International Journal of Pharmaceutics 636 (2023) 122798

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Anionic polysaccharides for stabilization and sustained release of antimicrobial peptides

Cristina Casadidio [a,b,*], Laura Mayol [c], Marco Biondi [d], Stefania Scuri [e,1], Manuela Cortese [a], Wim E. Hennink [b], Tina Vermonden [b], Giuseppe De Rosa [d], Piera Di Martino [f,g,*], Roberta Censi [a,g]

[a] School of Pharmacy, Drug Delivery Division, University of Camerino, CHiP Research Center, Via Madonna delle Carceri, 62032 Camerino (MC), Italy
[b] Department of Pharmaceutical Sciences, Division of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University 99, 3508 TB Utrecht, the Netherlands
[c] Department of Advanced Biomedical Sciences, School of Medicine and Surgery, University of Naples, Federico II, Via Pansini 5, 80131 Napoli (NA), Italy
[d] Department of Pharmacy, University of Naples Federico II, Via Domenico Montesano 49, 80131 Napoli (NA), Italy
[e] School of Pharmacy, Hygiene and Public Health Research Centre, University of Camerino, Via S. Agostino 1, 62032 Camerino (MC), Italy
[f] Department of Pharmacy, University of Chieti, Via dei Vestini 1, 66100 Chieti (CH), Italy
[g] Recusol Srl, Via del Bastione 16, 62032 Camerino (MC), Italy

ARTICLE INFO

Keywords:
Peptide stability
Polysaccharide hydrogels
Vancomycin
Daptomycin
Controlled release
Staphylococcal infections

ABSTRACT

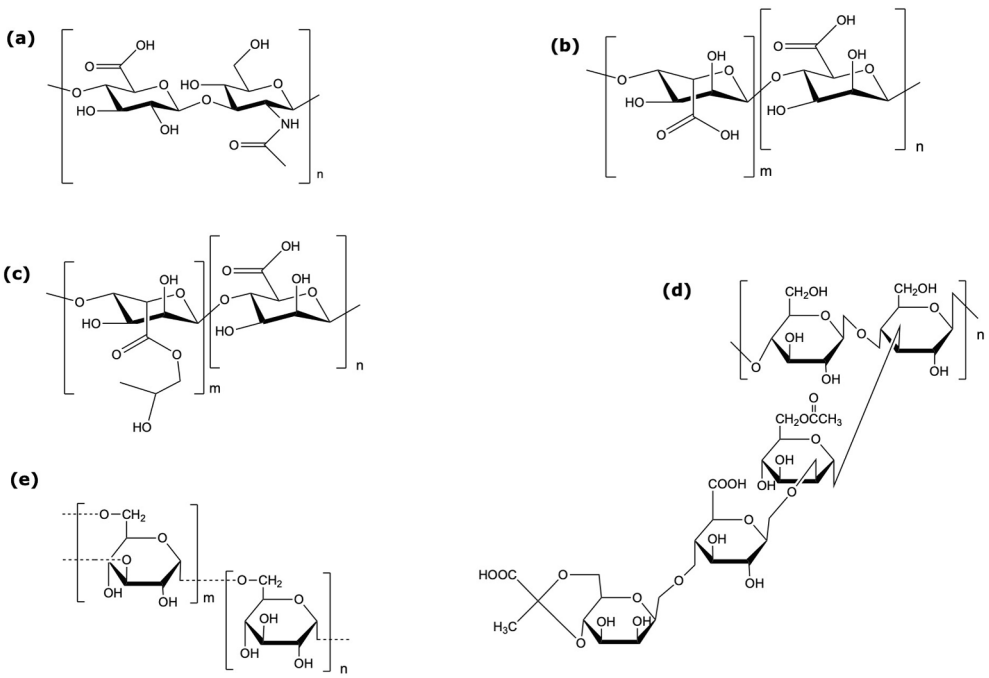
Chemical and enzymatic *in vivo* degradation of antimicrobial peptides represents a major challenge for their therapeutic use to treat bacterial infections. In this work, anionic polysaccharides were investigated for their ability to increase the chemical stability and achieve sustained release of such peptides. The investigated formulations comprised a combination of antimicrobial peptides (vancomycin (VAN) and daptomycin (DAP)) and anionic polysaccharides (xanthan gum (XA), hyaluronic acid (HA), propylene glycol alginate (PGA) and alginic acid (ALG)). VAN dissolved in buffer of pH 7.4 and incubated at 37 °C showed first order degradation kinetics with a reaction rate constant k_{obs} of 5.5×10^{-2} day^{-1} corresponding with a half-life of 13.9 days. However, once VAN was present in a XA, HA or PGA-based hydrogel, k_{obs} decreased to $(2.1–2.3) \times 10^{-2}$ day^{-1} while k_{obs} was not affected in an alginate hydrogel and a dextran solution (5.4×10^{-2} and 4.4×10^{-2} day^{-1}). Under the same conditions, XA and PGA also effectively decreased k_{obs} for DAP (5.6×10^{-2} day^{-1}), whereas ALG had no effect and HA even increased the degradation rate. These results demonstrate that the investigated polysaccharides (except ALG for both peptides and HA for DAP) slowed down the degradation of VAN and DAP. DSC analysis was used to investigate on polysaccharide ability to bind water molecules. Rheological analysis highlighted that the polysaccharides containing VAN displayed an increase in G' of their formulations, pointing that the peptides interaction act as crosslinker of the polymer chains. The obtained results suggest that the mechanism of stabilization of VAN and DAP against hydrolytic degradation is conferred by electrostatic interactions between the ionizable amine groups of the drugs and the anionic carboxylate groups of the polysaccharides. This, in turn, results in a close proximity of the drugs to the polysaccharide chain, where the water molecules have a lower mobility and, therefore, a lower thermodynamic activity.

1. Introduction

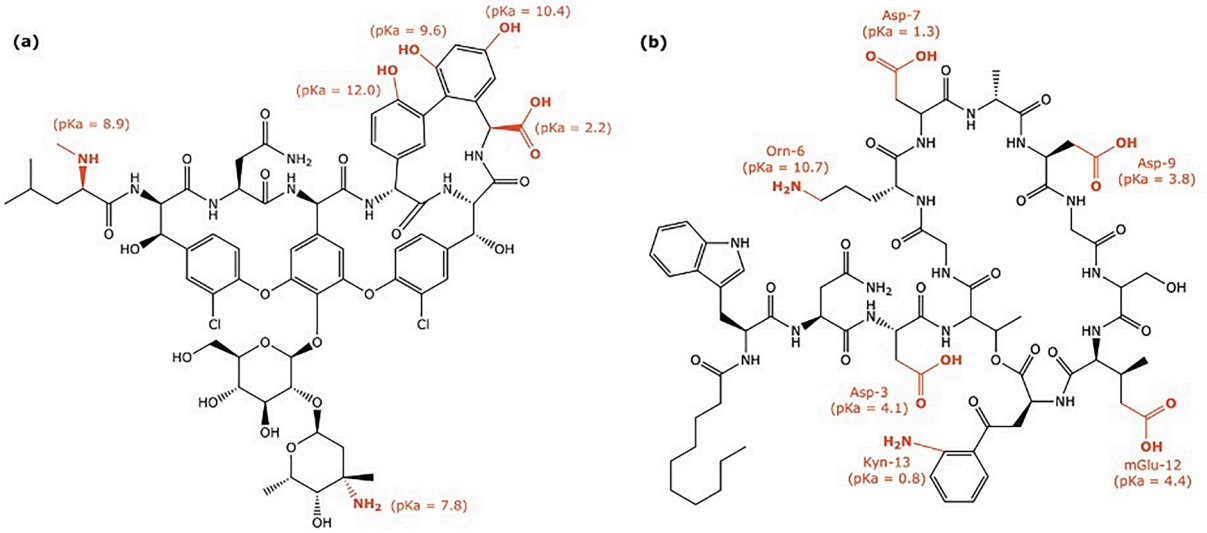
Over recent decades, biotherapeutic peptides have been thoroughly studied as a new class of drugs for the treatment of a variety of chronic and life-threatening diseases. Because of their short half-life, high doses have to be administered to reach therapeutic drug concentrations at the target site. These high doses, however, result in high initial blood concentrations which in turn are associated with side effects and systemic

* Corresponding authors at: Department of Pharmaceutical Sciences, Division of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, 99, 3508 TB Utrecht, The Netherlands (Cristina Casadidio). Department of Pharmacy, University of Chieti, Via dei Vestini 1, 66100 Chieti (CH), Italy (Piera Di Martino).
E-mail addresses: c.casadidio@uu.nl, piera.dimartino@unich.it (P. Di Martino).
[1] In Memoriam of our valued colleague and friend Stefania Scuri who contributed to this manuscript and tragically passed away.

https://doi.org/10.1016/j.ijpharm.2023.122798
Received 13 December 2022; Received in revised form 27 February 2023; Accepted 1 March 2023
Available online 7 March 2023
0378-5173/© 2023 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).



Scheme 1. Structures of the polysaccharides used in the present study: hyaluronic acid (a), alginic acid (b), propylene glycol alginic acid (c), xanthan gum (d) and dextran (e).



Scheme 2. Chemical structures of vancomycin (a) and daptomycin (b) with their respective pKa values. Vancomycin has 6 ionizable groups with different pKa values. At pH 7.4, three groups are not ionized, the amine (–NH_2) and methylamide (–$NHCH_3$) are positively charged whereas the –COOH group is negatively charged, resulting in a molecule with a net charge of + 1 at physiological pH (Takács-Novák et al., 1993). Daptomycin has two cationic, one belongs to the ornithine (pKa 10.7) and the other one to the kynurenine (pKa 0.8), and four anionic (three aspartic acids with pKa values of 1.3, 3.8 and 4.1 and one methylglutamic acid with pKa 4.4) groups. DAP has an isoelectric point of about 3.8 and a net charge of −3 at physiological pH. The single daptomycin positive charge at pH 7.4 is due to the primary aliphatic amine of the ornithine residue. The aromatic amine –NH_2 of the kynurenine residue is not protonated at physiological pH (Qiu et al., 2011).

toxicity (Koo and Seo, 2019; Fosgerau and Hoffmann, 2015; Lau and Dunn, 2018). Moreover, the physicochemical and biological characteristics of peptide therapeutics make them prone to rapid degradation upon administration due to both chemical and enzymatic action. For chemical degradation, the common pathways are deamidation, hydrolysis, isomerization, oxidation, racemization and/or elimination, leading to the formation of inactive products (Manning et al., 2010). To protect therapeutic peptides against degradation, their incorporation into polymeric delivery systems is undoubtedly an important and effective strategy (Agarwal and Rupenthal, 2013; Du and Stenzel, 2014; Yang et al., 2019; Cao et al., 2021). Particularly, hydrogels represent a class of polymeric matrices that enhance peptide stability by reducing the mobility of water molecules in the gels (Mikac et al., 2019; Yoshioka et al., 1995; Yoshioka et al., 1992; Sadhale and Shah, 1998). Water in hydrogels is present as free non-bound/freezing, strongly bound/non-freezing, and weekly bound/freezing water (V.M. Gun'ko, I.N. Savina, S.V. Mikhalovsky, Properties of water bound in hydrogels, Gels 3(4), 2017). The last two types of water molecules are involved in multiple interactions (hydrogen and ionic bonds) with the hydrogel network. This can result in a lower availability of free water molecules that

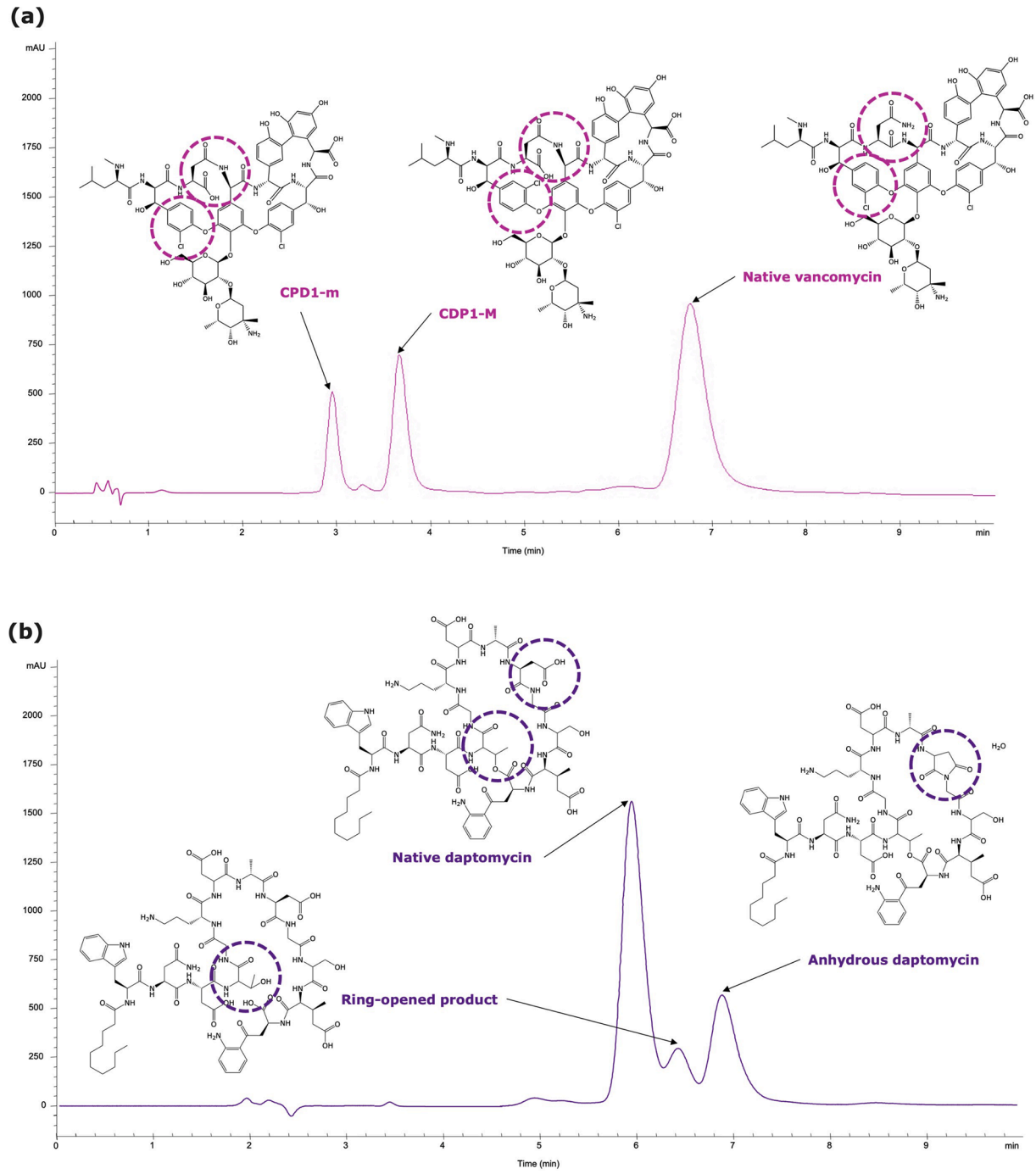


Fig. 1. Chromatograms, structures and chemical degradation products of VAN (a) DAP (b) formed in aqueous solution at pH 7.4 and 37 °C, after 5 days and 7 days of incubation respectively.

potentially can interact with the drug, favouring its slower degradation kinetics.

In the present work, four different anionic pH-responsive polysaccharides, namely xanthan gum, hyaluronic acid, propylene glycol alginate and alginic acid (structures shown in Scheme 1) were investigated for their capability to stabilize two antimicrobial peptides against hydrolytic degradation. These polysaccharides all contain carboxylic groups that are ionized to yield anionic polyelectrolytes when the pH is close to or above the pKa of these groups. The negative charges of these polysaccharides can be exploited to form ionic complexes with positive charges of antimicrobial peptides (Claudius and Neau, 1996; Dubashynskaya et al., 2021). Vancomycin and daptomycin (Scheme 2) are antibiotics active against Gram-positive bacteria and therefore used to treat a wide range of staphylococcal infections (Taylor and Palmer, 2016; Liu et al., 2011). Vancomycin is a glycopeptide antibiotic derived from Gram-positive bacteria like *Clostridium difficile* and *Staphylococcus aureus* and used to treat potentially lethal infections (Takács-Novák et al., 1993). On the other hand, daptomycin is an acidic lipopeptide active against *Staphylococcus aureus* Gram-positive bacteria (Zupančič et al., 2016; Qiu et al., 2011). The main limitation of these biotherapeutic drugs is their poor stability and both vancomycin and daptomycin possess amino acids that undergo chemical degradation at physiological conditions (Fig. 1) (Manning et al., 2010; Ghassempour and Aboul-Enein, 2008; Muangsiri and Kirsch, 2001). Moreover,

Table 1
Compositions of polysaccharide hydrogels and solutions. The concentration of VAN and DAP was 0.5 mg/ml for all the formulations. For the solutions, the PLS concentration used was 5 mg/mL. (Overlap concentrations present in the Supplementary Information, Table S1).

Polysaccharide (PLS)	Monomer unit (Da)	Acidic unit and pKa		PLS charge density (number of charges/kDa) at pH 4.5	7.4	9.0	Solutions: Molar ratio NH_2:COOH VAN:PLS at pH 7.4	Solutions: Molar ratio NH_2:COOH of DAP:PLS at pH 7.4	Hydrogels: PLS concentration (mg/mL)	Hydrogels: Molar ratio NH_2:COOH VAN:PLS at pH 7.4	Hydrogels: Molar ratio NH_2:COOH of DAP:PLS at pH 7.4
XA	889 Da	Glucuronic acid Pyruvic acid	3.1 2.9	−1.6	−1.7	−1.7	1: 8	1: 18	30	1: 50	1: 109
HA	378 Da	Glucuronic acid	3.0	−2.6	−2.6	−2.6	1: 20	1: 43	35	1: 138	1: 300
PGA	252 Da	Mannuronic acid	3.4	−1.6	−1.8	−1.8	1: 30	1: 64	50	1: 295	1: 643
ALG	192 Da	Guluronic acid	3.7	−4.7	−5.2	−5.2	1: 39	1: 85	100	1: 775	1: 1690

vancomycin and daptomycin have positively charged amine groups which potentially can electrostatically interact with the carboxylate groups of anionic polysaccharides (Costa et al., 2015; Sikwal et al., 2016). In our previous studies, chemically crosslinked hydrogels based on thermosensitive triblock copolymers of PEG-p(HPMAm-lac_{1-2}) and thiolated hyaluronic acid were used as matrices for the controlled release of vancomycin and daptomycin in the treatment of orthopaedic implant infections (Censi et al., 2019; Casadidio et al., 2018). These hydrogels, containing the negatively charged polysaccharide hyaluronic acid, improved the stability of the loaded antimicrobial matrix against hydrolytic degradation. In the present study, anionic hydrogels based on the above-mentioned polysaccharides were investigated as stabilizers. Differential scanning calorimetry and rheological studies were done to obtain mechanistic insight into the stabilization mechanism.

2. Materials and methods

2.1. Materials

Unless indicated otherwise, chemicals were purchased from Sigma-Aldrich (Stenheim, Germany) and used as received. Dextran from *Leuconostoc* spp., used as neutral polysaccharide, had a molecular weight (Mw) of 100–150 kDa, alginic acid sodium salt had a Mw of 120–190 kDa, hyaluronic acid sodium salt from *Streptococcus equi* had a Mw of 1500–1800 kDa. Xanthan gum (molecular weight specification not provided by the supplier) from *Xanthomonas campestris* had an acetylation degree of 100% and a pyruvation degree of 50%. Propylene glycol alginate with a degree of esterification 55% (Kelcoloid® K3B426, molecular weight not provided) was purchased from International Specialty Products (Köln, Germany). Vancomycin hydrochloride (MW = 1485.7 g/mol) and daptomycin (MW = 1619.7 g/mol), both in lyophilized form, were supplied by Hikma Farmacêutica (Terrugem, Portugal) and Novartis (Pharma Schweiz, Basel, Switzerland), respectively.

2.2. Composition of the buffers

Three buffers of different compositions were prepared, following the IUPAC standard guidelines (McNaught and Wilkinson, 1997). To prevent bacterial growth during the peptide stability studies, the buffers contained 0.02 wt% sodium azide. A monopotassium phosphate buffer (100 mM) was used as acidic buffer of pH 4.5. NaCl (136.9 mM), KH_2PO_4 (1.7 mM) and Na_2HPO_4 (13.4 mM) was used as pH 7.4 buffer. Borate buffer (101 mM) was used as basic buffer of pH 9.0. Ionic strengths were 100 mM for pH 4.5 buffer, 178.8 mM for pH 7.4 buffer and 101 mM for pH 9.0 buffer.

2.3. Preparation of the placebo and drug-loaded formulations

Intrinsic viscosity [η] and the overlap concentration (c^*) of the polysaccharides dissolved in water are reported in Table S1 (Supporting Information) and experimentally evaluated following the method described in the Supporting Information. The c^* was used as indicator to prove that the polysaccharide concentration used in the manuscript was above its c^*, therefore working in a semi-diluted system. Placebo polymer solutions were obtained via dissolution of the polysaccharides at a concentration of 5 mg/mL in the different buffer solutions (compositions in 2.2) at room temperature for 15 min under magnetic stirring. Polysaccharide placebo hydrogels were formed in the mentioned buffers at gel point concentrations (Table 1). The gelation point was assessed as the minimal polysaccharide concentration generating a no-flow system by the vial tilting method (Gil et al., 2017). Vancomycin and daptomycin-containing polysaccharide solutions and hydrogels were prepared by dissolving the different polysaccharides in solutions of VAN/DAP (0.5 mg/ml in the different buffers of pH 4.5, 7.4 and 9). The final concentrations of the different formulations are reported in Table 1. After preparation, the samples were incubated at 37 °C.

2.4. Chemical stability of vancomycin and daptomycin

The chemical stability of vancomycin and daptomycin dissolved in buffer, polysaccharidic solutions and hydrogels was evaluated during 22 days at 37 °C by sampling aliquots at regular time points. For VAN buffers of pH 7.4 and 9.0 and for DAP buffers of pH 4.5 and 7.4 were used. The different samples (100 μL for solutions and ~ 25 mg (accurately weighed) for hydrogels) were diluted with 300 μL PBS (pH 7.4) and then centrifuged at 12,000 rpm for 15 min at 4 °C (Scilogex D3024R, Rocky Hill, CT, USA). Supernatants were filtered through a 0.22 μm polytetrafluoroethylene filter (Agilent Captiva Econo Filter, Santa Clara, USA) and analyzed by HPLC-DAD (High Pressure Liquid Chromatography-Diode Array Detector, Agilent 1100 Series, Santa Clara, USA). Vancomycin and its degradation products were analyzed following the validated method described by Censi *et al.* (Censi et al., 2019), using a LiChrospher® RP 18 column (5 μm, 125 × 4.6 mm, Merck, Darmstadt, Germany) at 30 °C. Isocratic elution with eluent A (75%; water/ammonium formate 0.06 M, pH 7.7), eluent B (25%; methanol) at a flow rate of 0.8 mL/min was applied and 20.0 μL of the different samples was injected. Ultraviolet detection was performed at 230 nm with a retention time of 6.8 min for native VAN, while 2.9 and 3.6 min for its degradation products CPD1-m and CDP1-M (structures shown in Fig. 1), respectively. It is noted that these rotamers are separated because of their rigid structures. The chemical stability of daptomycin was investigated following the validated method developed by Casadidio *et al.* (Casadidio et al., 2018). Briefly, the concentration of native daptomycin in the different samples was determined using a

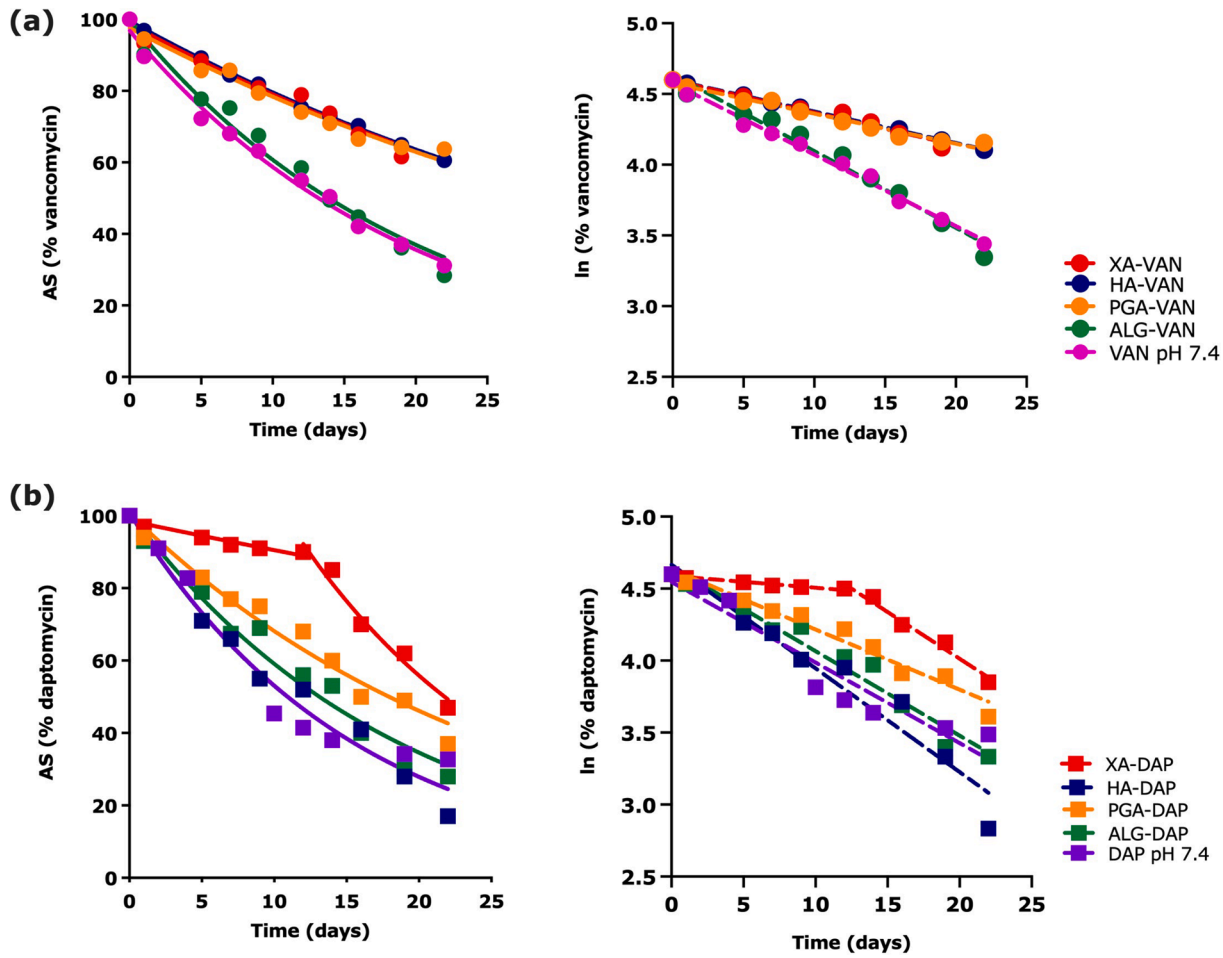


Fig. 2. Degradation of VAN and DAP under different conditions. Drug stability percentage (AS%) and ln concentration (ln %) of VAN (a) and DAP (b) in polysaccharide hydrogels formulation (XA, HA, PGA and ALG; concentrations reported in Table 1) and in buffer only, at pH 7.4 and 37 °C versus time. Fig. S1 (Supporting Information) shows the plots VAN and DAP in polysaccharide solutions.

Zorbax SB-AQ C18 column (5 μm, 150 × 4.6 mm column, Agilent Technologies Inc., Santa Clara, CA, USA) at 30 °C, using an isocratic elution of eluent A (52%; water/formic acid (15 mM)/triethylamine (pH 3.5)), eluent B (30%; acetonitrile) and eluent C (18%; methanol) at a flow rate of 1.0 mL/min. Samples of 5.0 μL were injected and detection was performed at the wavelengths of 223 and 245 nm with retention times of 6.0, 6.4 and 7.0 min for native daptomycin, ring-opened product and anhydrous product (structures shown in Fig. 1), respectively.

Antibiotic stability percentage (AS%) is defined as the percentage of residual concentration of native antimicrobial agent relative to its concentration at time 0, and calculated according to the following equation (1):

$$AS\% = \frac{A_t}{A_0} \text{ x } 100\% \quad (1)$$

where A_0 is the integral of the chromatographic peak of the native antibiotic and A_t is the integral of the chromatographic peak of the antibiotic at time *t* (Censi et al., 2019; Casadidio et al., 2018).

The observed first order reaction rate constants (k_{obs}) of VAN and DAP were calculated from the slope of ln (% of native drug) plotted against time (days).

2.5. Hydrogels thermal analyses

A differential scanning calorimeter (DSC Q20, TA Instruments, USA) equipped with a RCS90 refrigerated cooling system was used to investigate on interactions between water molecules, polysaccharides and drugs. Briefly, hydrogel samples (approximately 25 mg, accurately weighed) were loaded into aluminium pans which were subsequently hermetically sealed, cooled to −40 °C and after equilibration for 20 min heated to 40 °C at a heating rate of 2.5 °C/min, following the method reported by Mayol *et al.* (Mayol et al., 2014). The extrapolated onset melting temperature of frozen water (*T*m) was then determined (a representative thermogram shown in Fig. S3, Supporting Information). The enthalpy of the solid-to-liquid transition of water was obtained by integrating the endothermic melting peak and normalized with respect to the solid mass percentage of the gel (Biondi et al., 2012). The machine was calibrated with an indium standard, and an inert atmosphere was maintained by nitrogen purging at a 50 mL/min flow rate. To assess the validity of the method, different concentrations of polysaccharides were used to measure the resulting melting enthalpy (Fig. S4, Supporting Information) while the onset melting temperature of pure water obtained was 0.30 ± 0.06 °C, with a melting enthalpy (ΔH_m) equal to 334 ± 14 J/g, in agreement with literature data (Mayol et al., 2014; Mayol et al., 2014).

2.6. Rheological characterization of placebo and drug-loaded hydrogels

Rheological analyses of placebo and drug-loaded hydrogels were performed on a Kinexus rotational rheometer (Malvern, Malvern, United Kingdom). The viscoelastic properties of the gels in buffers of different pH's (compositions given in section 2.2) were studied by means of oscillatory shear experiments, at 37 °C, using a cone-plate geometry (CP

Table 2
VAN and DAP first order degradation rate constants in different formulations. The values present a R^2 value between 0.92 and 1.00.

Formulation	Vancomycin $k_{obs} \times 10^{-2}$ day^{-1}			Daptomycin $k_{obs} \times 10^{-2}$ day^{-1}		
	Hydrogel pH 7.4	Solution pH 7.4	Solution pH 9.0	Hydrogel pH 7.4	Solution pH 7.4	Solution pH 4.5
Soluble drug	n.a.	5.5	6.5	n.a.	5.6	6.9
XA	2.3	3.3	6.3	0.90*-5.6**	3.5	4.1
HA	2.2	2.3	7.7	7.2	8.7	4.9
PGA	2.1	2.7	3.7	4.2	2.0	4.2
ALG	5.4	4.7	7.4	5.9	5.8	3.7
DEX	n.a.	4.4	6.9	n.a.	6.1	5.5

*value taken for calculation until day 12, ** value taken for day 12–22; n.a. = not applicable.

40 mm), at 1% strain (at which linear viscoelasticity was attained) and 0.1–10 Hz oscillation frequency range. Thus, hydrogel mechanical properties were determined by measuring the shear storage (or elastic) modulus, G', and the shear loss (or viscous) modulus, G'', as a function of oscillation frequencies.

3. Results

3.1. Chemical (in)stability of vancomycin and daptomycin

The degradation pathway of vancomycin (VAN) in aqueous solution starts with deamidation of the asparagine residue followed by a rearrangement into two zwitterionic rotamers named CDP1-m and CDP1-M (Fig. 1a) (A.S. Antipas, D. Vander Velde, V.J. Stella, Factors affecting the deamidation of vancomycin in aqueous solutions, International journal of pharmaceutics 109(3), 1994). The degradation of daptomycin (DAP) in aqueous solution occurs via two pathways, namely ester hydrolysis, with the formation of ring-opened product, and aspartyl transpeptidation, via the reversible formation of anhydrous daptomycin and β-asp daptomycin (Fig. 1b) (Muangsiri and Kirsch, 2001).

Fig. 2 shows that VAN and DAP dissolved in phosphate buffer of pH 7.4 and incubated at 37 °C, degraded following first order kinetics, in agreement with previous studies (Muangsiri and Kirsch, 2001; A.S. Antipas, D. Vander Velde, V.J. Stella, Factors affecting the deamidation of vancomycin in aqueous solutions, International journal of pharmaceutics 109(3), 1994; Kirsch et al., 1989; El-Din et al., 2018), with k_{obs} values for VAN and DAP of 5.1 × 10^{-2} day^{-1} and 5.6 × 10^{-2} day^{-1} respectively. The k_{obs} value of VAN is in the ballpark of values found under similar conditions in the literature (Cao et al., 2018; Claudius and Neau, 1998). For the degradation of DAP at pH 7.4, no data are reported in the literature.

The capability of polysaccharidic xanthan gum (XA), hyaluronic acid (HA), propylene glycol alginate (PGA) and alginic acid (ALG) to stabilize the two peptide antibiotics against hydrolytic degradation was investigated. Based on previous publications (Censi et al., 2019; Casadidio et al., 2018), it is hypothesized that the hydrolytic degradation of VAN and DAP is slowed down by ionic interactions between the cationic site (s) of the drugs and the anionic ones of the polysaccharides (as indicated in Scheme 1 and 2). Therefore, their chemical stability was investigated in Newtonian viscous solutions (polysaccharide concentration of 5 mg/mL) at different pH values (9.0, 7.4 and 4.5). The studies were done in formulation with an excess of carboxylate anions of the polysaccharides over amine groups of VAN and DAP (ratios expressed in Table 1). As control, dextran was included as non-ionic and thus neutral polysaccharide.

Fig. 2a shows that VAN in the different polysaccharide hydrogels at pH 7.4 and at 37 °C degraded following a first order kinetics. Table 2 reports that at pH 7.4 the polysaccharides XA, PGA and HA both in solution (degradation kinetics plots shown in Fig. S1, Supporting Information) and in the form of hydrogels retarded the degradation of VAN with a decrease in k_{obs} of ~ 50% (from 5.5 × 10^{-2} to (2.3–3.3) × 10^{-2} day^{-1}). For VAN-ALG and VAN-DEX systems no effect on hydrolytic degradation was observed. Table 2 also shows that regardless the polysaccharide used, the k_{obs} values of native and loaded VAN at pH 9.0 are in the same range (6–7 × 10^{-2} day^{-1}, close to the one found by Claudius *et al.* of 5.0 × 10^{-2} day^{-1} (Claudius and Neau, 1998) except for PGA that slowed down its degradation (3.7 × 10^{-2} day^{-1}). The ln concentration versus times plots are shown in Fig. S2, Supporting Information.

Table 2 and Fig. 2b show that soluble PGA and XA slowed down the degradation of DAP (with k_{obs} of 2.0 × 10^{-2} and 3.5 × 10^{-2} day^{-1} respectively) whereas HA accelerated its degradation (k_{obs} = 8.7 × 10^{-2} day^{-1}). On the other hand, ALG and DEX had no effect on DAP stability. Moreover, it is observed that k_{obs} values for VAN in the presence of polysaccharides is the same below and above the gel point. Further, DAP in XAN hydrogel showed a biphasic degradation kinetics. However, no explanation is available for this observation.

Table 2 shows that at pH 4.5, DAP k_{obs} values are in the range between 3.7 and 5.5 × 10^{-2} day^{-1} (ln concentration versus time plots are shown in Fig. S2, Supporting Information), which are lower than the value of free DAP under the same conditions (k_{obs} = 6.9 × 10^{-2} day^{-1}).

Table 3
Water melting temperatures and melting enthalpy of placebo and drug-loaded polysaccharide hydrogels at pH 7.4 (n = 3, mean ± SD).

Polysaccharide	Formulation	*Tm* (°C)	ΔH_m* (J/g)
XA	Placebo	−1.93 ± 0.05	289 ± 10
(30 mg/mL)	VAN	−1.98 ± 0.26	285 ± 12
	DAP	−2.05 ± 0.17	279 ± 18
HA	Placebo	−2.62 ± 0.18	288 ± 5
(35 mg/mL)	VAN	−2.22 ± 0.17	285 ± 12
	DAP	−2.26 ± 0.07	289 ± 18
PGA	Placebo	−2.77 ± 0.02	284 ± 5
(50 mg/mL)	VAN	−2.54 ± 0.15	278 ± 6
	DAP	−2.54 ± 0.06	277 ± 8
ALG	Placebo	−2.73 ± 0.05	290 ± 10
(100 mg/mL)	VAN	−2.69 ± 0.19	282 ± 9
	DAP	−2.79 ± 0.25	278 ± 18
	Placebo	−3.21 ± 0.04	296 ± 3
DEX	VAN	−3.24 ± 0.01	292 ± 2
(100 mg/mL)	DAP	−3.17 ± 0.01	292 ± 2
Pure water		0.35 ± 0.10	334 ± 14
Water with PBS		−3.16 ± 0.16	299 ± 2

* values were normalized for solid mass fraction of the gels.

3.2. Thermal analysis of hydrogels

The DSC thermograms of the different polysaccharide hydrogels exhibited one endothermic peak (a typical example is shown in Fig. S3, Supporting Information). Table 3 summarizes the onset melting temperatures (*Tm*) and the melting enthalpies (ΔH) for frozen water in the different samples at pH 7.4 (placebo and drug loaded hydrogels, compositions given in Table 1). A very slight depression in the melting temperature of water ranging from −3.21 °C to −1.93 °C was observed for the hydrogels. Moreover, no contribution of DAP and VAN to the

Table 4
Storage and loss moduli of placebo, VAN- and DAP-loaded polysaccharide hydrogels at 0.1 Hz and 37 °C. The loading of DAP/VAN was 0.5 mg/mL ($n = 3$, mean ± SD).

Polysaccharide	Formulation	G' (Pa)	G'' (Pa)
XA	Placebo	217 ± 7	42 ± 7
	VAN	335 ± 23	48 ± 8
	DAP	327 ± 27	40 ± 7
HA	Placebo	184 ± 47	214 ± 44
	VAN	652 ± 10	402 ± 9
	DAP	168 ± 3	218 ± 2
PGA	Placebo	20 ± 4	10 ± 1
	VAN	129 ± 73	37 ± 19
	DAP	30 ± 3	13 ± 1
ALG	Placebo	17 ± 5	22 ± 2
	VAN	24 ± 5	27 ± 5
	DAP	28 ± 10	25 ± 2

melting temperature depressions was observed. Thermal analyses were conducted also at pH 9.0 and pH 4.5 (Table S2 and S3, Supporting Information).

3.3. Rheological characterization of placebo and drug-loaded hydrogels

Table 4 and Fig. 3 show the rheological properties of both placebo and drug-loaded hydrogels at 37 °C and pH 7.4. The rheological behaviour of the PGA placebo hydrogel is that typical of a weak gel, with G'>G'' over the whole frequency range analysed and G' < 100 Pa (Fig. 3a). The presence of VAN in the PGA gel resulted in an increment of both viscoelastic moduli and, particularly, at a frequency value of 0.1 Hz, the storage modulus increased from 20 ± 4 to 129 ± 73 Pa. In contrast, DAP did not significantly influence the rheological behaviour of the PGA hydrogel. HA formulations (Fig. 3b) showed a prevalently viscous character (G''> G') at low frequencies, but, with increasing frequency, the elastic modulus G' became larger than the viscous one G''. More in detail, at 0.2 Hz, the G' curve crossed the G'' one at the so-called cross-over frequency (ω_c), after which the elastic modulus prevailed on the viscous one and the system showed a predominant elastic character. This rheological behaviour is typical for viscoelastic material (Ambrosio et al., 1999). The presence of VAN in the HA solution caused a transition to a gel-like rheological behaviour with G' values ranging from588 to 652 Pa. Differently, in the presence of DAP in HA solution, only a shift of ω_c was observed from 0.2 Hz for HA to 0.13 Hz for DAP-HA. XA formulation mechanical properties showed a gel like rheological behaviour with G' always higher than G'', of about one order on magnitude, in all the frequency range examined (Fig. 3c). When loaded with VAN or DAP, an evident increase of elastic modulus of XA-based gels was recorded. In detail, at 0.1 Hz, an increase of the storage modulus from 217 ± 7 Pa for placebo XA to 335 ± 23 Pa for VAN-XA and to 327 ± 27 for DAP-XA was recorded. No significant differences between placebo and drug loaded ALG gels were observed, and all the formulations presented a rheological behaviour typical of a viscous solution, with G''>G' over the whole frequency range analysed (Fig. 3d).

Figure S5 (Supplementary Information) shows that the placebo hydrogels and VAN-loaded ones displayed the same rheological behaviour at pH 9.0. Fig. S6 (Supplementary Information) shows that DAP-loaded formulations displayed at pH 4.5 basically the same rheological properties compared to placebo gels. Only a slight increase of elastic modulus of DAP-XA gel and a decrease of both moduli of DAP-loaded alginate and hyaluronate gels were found.

4. Discussion

The aim of this study was to get insight into the mechanism(s) responsible foranionic polysaccharides stabilization of the antimicrobial peptides VAN and DAP against hydrolytic degradation. To this end, the degradation kinetics of these two peptides were investigated in buffer as well as in aqueous solutions and gels made up of anionic polysaccharides, such as XA, HA, PGA and ALG. Fig. 2 and Table 2 show that at pH 7.4 the degradation of VAN was substantially retarded once dissolved in solutions and gels of XA, HA, PGA, while only a slight decrease

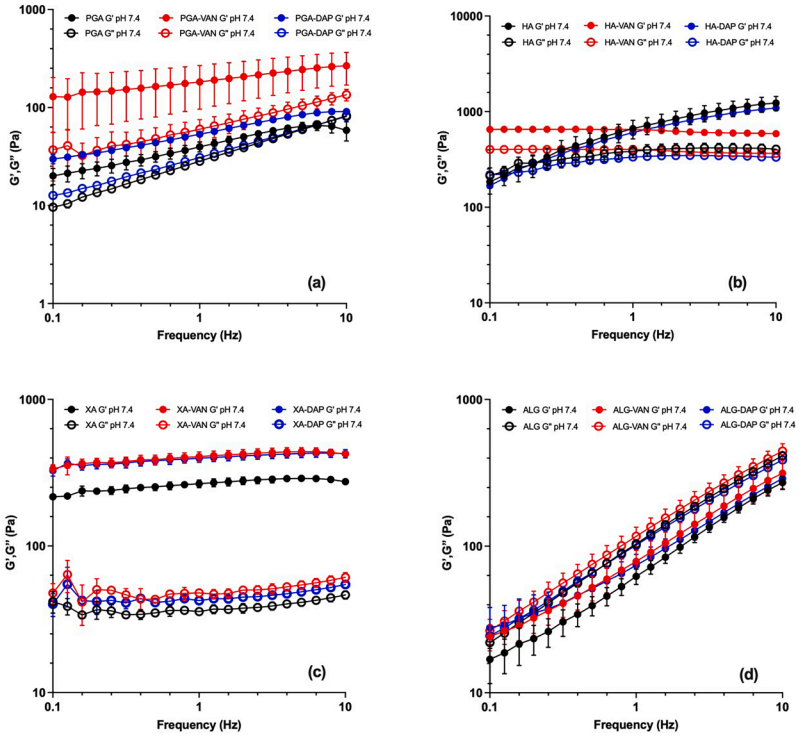


Fig. 3. Storage (G') and loss (G'') moduli at 37 °C as a function of frequency of placebo (black), VAN (red) and DAP (blue) polysaccharidic formulations at pH 7.4. (a) PGA, (b) HA, (c) XA and (d) ALG. Concentrations reported in Table 1 (n = 3, mean ± SD).

Table 5
Calculated number of weekly bound water molecules per polysaccharidic unit. The calculations were based following the DSC results presented in Table 3.

Polysaccharide (PLS)	PLS concentration (mg/mL) in H_2O with PBS (pH 7.4)	Molar ratio Monomer PLS unit: H_2O
XA	30	1: 55
HA	35	1: 22
PGA	50	1: 14
ALG	100	1: 3
DEX	100	1: 1

in VAN degradation kinetics at the same pH was observed when formulated in ALG and DEX. The observations point that stabilization of VAN by the polysaccharides is due to their electrostatic interactions with VAN. As shown in Fig. 5 and Table 4, the VAN containing hydrogels showed higher G' and G'' than the placebo gels. This can be ascribed to the fact that VAN possesses two positive charges at physiological pH (overall + 1 net charge) and can therefore act as crosslinker of the polysaccharide chains. Consequently, the ionic interactions between the -COO^- groups of the polysaccharides and the two amines of VAN brings this drug in close proximity of the polysaccharide chain.

As mentioned in the introduction, in aqueous solutions polysaccharides are surrounded by strongly and weekly bound water molecules (V.M. Gun'ko, I.N. Savina, S.V. Mikhalovsky, Properties of water bound in hydrogels, Gels 3(4), 2017). The thermograms represented in Fig. S3 (Supporting Information) show that the nature of the bound water in the polysaccharides studied can be classified as weekly bound. Using the DSC data, the number of weekly bound water molecules surrounding polysaccharidic units were calculated (Table 5). Rheological analysis corroborated the hypothesis that the amine groups of VAN can form salt bridges with the -COO^- groups of these polysaccharides. It is therefore concluded that the degradation of VAN is retarded because the labile groups are surrounded by water molecules with limited mobility and thus lower reactivity towards hydrolytic reaction, as schematically depicted in Fig. 4. Interestingly, for ALG, the low number of bound water molecules found in our experiments (Table 5) likely explains the observation that this polysaccharide did not protect VAN against degradation. In line herewith, although also dextran is capable to bound water molecules, it does not retard the degradation of VAN because this polysaccharide lacks anionic carboxylate groups.

To support the mechanism as schematically shown in Fig. 4, the stability of VAN in the different polysaccharide solutions was also investigated at pH 9.0. To explain, this pH is higher than the respective pKa's of its amine groups (Scheme 2) and the extent of protonation of this drug is consequently reduced as compared to pH 7.4, resulting in a decrease of its net charge (close to VAN isoelectric point of 8.3). The degradation data at pH 9.0 support the hypothesis that, when the interactions of VAN and the polysaccharides are minimized, the polysaccharides do not protect VAN against hydrolytic degradation (Fig. 2 and Table 2). Also rheological data proved that the interactions between polysaccharides and VAN are reduced at pH 9 (Fig. S6, Supporting Information).

Regarding DAP, at pH 7.4 the molecule is overall negatively charged (net charge −3) and indeed its presence in hydrogels of HA and ALG hardly affected their rheological characteristics (Fig. 3). In line herewith no retardation of DAP degradation in these gels was observed (Fig. 2 and Table 2). On the other hand, for XA and PGA gels loaded with DAP an increase in G' was observed compared to the placebo gels (Table 4). This might support the hypothesis that the proximity of the drug in the polysaccharide backbone enhances DAP stability in XAN and PGA due to the lower mobility and reactivity of the water molecules. To further elucidate the polysaccharide stabilization mechanism on DAP degradation, an acidic buffer of pH 4.5 was selected under conditions at which the electrostatic repulsions between the drug and polysaccharide are minimized. At this acidic pH, the overall charge of DAP is approximately −2, because ~ 80% of the -COOH groups of Asp-3, 7, 9 and mGlu-12 are negatively charged whereas the Orn-6 amine carries one positive charge. Table 2 shows that at pH 4.5, DAP is stabilized by all polysaccharides used. To be mentioned, no relation is observed between the charge density of the polysaccharides and their capability to stabilize DAP against hydrolytic degradation in acidic environment, as supported by the rheological measurements as well (Fig.S6, Supporting Information). Therefore, it is assumed that DAP stability in acidic conditions is due to the intrinsic properties of the drug, such as its hydrophobic nature and capability to self-assembles into nanostructures (Kirkham et al., 2016; Qiu and Kirsch, 2014).

The polysaccharide antibiotic formulations were also evaluated as *in situ*-forming drug delivery systems for the inhibition of *Staphylococcus*

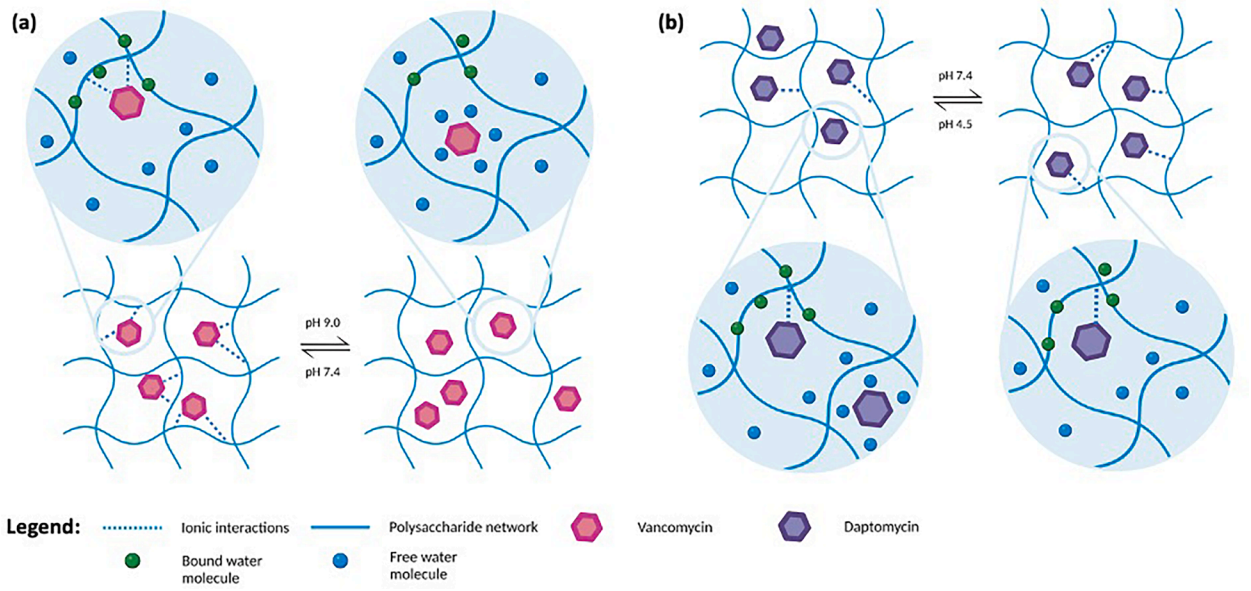


Fig. 4. Proposed mechanism of protection of VAN (a) and DAP (b) against hydrolytic degradation in the presence of anionic polysaccharides. Ionic interactions between positive charge(s) of the VAN/DAP and anionic polysaccharidic chains occur which, in turn, result in a lower mobility and thermodynamic activity of the water molecules in proximity of polysaccharide backbone surrounding the hydrolytically sensitive groups in VAN/DAP.

aureus growth (Fig. S7-8, Supporting Information). Release studies and antimicrobial tests demonstrated that these polysaccharide-drug formulations proved to be attractive materials for the release of pharmaceutical peptides and as potential substrates in the prevention and treatment of topical staphylococcal infections. From a clinical point of view, freeze-dried powders of polysaccharide-VAN/DAP formulations can be administered as coating onto an implantable device (i.e. urinary catheters) to prevent staphylococcal infections and prevent oncoming biofilm formation or, as treatment for burns and pressure ulcers.

5. Conclusion

In this study, we suggested that the mechanism explaining the enhanced stability of the peptides in hydrogel matrices, as well as in solutions of an anionic polysaccharides, relies on electrostatic interactions between antibiotic and polysaccharide. This results in a close proximity of the drugs to the polysaccharide chain, where the water molecules have a lower mobility and, therefore, a lower activity to hydrolytically degrade the antibiotics. According to the obtained findings, XA and PGA are the polysaccharides that can stabilize the cargo better than HA and ALG, regardless of the nature of the antimicrobial peptide used. In conclusion, these designed formulations have proven to be promising candidates for stabilization and controlled release of antimicrobial peptides intended for functional coating in the treatment and prevention of wounds and implant-associated infections.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

The authors gratefully acknowledge Francesco Palmieri, Bas G. P. van Ravensteijn, Carl C. L. Schuurmans (Utrecht University), Roberta Morabito (University of Naples Federico II), Maria Cristina, Dolores Vargas Peregrina and Lucrezia Di Nicolantonio (RECUSOL S.R.L.) for their precious contribution to this project. Authors acknowledge receipt of funding from Regione Marche (Italy) POR MARCHE FESR 2014–2020 - Asse 1 – OS 2 – Azione 2.1. (https://www.marche-biobank.it)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2023.122798.

References

A.S. Antipas, D. Vander Velde, V.J. Stella, Factors affecting the deamidation of vancomycin in aqueous solutions, International journal of pharmaceutics 109(3) (1994) 261-269.

Agarwal, P., Rupenthal, I.D., 2013. Injectable implants for the sustained release of protein and peptide drugs. Drug discovery today 18 (7–8), 337–349.

L. Ambrosio, A. Borzacchiello, P. Netti, L. Nicolais, Rheological study on hyaluronic acid and its derivative solutions, Journal of Macromolecular Science—Pure and Applied Chemistry 36(7-8) (1999) 991-1000.

Biondi, M., Fusco, S., Lewis, A.L., Netti, P.A., 2012. New insights into the mechanisms of the interactions between doxorubicin and the ion-exchange hydrogel DC Bead™ for use in transarterial chemoembolization (TACE). Journal of Biomaterials Science, Polymer Edition 23 (1–4), 333–354.

Cao, M., Feng, Y., Zhang, Y., Kang, W., Lian, K., Ai, L., 2018. Studies on the metabolism and degradation of vancomycin in simulated in vitro and aquatic environment by UHPLC-Triple-TOF-MS/MS. Scientific Reports 8 (1), 1–13.

Cao, S., Lv, Z., Guo, S., Jiang, G., Liu, H., 2021. An Update-Prolonging the action of protein and peptide drugs. Journal of Drug Delivery Science and Technology 61, 102124.

Casadidio, C., Butini, M.E., Trampuz, A., Di Luca, M., Censi, R., Di Martino, P., 2018. Daptomycin-loaded biodegradable thermosensitive hydrogels enhance drug stability and foster bactericidal activity against Staphylococcus aureus. European Journal of Pharmaceutics and Biopharmaceutics 130, 260–271.

Censi, R., Casadidio, C., Dubbini, A., Cortese, M., Scuri, S., Grappasonni, I., Golob, S., Vojnovic, D., Sabbieti, M.G., Agas, D., 2019. Thermosensitive hybrid hydrogels for the controlled release of bioactive vancomycin in the treatment of orthopaedic implant infections. European Journal of Pharmaceutics and Biopharmaceutics 142, 322–333.

Claudius, J.S., Neau, S.H., 1996. Kinetic and equilibrium characterization of interactions between glycopeptide antibiotics and sodium carboxymethyl starch. International journal of pharmaceutics 144 (1), 71–79.

Claudius, J.S., Neau, S.H., 1998. The solution stability of vancomycin in the presence and absence of sodium carboxymethyl starch. International journal of pharmaceutics 168 (1), 41–48.

Costa, J., Silva, N., Sarmento, B., Pintado, M., 2015. Potential chitosan-coated alginate nanoparticles for ocular delivery of daptomycin. European Journal of Clinical Microbiology & Infectious Diseases 34 (6), 1255–1262.

Du, A.W., Stenzel, M.H., 2014. Drug carriers for the delivery of therapeutic peptides. Biomacromolecules 15 (4), 1097–1114.

Dubashynskaya, N.V., Raik, S.V., Dubrovskii, Y.A., Shcherbakova, E.S., Demyanova, E.V., Shasherina, A.Y., Anufrikov, Y.A., Poshina, D.N., Dobrodumov, A.V., Skorik, Y.A., 2021. Hyaluronan/colistin polyelectrolyte complexes: Promising antiinfective drug delivery systems. International Journal of Biological Macromolecules 187, 157–165.

M.K.S. El-Din, F. Ibrahim, A.K. El-Deen, K. Shimizu, Stability-indicating spectrofluorimetric method with enhanced sensitivity for determination of vancomycin hydrochloride in pharmaceuticals and spiked human plasma: Application to degradation kinetics, journal of food and drug analysis 26(2) (2018) 834-841.

Fosgerau, K., Hoffmann, T., 2015. Peptide therapeutics: current status and future directions. Drug discovery today 20 (1), 122–128.

Ghassempour, A., Aboul-Enein, H.Y., 2008. Vancomycin degradation products as potential chiral selectors in enantiomeric separation of racemic compounds. Journal of Chromatography A 1191 (1–2), 182–187.

Gil, M.S., Cho, J., Thambi, T., Phan, V.G., Kwon, I., Lee, D.S., 2017. Bioengineered robust hybrid hydrogels enrich the stability and efficacy of biological drugs. Journal of Controlled Release 267, 119–132.

Kirkham, S., Castelletto, V., Hamley, I.W., Inoue, K., Rambo, R., Reza, M., Ruokolainen, J., 2016. Self-assembly of the cyclic lipopeptide daptomycin: spherical micelle formation does not depend on the presence of calcium chloride. ChemPhysChem 17 (14), 2118–2122.

Kirsch, L.E., Molloy, R.M., Debono, M., Baker, P., Farid, K.Z., 1989. Kinetics of the aspartyl transpeptidation of daptomycin, a novel lipopeptide antibiotic. Pharmaceutical research 6 (5), 387–393.

Koo, H.B., Seo, J., 2019. Antimicrobial peptides under clinical investigation. Peptide Science 111 (5), e24122.

Lau, J.L., Dunn, M.K., 2018. Therapeutic peptides: Historical perspectives, current development trends, and future directions. Bioorganic & medicinal chemistry 26 (10), 2700–2707.

Liu, C., Bayer, A., Cosgrove, S.E., Daum, R.S., Fridkin, S.K., Gorwitz, R.J., Kaplan, S.L., Karchmer, A.W., Levine, D.P., Murray, B.E., 2011. Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant Staphylococcus aureus infections in adults and children. Clinical infectious diseases 52 (3), e18–e55.

Manning, M.C., Chou, D.K., Murphy, B.M., Payne, R.W., Katayama, D.S., 2010. Stability of protein pharmaceuticals: an update. Pharmaceutical research 27 (4), 544–575.

Mayol, L., Biondi, M., Russo, L., Malle, B.M., Schwach-Abdellaoui, K., Borzacchiello, A., 2014. Amphiphilic hyaluronic acid derivatives toward the design of micelles for the sustained delivery of hydrophobic drugs. Carbohydrate polymers 102, 110–116.

Mayol, L., De Stefano, D., Campani, V., De Falco, F., Ferrari, E., Cencetti, C., Matricardi, P., Maiuri, L., Carnuccio, R., Gallo, A., 2014. Design and characterization of a chitosan physical gel promoting wound healing in mice. Journal of Materials Science: Materials in Medicine 25 (6), 1483–1493.

A.D. McNaught, A. Wilkinson, Compendium of chemical terminology, Blackwell Science Oxford (1997) 1669.

Mikac, U., Sepe, A., Gradišek, A., Kristl, J., Apih, T., 2019. Dynamics of water and xanthan chains in hydrogels studied by NMR relaxometry and their influence on drug release. International journal of pharmaceutics 563, 373–383.

Muangsiri, W., Kirsch, L.E., 2001. The kinetics of the alkaline degradation of daptomycin. Journal of pharmaceutical sciences 90 (8), 1066–1075.

Qiu, J., Kirsch, L.E., 2014. Evaluation of lipopeptide (daptomycin) aggregation using fluorescence, light scattering, and nuclear magnetic resonance spectroscopy. Journal of pharmaceutical sciences 103 (3), 853–861.

Qiu, J., Yu, L., Kirsch, L.E., 2011. Estimated pKa values for specific amino acid residues in daptomycin. Journal of pharmaceutical sciences 100 (10), 4225–4233.

Sadhale, Y., Shah, J.C., 1998. Glyceryl monooleate cubic phase gel as chemical stability enhancer of cefazolin and cefuroxime. Pharmaceutical development and technology 3 (4), 549–556.

Sikwal, D.R., Kalhapure, R.S., Rambharose, S., Vepuri, S., Soliman, M., Mocktar, C., Govender, T., 2016. Polyelectrolyte complex of vancomycin as a nanoantibiotic:

preparation, in vitro and in silico studies. Materials Science and Engineering: C 63, 489–498.
Takács-Novák, K., Noszál, B., Tókés-Kövesdi, M., Szász, G., 1993. Acid-base properties and proton-speciation of vancomycin. International journal of pharmaceutics 89 (3), 261–263.
Taylor, S.D., Palmer, M., 2016. The action mechanism of daptomycin. Bioorganic & medicinal chemistry 24 (24), 6253–6268.
V.M. Gun'ko, I.N. Savina, S.V. Mikhalovsky, Properties of water bound in hydrogels, Gels 3(4) (2017) 37.
Yang, Y., Chen, Q., Lin, J., Cai, Z., Liao, G., Wang, K., Bai, L., Zhao, P., Yu, Z., 2019. Recent advance in polymer based microspheric systems for controlled protein and peptide delivery. Current medicinal chemistry 26 (13), 2285–2296.
Yoshioka, S., Aso, Y., Terao, T., 1992. Effect of water mobility on drug hydrolysis rates in gelatin gels. Pharmaceutical research 9 (5), 607–612.
Yoshioka, S., Aso, Y., Otsuka, T., Kojima, S., 1995. Water Mobility in Poly (ethy1ene glycol)–, Poly (vinylpyrro1idone)–, and Gelatin-Water Systems, as Indicated by Dielectric Relaxation Time. Spin-Latice Relaxation Time, and Water Activity, Journal of pharmaceutical sciences 84 (9), 1072–1077.
Zupančič, O., Partenhauser, A., Lam, H.T., Rohrer, J., Bernkop-Schnürch, A., 2016. Development and in vitro characterisation of an oral self-emulsifying delivery system for daptomycin. European Journal of Pharmaceutical Sciences 81, 129–136.