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Polyvinyl Alcohol–Pectin Cryogel Films for Controlled Release of Enrofloxacin

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Abstract The release of enrofloxacin entrapped in polyvinyl alcohol (PVA) cryogel at pH 5.5 showed a first-order kinetic, releasing 69.7% of the antibiotic after 4.5 h at 37 °C. In order to slow down the fluoroquinolone release rate, high-methoxylated pectin was added into the cryogel (PVA–P). A film containing 1.0% (*w*/*v*) HM pectin and 5.0 μg/ml enrofloxacin released only 3.7% of the antibiotic after 4.5 h. Since the FTIR spectrum showed that most of the interactions between PVA–P matrix and enrofloxacin were due to polar groups (carboxylate and amine), a two-layer film system was designed to modulate the releasing rate of the drug. The top film equilibrated with 0.75 or 1.5 M NaCl release up to 41.9% and 89.0% of the enrofloxacin in 4 h, respectively. The release rate of enrofloxacin was found dependent on NaCl concentration in the upper gel layer. The two-layer cryogel system showed attractive features for transcutaneous antibiotic delivery.

Keywords Transcutaneous delivery · Enrofloxacin · PVA · Pectin · Films

Introduction

Enrofloxacin is a synthetic fluoroquinolone antibiotic with activity against a broad spectrum of Gram-negative and Gram-positive bacteria widely used in veterinary medicine. Enrofloxacin is frequently used in poultry production to treat respiratory and enteric bacterial infections. However, because of its high toxicity, enrofloxacin is banned for humans. Fluoroquinolone is characterized by low water solubility and low bioavailability and

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biodisponibility [1, 2]. Enrofloxacin is generally administered at high concentrations which can cause molecular stacking and crystal formation affecting many organs with undesirable side consequences [3]. Films constitute interesting drug delivery systems to achieve a systemic effect throughout the transdermic path by its application onto skin or bounded areas in the case of antimicrobial compounds. For this reason, the combination of enrofloxacin controlled release and transdermal drug delivery could be a useful alternative to explore because of the following advantages: reducing antibiotic peaks in plasma, keeping the antibiotic at a constant level inside the therapeutic window for an established period of time and decreasing the antibiotic administration frequencies, which will allow to diminish the adverse side effects in the body.

Polyvinyl alcohols (PVAs) are hydrophilic polymers able to gel per se forming films under prolonged shelf storage by the synthesis of a non-covalent spatial network. Currently, PVA is being used in many fields such as paper, textile, cosmetics, and others. PVA films have been found to be non-immunogenic and non-carcinogenic in humans with many biomedical potential applications [4]. PVA cryogels performed by cycles of freezing and thawing have the advantage of no additional chemical crosslinker agents (e.g., glutaraldehyde, EDC, etc.), and consequently, no further purification steps are required which is essential for biomedical or pharmacological applications [5]. When a PVA solution is subjected to cycles of freezing–thawing (F/T), water initially crystallizes while the solute stays in the liquid part of the material. Such state leads to stronger polymer–polymer chain interactions leading to a stable gel structure even at room temperature and beyond [6].

Pectins are structural polysaccharides present in plant cell walls composed by blocks of homogalacturonans and rhamnoglacturonans bearing neutral sugar side chains. Pectins are regularly used as thickeners, stabilizers, and gelling and emulsifying agents in the food industry. According to the degree of methyl esters present in the pectin molecule, pectins can be classified into low-, medium-, and high-methoxylated pectins (LM, MM, and HM, respectively). All pectins are able to form gels by ionotropic gelation: LM and MM pectins can be cross-linked by multivalent cations, but HM pectins under acid conditions in the presence of solutes [7].

Interpenetrating polymer networks (IPNs) are materials where at least one component is polymerized and/or cross-linked in the presence of the other [8]. Blending different polymers and yet conserving their individual properties in the final mixture is an extremely attractive, inexpensive, and advantageous way for obtaining new structural devices [9]. The advantages of polymer blends to generate new applications may include easy fabrication and modification of the film properties like hydration, mechanical strength, binding capacity, and so on. Therefore, the performed material will influence the delivery rate of the entrapped drugs. In previous works, the addition of alginate into PVA solution before the cycles of freeze–thawing rendered a cryogel that slows down drug diffusion. Besides, by increasing alginate concentration, high mechanical stability in PVA gels was found [10]. Additionally, films synthesized from polymer blends made by different ratios of PVA–pectin were previously reported. The works found that pectin and PVA polymers can be mixed in all proportions, and increasing the PVA content in the blends reduces the glass transition temperature and increases the toughness of the films [11, 12]. Recently, an interesting photostable pectin film containing PVA for storage and outdoor applications was reported [13].

The aim of the present work is to develop a controlled release gel matrix for enrofloxacin based on a two-layer cryogel of polyvinyl alcohol/pectin (PVA–P) with different ionic strength. Gel morphologies by scanning electron microscopy and the interaction between enrofloxacin and PVA–P by FTIR are also reported.

Materials and Methods

Preparation of Aqueous Solution Component

PVA with an average molecular weight of 13–23 kDa (98–99% hydrolyzed) was supplied by Sigma-Aldrich (Buenos Aires, Argentina). Solutions of 15% or 30% (*w*/*v*) PVA were prepared in distilled water. Dissolution was achieved by heating the mixture at 80 °C for 90 min, while slowly stirring. Pectin with different ranges of methoxylation degree (35.5%, LM; 62.5%, MM; and 71.1%, HM) was kindly supplied by CPKelco. Solutions of 1.0–2.0% (*w*/*v*) pectin were prepared in distilled water with slow stirring. Enrofloxacin was supplied by Sigma-Aldrich (Buenos Aires, Argentina), and a stock solution of 500 μg/ml was prepared on 1.0 M HCl or citrate buffer 50 mM (pH=3.5).

Screening of Pectin–Enrofloxacin Interaction

Enrofloxacin 10 μg/ml solution was incubated with 1.0% (*w*/*v*) pectin solution with different degrees of methoxylation (35.5%, 62.5%, and 71.7%) during 30 to 90 min. Then, cold ethanol 96.0% (*v*/*v*) was added and then centrifuged at 10,000×*g* for 10 min (Eppendorf). The supernatant was collected, and the absorbance was measured at 279 nm.

Preparation of Film Component

Equals amounts of 30.0% (*w*/*v*) PVA and 0.2% or 2.0% (*w*/*v*) pectin solutions (previously incubated with 10.0 to 70.0 μg/ml enrofloxacin) were mixed to produce a film of 15.0% (*w*/*v*) PVA and 0.1% or 1.0% (*w*/*v*) pectin containing 5.0 to 35.0 μg/ml enrofloxacin. The solution was cast into Petri plates frozen at −18°C for 20 h and then thawed to room temperature (25°C) for 8 h. These cycles of freezing/thawing (F/T) were repeated three to five times. The films were dried at 25°C in a fume hood and then stored in 96.0% (*v*/*v*) ethanol.

Scanning Electron Microscopy

The morphology of the cryogel blends was analyzed by a SEM Jeol JSM-840A with cryo-attachment. Cryogels (PVA and PVA–pectin) were incubated 1 h in a solution of 1.0% (*v*/*v*) glutaraldehyde in 100 mM HCl. Fixed cryogels were washed with deionized water and incubated for 1 h with 1.0% (*w*/*v*) OsO_4 solution. The OsO_4 solution was discarded, and the cryogels were dehydrated with increasing concentrations of ethanol (25–100%, *v*/*v*) during 20 min in a rocking shaker.

FTIR Analysis

FTIR spectra were analyzed by ATR-FTIR at the SMIS beamline (Soleil French National Synchrotron Facility, France) and also in Jasco FTIR 4200 spectrometer. Samples were lyophilized and mixed with KBr.

Release of Enrofloxacin from PVA–P Cryogels

Release experiments were done by placing the cryogels in 1.5 ml of acetate buffer at pH=5.5 and 37°C. An aliquot was taken from the solution at different times and replenished with the

same volume of fresh buffer. The released antibiotic was measured by fluorescence in a PerkinElmer LS 50B spectrofluorimeter with an λ_{ex} 275 nm and λ_{em} 437 nm.

Two-Layer PVA–P Cryogel

The release of enrofloxacin contained in the two-layer film was tested using a diffusion cell [14, 15] holding 1-cm^2 (total volume: 0.78 cm^3) films onto a cellophane dialysis membrane (cutoff, 5 kDa) previously soaked for 24 h in 50 mM sodium citrate buffer (pH 5.5) (Fig. 1). The effect of a second layer in the system was tested adding a top gel of PVA–P previously equilibrated with different concentrations of NaCl at 37.0±0.5°C for 12 h. Aliquots of 300-μl samples were withdrawn from the receptor medium at different time intervals and replaced with equal volumes of fresh buffer (50 mM sodium citrate, pH 5.5). Enrofloxacin was determined spectrophotometrically using a UV–vis microtiter plate reader (EPOCH-BIOTEK) at 275 nm.

Results and Discussion

Enrofloxacin release was evaluated in a matrix of PVA cryogels containing 5.0 μg/ml of the antibiotic at pH 5.5. The antibiotic release was following a first-order kinetic with 70% release in 4.5 h (Fig. 2). The highly hydrophilic matrix and the aromatic character of the bioactive molecule would be a possible explanation for the free diffusion of enrofloxacin from the gel matrix to the medium. It has been reported that engineering the cryogel by adding co-polymer additives leads to IPNs with differential properties [10–13].

Interactions of fluoroquinoles with several biopolymers were tested in our laboratory, and pectins were selected because of 70% antibiotic binding (data not shown). Furthermore, pectins with different methoxylation degrees from 35.5% (LM), 62.5% (MM) to 71.1% (HM) were screened with enrofloxacin. HM pectin was selected because it binds 20% more of the fluoroquinolone compared to the other pectins (Fig. 3). Interestingly, considering the different p*K*a values of pectin (p*K*a=3.5) and enrofloxacin (p*K*a=7.7) under slightly acidic dispersion similar to the skin (pH 5.5), most of the unesterified carboxyl groups of pectin would be partially ionized, and consequently, the electrostatic interaction with the polymer would be favored.

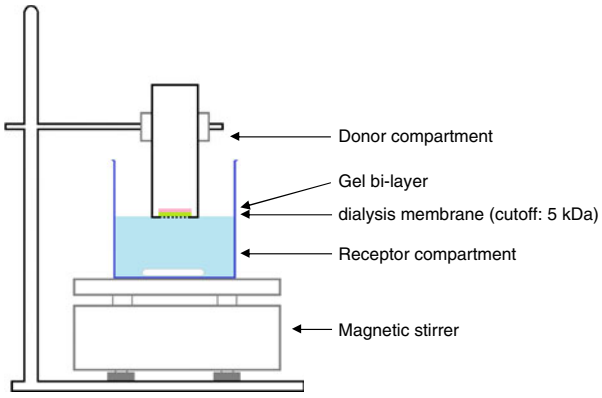

Fig. 1 Diffusion cell holding a two-layer 1-cm^2 PVA–P cryogel containing sodium chloride in the upper gel and enrofloxacin in the bottom gel. The cryogels are settled on a dialysis membrane. The receptor compartment containing 1.65 ml of 50 mM sodium citrate buffer pH 5.5 was stirred at 100 rpm

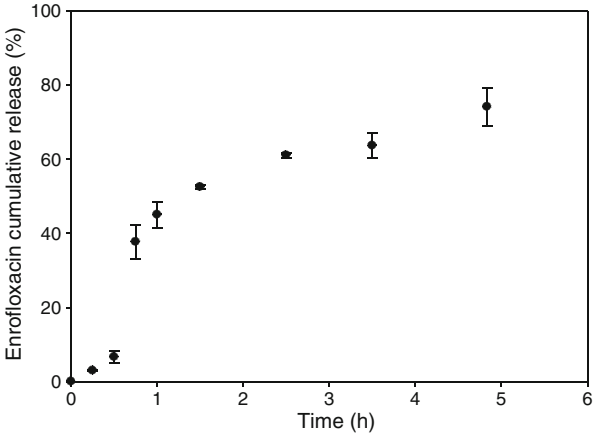

Fig. 2 Release of enrofloxacin from PVA cryogel

Cryogel patches containing 0.1% and 1.0% (*w*/*v*) HM pectin with enrofloxacin were developed for the study of antibiotic release. The antibiotic (5.0 μg/ml) was incorporated into the PVA–P cryogel just before the cycles of F/T. PVA–P films containing 0.1% HM pectin released up to 70% of antibiotic in 12 h. Besides, when the enrofloxacin concentration was double (10.0 μg/ml) in the film, the release of fluoroquinolone under the same experimental conditions was only 7.5%. The two-time increase in enrofloxacin concentration in the film and the unexpected drastic antibiotic binding to the PVA–P film could be attributed to the molecular stacking of the antibiotic. Molecular stacking is one of the main problems in fluoroquinolone dosages and should be avoided in order to reduce severe toxic side effects [3]. However, PVA films containing 1.0% (*w*/*v*) pectin and 5.0 μg/ml enrofloxacin released only 3.7% of the antibiotic after 4.5 h of incubation at 37°C. It is remarkable that PVA–P films containing 1.0% HM pectin release 14 times less antibiotic to the medium compared with 0.1% HM pectin PVA films. Figure 4 is showing the 5.0 μg/ml enrofloxacin cumulative release kinetic from PVA and PVA–pectin 0.1% and 1.0% matrix. These results are indicating that the interaction between the matrix containing pectin and the antibiotic is stronger due to more HM pectin molecules available in the gel. The presence of HM pectin in the matrix is crucial to develop enrofloxacin controlled release in the PVA system. Also, the hydrophobic character of enrofloxacin and the increase of HM pectin concentration on

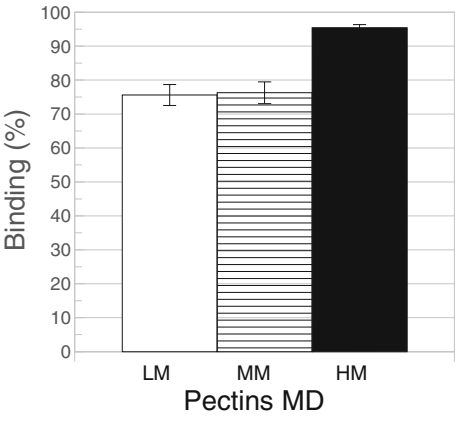

Fig. 3 Effect of methoxylation degree (MD) of pectins on the binding capacity of enrofloxacin. Pectin MD: 35.5% (*white bar*), 62.5% (*striped bar*), and 71.7% (*black bar*)

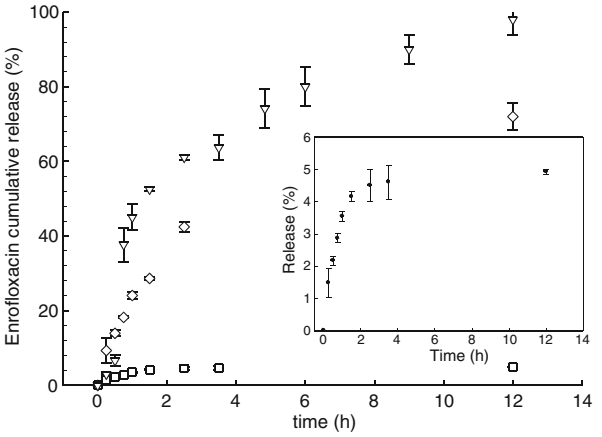

Fig. 4 Enrofloxacin cumulative release was studied in PVA (*inverted triangles*), PVA–pectin 01% (*diamonds*), and PVA–pectin 1.0% (*squares*) matrixes

the film did not affect the mechanical properties of the PVA–P gel as it was similarly described for PVA cryogels containing atenolol [16]. Additionally, the interaction among PVA and the free carboxylic groups of pectin resulted in a stable and strengthened three-dimensional cryogel network probably attributed to extensive hydrogen bonding as previously reported [12, 13].

Another parameter to be taken into account is the number of F/T cycles; the cross-linking of PVA in the gel matrix could be affected and, consequently, the controlled release of enrofloxacin. Three and five F/T cycles were analyzed for the release of enrofloxacin in all tested systems. No significant differences in the release antibiotic profiles ($p>0.0.5$) were found for PVA and both PVA–pectin films (data not shown). SEM images obtained for the PVA cryogel are in agreement with a previous description where the increment in the number of cycles of F/T leads with the higher surface of the gel and the stiffness of the gel network (Figs. 5a and b). Herein, the addition of pectin showed a highly porous structure that was even higher with five cycles of F/T (Figs. 5c and d).

In order to determine the interaction type of enrofloxacin and the matrix components, FTIR analysis of the films were performed. The FTIR spectrum of free and entrapped enrofloxacin on the PVA–pectin gel matrix is shown in Fig. 6. IR spectra show characteristic CH aromatic vibrational modes at >3,000 cm^{-1}, skeleton vibrations at 1,500 cm^{-1}, and deformation vibrations at 1,400 cm^{-1} (Fig. 6b) of enrofloxacin. FTIR spectra show a red shift of the σ-$_{COOH}$ group from 1,733 cm^{-1} of enrofloxacin to 1,728 cm^{-1} in the formulation. Also, the peak assigned to the tertiary amine showed batochromic (red) shift from 1,154 to 1,142 cm^{-1} of free quinolone to the enrofloxacin inside the gel matrix. On the contrary, the peaks assigned to nonsubstituted benzene and the F–C interaction showed a hypsochromic (blue) shift among the bands from free and entrapped enrofloxacin (Table 1). The FTIR results suggest that the interaction among the antibiotic and the matrix is mostly related to the polar groups present in the enrofloxacin.

Considering the FTIR results and the low enrofloxacin release from PVA–pectin (1.0%) films, a procedure modifying the ionic strength by means of a second-layer film was explored for fine tuning the controlled release of enrofloxacin. Laminated polymer matrixes were investigated to design and model drug delivery devices capable of constant release rates [6]. Further design of such systems has been investigated previously in order to achieve a constant rate control for drugs and proteins [17, 18]. The mathematical analysis showed that drug release from such devices could be done under zero-order conditions (Fig. 7). The

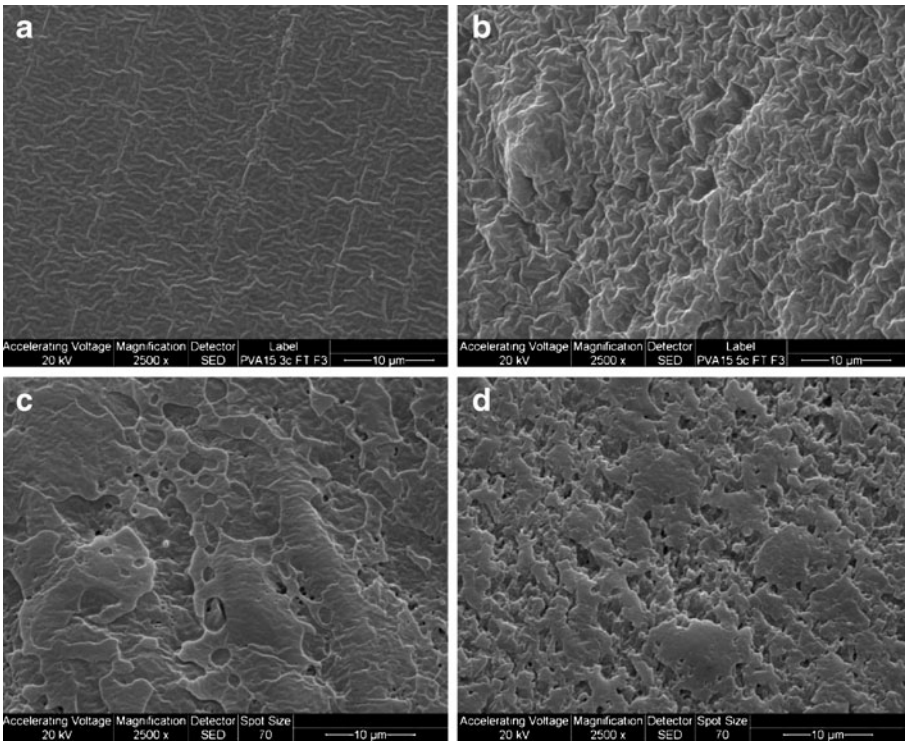

Fig. 5 SEM picture of PVA cryogel containing 1.0% pectin after three and five cycles of freezing and thawing. **a** PVA three cycles of F/T, **b** PVA five cycles of F/T, **c** PVA–P three cycles of F/T, **d** PVA–P five cycles of F/T

ability of multilayer structures was attractive for both small molecules and macromolecular drug delivery over a period of hours to days [17]. The system was designed to modulate the releasing rate of enrofloxacin from a PVA–P cryogel, and it was assessed in a diffusion cell (Fig. 1). The top PVA–P layer contained a given concentration of NaCl, and the bottom film contained 35.0 μg/ml enrofloxacin. The two-layer system was found to release up to 89.8% of the drug in 4 h when the top gel contained 1.5 M NaCl, comparable to the first-order

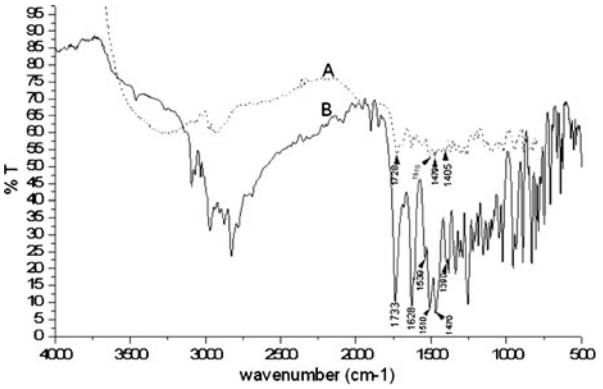

Fig. 6 FTIR spectra enrofloxacin–PVA–pectin (**a**) and free enrofloxacin (**b**)

Table 1 FTIR assignments of free and entrapped enrofloxacin in pectin–PVA cryogel

Wavenumber (cm^{-1})		$\Delta\upsilon$ (cm^{-1})	Assignments
Enrofloxacin	Formulation		
1,733	1,728	+5	COOH
1,628	1,631	−2	C=O pyridine
1,510	1,510	0	C=C N=C pyridine
1,470	1,479	−9	Monosubstituted benzene
1,390	1,405	−15	C–F
1,154	1,142	+12	Tertiary amine

$\Delta\upsilon = \upsilon_{\text{enrofloxacin}} - \upsilon_{\text{formulation}}$

kinetic obtained without pectin (Fig. 7). The release was reduced to 41.9% when 0.75 M NaCl was applied in the top gel. The results are showing that the release rate of enrofloxacin was not only dependent on pectin quantities inside the gel but also on the NaCl concentration in the upper gel layer, implying that the antibiotic release can be modulated just by changing the salt concentration in the top gel. Also, the enrofloxacin stacking problem in the PVA–P gel was avoided which is another relevant advantage of the double-layer gel. So, the two-layer cryogel system showed attractive features for transcutaneous drug delivery.

Conclusions

The manufacture of PVA films by F/T is a simple technique that can be easily scaled up. However, the addition of pectin is a prerequisite to hold enrofloxacin inside the film. The analysis of the enrofloxacin FTIR spectrum showed shifts both in polar and non-polar peaks when the antibiotic is entrapped in the PVA–pectin matrix. Most of the observed interactions with the matrix can be attributed to polar groups, carboxylate and amine; on the contrary, hydrophobic motifs were blue shifted indicating no interaction with the matrix components. Based on the slow release of enrofloxacin from the PVA–(1.0%)P film, a second polar layer of PVA containing NaCl was developed, and the change of NaCl concentration can be used

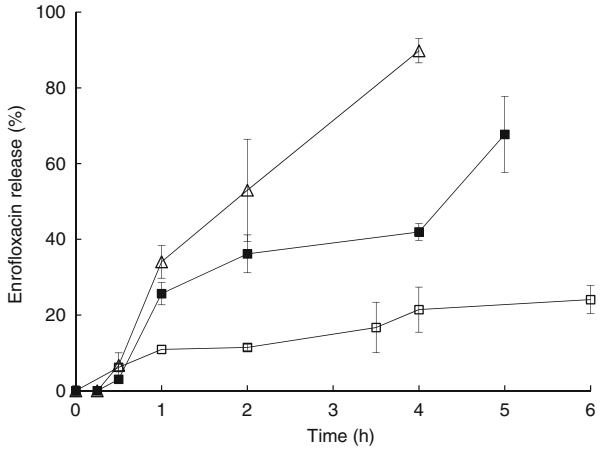

Fig. 7 Controlled release of enrofloxacin performed by two-layer patch of PVA–P cryogel at 37 °C. 1.5 M NaCl (*white triangles*), 0.75 M NaCl (*black squares*), and without NaCl (*white squares*)

as modulator agent for time release of the antibiotic.

As a concluding remark, the system developed in the present work is very versatile since it poses two ways to tune enrofloxacin: pectin which is directly related to the ability of keeping the antibiotic inside the matrix and control of the cargo amount in the gel. On the other side is the NaCl or high-ionic-strength-type compound which could be able to determine the kinetic enrofloxacin release. By modifying one or both parameters, an appropriate gel matrix can be used for different infectious pathologies and/or treatments.

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