64].

Disks of hydrogel and positive control disk, with the same concentration of vancomycin (0.1% w/w) were directly exposed to *S. aureus* coated agar and after the incubation period, the Zone of Inhibition (ZOI) was measured.

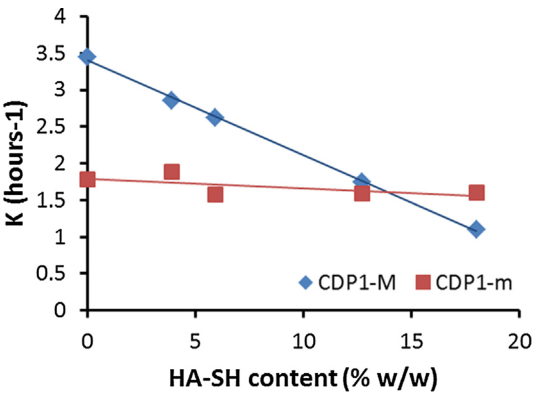


Fig. 7. First order kinetic constants of CDP1-m and CDP1-M as a function of HA-SH solid content.

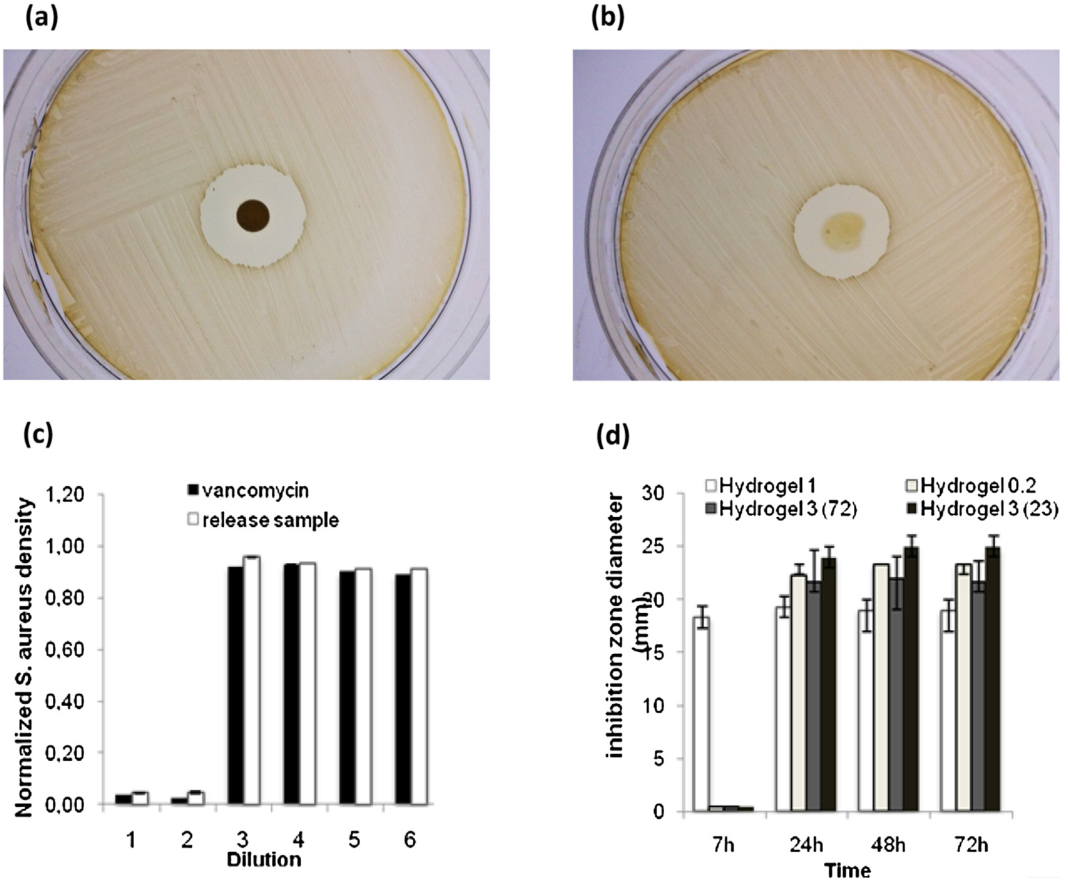


Fig. 8. *Staphylococcus aureus* growth inhibition. (a) Mueller Hinton Agar coated with *S. aureus* exposed to an Antimicrobial Susceptibility Test Disc (6 mm) loaded with 0.1 mg/ml vancomycin (positive control). Zone of inhibition surrounding vancomycin disc control (inhibition zone diameter = 20 mm). (b) Mueller Hinton Agar coated with *S. aureus* exposed to a 0.1% w/w vancomycin loaded 15% w/w TC_Met/HA-SH_72 hydrogel disc (7 mm). Zone of inhibition surrounding TC_Met/HA-SH_72 hydrogel disc (inhibition zone diameter = 19 mm). (c) Normalized *S. aureus* density after exposure to dilutions of Vancomycin released from 15% w/w TC_Met/HA-SH_72 hydrogel and a control of non-released vancomycin (the range dilution showed is between 1 = 6,25 µg/l and 6 = 0,19 µg/l). (d) Inhibition zone diameter for each vancomycin loaded hydrogel tested against *S. aureus* at different time intervals. Comparison between 10% w/w TC_Met/HA-SH_72 (hydrogel 1), 10% w/w TC_Met/HA-SH_23 (Hydrogel 0.2), 15% w/w TC_Met/HA-SH_72 (Hydrogel 3(72)) and 15% w/w TC_Met/HA-SH_23 (Hydrogel 3 (23)).

Fig. 8a and b show a clear Zone of Inhibition around the vancomycin control (a) and around the vancomycin loaded 15% w/w TC_Met/HA-SH_72 hydrogel disk (b) exposed to *S. aureus* coated agar, in a modified Kirby-Bauer test. Comparing the ZOI of the hydrogel with that of positive control we could establish that the hydrogel had the same activity of positive control discs. In fact, the hydrogel produced an inhibition zone of 19 ± 1 mm, very close to the positive control disk (20 ± 1 mm). This result showed that the vancomycin formulated and released from hydrogels maintained its activity against *S. aureus*.

Fig. 8c shows the comparison between the normalized density of *S. Aureus* exposed to progressive dilutions of vancomycin released from 15% w/w TC_Met/HA-SH_72 and that of a standard solution of freshly dissolved vancomycin. This figure demonstrates that the MIC of the standard solution and the MIC of released samples are equal and in the range of 1.56–3.12 µg/ml, in agreement to what reported in literature [64–67]. Fig. 8d compares the inhibition zone diameters of vancomycin released at different timepoints from four different hydrogel formulations. It was found that, in line with stability and release data, hydrogels containing higher content of HA-SH showed slightly higher inhibitory activity than those containing smaller amounts of HA-SH (10% w/w TC_Met/HA-SH_72 (hydrogel 1) < 15% w/w TC_Met/HA-SH_72 (Hydrogel 3(72)) < 10% w/w TC_Met/HA-SH_23 (Hydrogel 0.2) < 15% w/w TC_Met/HA-SH_23 (Hydrogel 3(23)).

4. Conclusions

In this paper we reported on Michael type cross-linked hydrogels based on methacrylate, acrylate and vinyl sulfone bearing thermosensitive triblock copolymers of PEG-p(HPMAm-lac$_{1\text{-}2}$) and thiolated hyaluronic acid. Polymers modified with cross-linkable moieties were successfully synthesized at a degree of substitution of approximately 30% for all polymers. The combination of such polymers with thiolated hyaluronic acid yielded mechanically stable chemical networks displaying different gelation rates according to gel composition (vinyl sulfone > acrylate > methacrylate and HA-SH_23 > HA-SH_72). Vancomycin delivery studies *in vitro* revealed that the glycopeptidic antibiotic can be continuously released for at least 5 days with release mechanisms depending on diffusion and ionic dissociation of the positively charged vancomycin from the negatively charged hyaluronic acid. The release profiles could be tailored by initial hydrogel solid content, HA-SH thiolation degree and concentration. Hydrogels were demonstrated efficacious in preserving the structural and functional integrity of the encapsulated vancomycin, decreasing its deamidation rate. The vancomycin tendency to deamidation was particularly prevented in hydrogels of higher HA-SH content. Similarly, antimicrobial activity and minimal inhibitory concentration of vancomycin were found similar to those of vancomycin aqueous solutions, with slightly higher antimicrobial activity observed for hydrogels containing higher HA-SH content. In conclusion, we demonstrated that the physical immobilization in the hydrogel network and the ionic interaction at physiological pH between the drug and the polymer lead to the preservation of the chemical and biological integrity of the drug, that is controllably released from the delivery system. Interestingly, a direct correlation between polymer content, charge density and drug stability was found. These findings strengthen the hypothesis that jelling systems, able to immobilize protein drugs through a double mechanism of physical entrapment and ionic interaction, are promising candidates as releasing matrices for the local and controlled release of protein drugs susceptible to degradation. The vancomycin loaded injectable thermosensitive hydrogel developed in this work may have potential application in the field of orthopaedics, as safe and efficacious local delivery system in the treatment and/or prevention of implant-associated infections.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.07.006.

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