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Research paper

Syringeable Pluronic–α-cyclodextrin supramolecular gels for sustained delivery of vancomycin

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ABSTRACT

The ability of Pluronic® F127 to form supramolecular gels in the presence of αCD has been explored as a way to design syringeable gel formulations able to sustain drug release while using the lowest proportion of both components. The effects of αCD concentration range (0–9.7% w/v) in copolymer (6.5%, 13% and 20%) gel features were evaluated at 4, 20 and 37 °C. An effective complexation of Pluronic and αCD was evidenced as a change in the surface pressure of the π-A isotherm of Pluronic on a subphase of CD solution and the apparition of new peaks in the X-ray spectra. Once the Pluronic and αCD solutions were mixed, the systems became progressively turbid solutions or white gels. The greater the αCD concentration was, the faster the gel formation. The supramolecular hydrogels were thixotropic and those containing 5% or more αCD had G′ values above G″ at room temperature, but they were still easily syringeable. The values of both moduli increased as temperature raised; the effect being more evident for 13% and 20% w/v copolymer. The gels prepared with low proportions of αCD exhibited phase separation in few days, particularly when stored at 4 or 37 °C. By contrast, those prepared with 6.5% copolymer were stable for at least two months when stored at 20 °C. The gels were able to sustain vancomycin release for several days; the higher the αCD proportion, the slower the release was. Furthermore, the drug-loaded gels showed activity against *Staphylococcus aureus*. The results obtained highlight the role of the αCD concentration on the tuning of the rheological features and drug release profiles from Pluronic gels.

1. Introduction

Injectable systems capable of forming polymeric matrices *in situ* represent an attractive approach for minimally invasive and patient-friendly implantation of prosthesis and drug depots, avoiding the risk of infection inherent to surgical maneuvers [1,2]. Two main types of syringeable systems may be distinguished: (i) low-viscosity formulations that undergo a transition to gel under physiological conditions [3] and (ii) reversible (highly thixotropic) gels that can flow when a certain pressure is applied (e.g. pushing the plunger of a syringe) and that restore their conformation at rest [4]. Any way, the development of therapeutically useful products is a quite complex issue, namely it involves the design of formulations that exhibit optimal kinetics of *in vivo* formation/regeneration of the gel and that provide adequate drug release profiles. Furthermore, the list of biocompatible materials suitable for injectable gels is quite short and, therefore, an intense research is being carried out for synthesizing new polymers and gelling modulators [5–7] or, more preferably from the regulatory point of view, for designing adequate combinations of already approved materials [5,8,9].

Several attempts have been made to modulate the gel temperature and strength of poloxamers (Pluronic® or Lutrol®) aqueous dispersions. This family of amphiphilic poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO) triblock copolymers is one of the most typical temperature-responsive materials and some varieties (mainly Pluronic F127) are components of FDA and EMA-approved oral and parenteral products [10]. Self-association of poloxamers in water due to hydrophobic interactions among PPO blocks leads to micelle formation [11]. The number of micelles depends on both the copolymer concentration and the temperature. As temperature increases, the micelles pack to form 3D-aggregates, causing reversible sol-to-gel transitions [12,13]. Nevertheless, high Pluronic concentration is required to form

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gels *in vivo* able to remain in the application site and to sustain the release for a long time [10]. This is an important drawback not only from a technological point of view but also from safety issues. In fact, the risk of lipid metabolism alteration resulting in hypertriglyceridemia and hypercholesterolemia notably raises when high doses of Pluronic F127 are used [10]. Thus, approaches for increasing gel strength while reducing Pluronic concentration should render more safe systems with improved drug release performance. The gelling features of Pluronic solutions can be modulated by combining several poloxamers, other polymers or additives [14–21] or forming supramolecular structures with cyclodextrins (CDs) [22–24]. Self-assembled supramolecular hydrogels based on the ability of CDs to form inclusion complexes are attracting a high attention [22]. CDs can act as reversible cross-linkers of quite diverse polymer chains, rendering networks that can host the drug at the same time they are being formed. The process takes place at room temperature without using organic solvents, and removal of non-reacted substances is not needed afterwards (green chemistry). Since no covalent links between the CDs and the polymers are involved, significant changes in bioelimination/biodegradability of the components are not expected. Most information available on supramolecular gels focuses on thixotropic networks formed between polyethylene glycol (PEG) and αCD. A minimum length of PEG chain of 200 Da is required for the formation of stable inclusion complexes with stoichiometry 2EO:1αCD [25]. The increase in viscosity caused by the network formation has been shown as a very promising way to regulate diffusion of therapeutic substances, namely large-molecular-weight macromolecules such as proteins [24,26]. αCD could aid the formation of Pluronic gels at low copolymer concentrations due to the interactions between αCD units that are forming inclusion complexes with different PEO blocks [27,4]. Such a behavior is the opposite to that observed when PPO blocks of Pluronics are threaded through βCD or its derivatives, which results in necklace-like polypseudorotaxanes that exhibit an increase in gel temperature due to the shield of the hydrophobic blocks [28]. Nevertheless, most research carried out with Pluronic:αCD systems dealt with copolymer varieties of lower molecular weight, much shorter PEO blocks and, consequently, higher gel temperature than Pluronic F127 [27,29]. Interestingly, it was found that αCD can thread onto PEO chains even in the case of reverse Pluronics by sliding over the end PPO blocks [30,31]; the resultant polypseudorotaxane being promising for gene delivery [32].

The aim of this work was to elucidate the possibilities of combining Pluronic F127 and αCD to render syringeable supramolecular gel depots for controlled release of vancomycin. Vancomycin is a tricyclic glycopeptide useful for treating serious infections, including osteomyelitis, caused by Gram-positive bacteria such as *Staphylococcus aureus* [33,34], but it is safer for osteoblasts and skeletal cells compared with other commonly used antimicrobial agents [35,36]. Furthermore, vancomycin does not interfere in bone fracture healing [37]. Pluronic F127–αCD systems may offer an alternative to the systemic treatment of osteomyelitis if they are able to provide vancomycin levels well above the minimum inhibitory concentration for time enough to eradicate the infection [38]. Previous experiments carried out with 25% Pluronic gels saturated with vancomycin showed that after subcutaneous implantation, local levels above the minimal inhibitory concentration (1–4 mg/L) for most *S. aureus* isolates were achieved [39]. Vancomycin hydrochloride molecules can be entrapped into the Pluronic F127–αCD gel and, since vancomycin is too large to form inclusion complexes with αCD, no interference in the copolymer-αCD supramolecular gel formation is expected. Compared with PEG–αCD systems that show a marked drop in viscosity during injection and require even hours in being restored [4], the self-associative features of PPO in Pluronic may enable a faster recovery of the supramolecular structure at body temperature. That property may avoid premature clearance of the formulation from the injection site. The effect of the concentration of Pluronic F127 and αCD on the syringeability, thixotropy, viscoelasticity, temperature responsiveness and vancomycin release properties was analysed in detail. The physical stability of the formulations during storage was also taken into account. To the best of our knowledge, the effect of temperature on the aggregation of Pluronic/αCD polypseudorotaxanes and the evolution of the physical networks has not been studied yet. The information obtained should be helpful to identify Pluronic F127–αCD mixtures that render syringeable systems with the minimal content in both pharmaceutically acceptable components that still enables sufficient control of drug release.

2. Materials and methods

2.1. Materials

Pluronic® F127 (EO_{100}–PO_{69}–EO_{100}, 12,600 Da) was from Sigma–Aldrich (St. Louis, MO, USA), vancomycin HCl from Roig Farma (Barcelona, Spain), and αCD from Wacker (Burghausen, Germany). Purified water with a resistivity above 18.2 MΩ cm^{-1} was obtained using reverse osmosis (MilliQ®, Millipore, Barcelona, Spain). Other reagents were analytical grade.

2.2. π-A isotherms

The pressure-area profiles of Pluronic F127 on water or αCD solution (0.001–0.1%, 450 ml) were recorded with the accuracy of ±0.1 mN/m, using a Wilhelmy plate made from chromatography paper (Whatman Chr1, UK) as a pressure sensor, in a single barrier NIMA 611 (UK) surface balance with total area 550 cm^2 at 15 cm^2 min^{-1} compression rate. Prior to experiments, the trough was cleaned with chloroform and ethanol and rinsed with water. The temperature was kept at 25 °C. Pluronic F127 solution in chloroform (70 μl, 0.10 mg/ml) was deposited by means of a syringe (Hamilton, USA) and allowed to stand for at least 10 min in order to ensure complete evaporation of the solvent. The monolayer stability was verified by monitoring the change in surface pressure while holding the area constant. The π-A isotherms of αCD solution (0.001–0.1%) included in the subphase were previously recorded at different times to ensure the attainment of the equilibrium before the addition of Pluronic F127 to the air–water interface. Additional tests were carried out by preparing a Pluronic F127–αCD solution in ethanol which was diluted with chloroform (1:10) in order to render Pluronic F127–αCD 3:1 weight ratio (i.e., copolymer and αCD concentrations 0.10 and 0.033 mg/ml, respectively). The π-A isotherms were recorded on water as described earlier.

2.3. Gel preparation

Pluronic F127 solutions in water were prepared according to the cold method [40]. Briefly, a weighed amount of copolymer was added to water under stirring, and the dispersions were kept at 4 °C for further 12–24 h until a clear solution was obtained. Separately, αCD solutions containing vancomycin were prepared in water. Pluronic and αCD solutions were mixed at different volume ratios to obtain 6.5%, 13% or 20% w/v copolymer and 0% (control gels), 2.5%, 5.0%, 7.0% and 9.7% w/v αCD systems. The detailed composition of each system is given in Table 1. After vortexing the mixed solutions, replicates of each system were stored at 4, 25, or 37 °C.

2.4. X-ray diffraction and FTIR

X-ray diffraction was used to characterize the crystalline structure of the aggregates in the systems formed by 13% Pluronic F127 and 5% αCD. The gels were dried at 50 °C for 5 days and then

Table 1
Composition and appearance after 24 h of the Pluronic–αCD formulations loaded with vancomycin.

Formulation code	F127 (% w/v)	αCD (% w/v)	αCD:EO molar ratio	Vancomycin HCl (mg/ml)	4 °C	25 °C	37 °C
P-6.5	6.5	0	0	0	Sol	Sol	Sol
P-6.5-0	6.5	0	0.000	5.5	Sol	Sol	Sol
P-6.5-2.5	6.5	2.5	0.025	5.5	Sol	Sol	Sol
P-6.5-5	6.5	5	0.050	5.5	Gel	Gel	Sol[b]
P-6.5-7	6.5	7	0.070	5.5	Gel	Gel	Gel
P-6.5-9.7	6.5	9.7	0.097	5.5	Gel	Gel	Gel
P-13	13	0	0	0	Sol	Sol	Sol
P-13-0	13	0	0.000	5.5	Sol	Sol	Sol
P-13-2.5	13	2.5	0.012	5.5	Sol[b]	Sol	Sol
P-13-5	13	5	0.025	5.5	Gel	Gel	Sol[b]
P-13-7	13	7	0.035	5.5	Gel	Gel	Gel
P-13-9.7	13	9.7	0.048	5.5	Gel	Gel	Gel
P-20	20	0	0	0	Sol	Sol	Sol
P-20-0	20	0	0.000	5.5	Sol	Sol	Sol
P-20-2.5	20	2.5	0.008	5.5	Sol[a,b]	Sol	Sol
P-20-5	20	5	0.016	5.5	Gel[b]	Gel	Sol[a,b]
P-20-7	20	7	0.023	5.5	Gel[b]	Gel	Gel
P-20-9.7	20	9.7	0.031	5.5	Gel[b]	Gel	Gel

[a] Turbid white solution.
[b] Phase separation was observed after 8 days.

scanned from 5° to 30° at a speed of 0.4° per minute in a Philips PW 1710 using Ni-filtered Cu Ka radiation. Infrared spectra of the sample dispersed in KBr were recorded on a Bruker IFS 66 V FTIR spectrometer (Germany) in the 400–4000 cm^{-1} range, under resolution of 4 cm^{-1}.

2.5. Gel appearance and syringeability

Changes in turbidity and phase separation were evaluated through visual inspection of the systems while keeping stored at 4, 25 and 37 °C for several days after preparation. Gel formation was monitored applying the inverted-tube test. The syringeability of formulations stored for 8 days at 4 °C was determined in duplicate using a TA-TX Plus Texture Analyzer (Stable Micro Systems Ltd., Surrey, UK). In brief, formulations were transferred into fine-dosage polypropylene syringes (1 ml, Omnifix®-F, B. Braun Melsungen AG, Melsungen, Germany). Half of the content of each syringe was emptied using the texture analyzer in compression mode displacing the plunger at a constant rate of 2 mm/s. The syringeability was estimated as the work required for the process (i.e., the area under the force vs. displacement curve).

2.6. Rheological properties

Viscoelastic behavior of Pluronic–αCD systems previously stored at 4 °C for 5 days was evaluated at 10 (the lowest temperature affordable with the available equipment), 25 and 37 °C in a Rheolyst AR-1000 N rheometer (TA Instruments, New Castle, UK) equipped with an AR2500 data analyzer, and fitted with a Peltier plate. The storage (G′) and the loss (G″) moduli were recorded at 0.5 Pa in the 0.5–50 rad/s angular frequency interval using a cone-plate geometry (diameter 6 cm, angle 2°). The dependence of G′ and G″ on temperature was evaluated at 1 rad/s and 0.1 Pa in the 10–50 °C range. Viscosity and thixotropy of the formulations were recorded applying a flow cycle consisting in a continuously increasing shear rate ramp at 20 °C from 0.05 to 10 s^{-1} for 10 min, a conditioning step at 37 °C for 10 s, and a continuously decreasing shear rate ramp at 37 °C from 10 to 0.05 s^{-1} for 40 min.

2.7. Vancomycin release

Vancomycin release from the gels was evaluated using Franz-Chien vertical diffusion cells. The donor compartment was filled with 1 g of the Pluronic–αCD formulation, while the receptor compartment was filled with water (5.5 ml) and kept at 37 °C. The compartments were separated by a cellulose acetate membrane filter (0.45 μm, Albet®, Barcelona, Spain). The surface available for diffusion was 0.785 cm^2. At various times, 0.7 ml aliquots were withdrawn from the receptor compartment, the absorbance measured at 280 nm (Agilent 8453, Waldbronn, Germany) and the amount of drug released determined. During the first three hours of the test, the aliquots were immediately returned to the corresponding cell. Beyond that time, the sample aliquots were replaced by fresh medium. Diffusion coefficients were estimated by fitting the Higuchi (1962) equation:

$$Q/A = 2C_0(D \cdot t/\pi)^{1/2}$$

where Q is the amount of vancomycin (mg) released by time t (s), A is diffusion area (cm^2), C_0 is the initial concentration of vancomycin in the formulation (mg/ml), and D is the diffusion coefficient (cm^2/s) [41]. Statistical analysis of the dependence of D values on the gel composition was carried out by multiple regression using

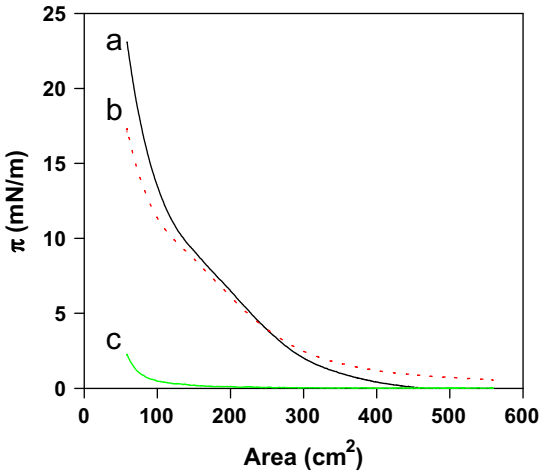

Fig. 1. π-A isotherms recorded for Pluronic F127 on water (a) or on the αCD solutions (b) and for the αCD solutions used as subphase (c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

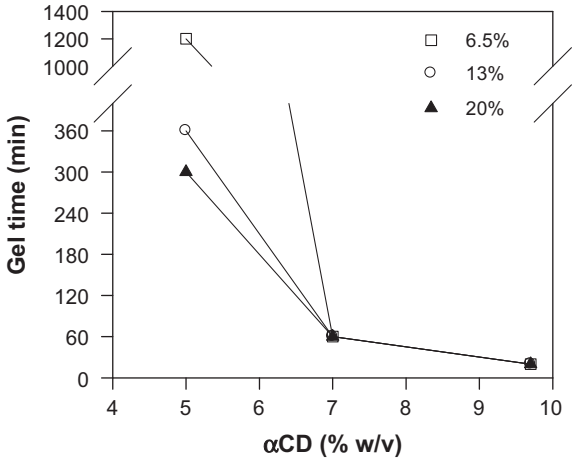

Fig. 2. Time required for the Pluronic:αCD aqueous systems to become a gel (estimated using the inverted-tube test) when stored at 20 °C.

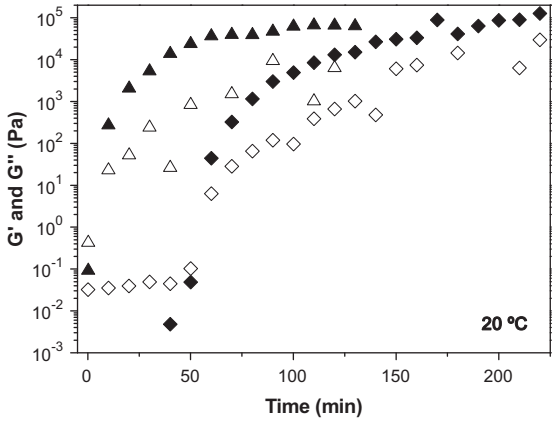

Fig. 3. Evolution of storage (G′, full symbols) and loss (G″, open symbols) moduli of Pluronic:αCD formulations containing 13% Pluronic F127 and 7% (up triangles) or 9.7% (diamonds) αCD.

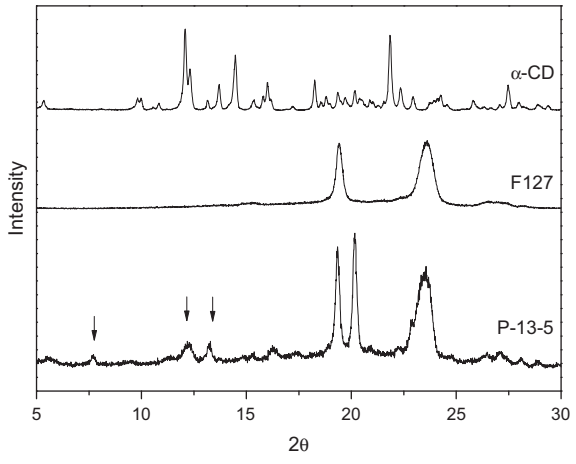

Fig. 4. X-ray diffraction patterns of αCD, Pluronic F127 and the Pluronic F127 (13%)–αCD (5%) gel.

Statgraphics Plus 5.1 software (Statpoint Technologies, Inc., Warrenton, Virginia USA).

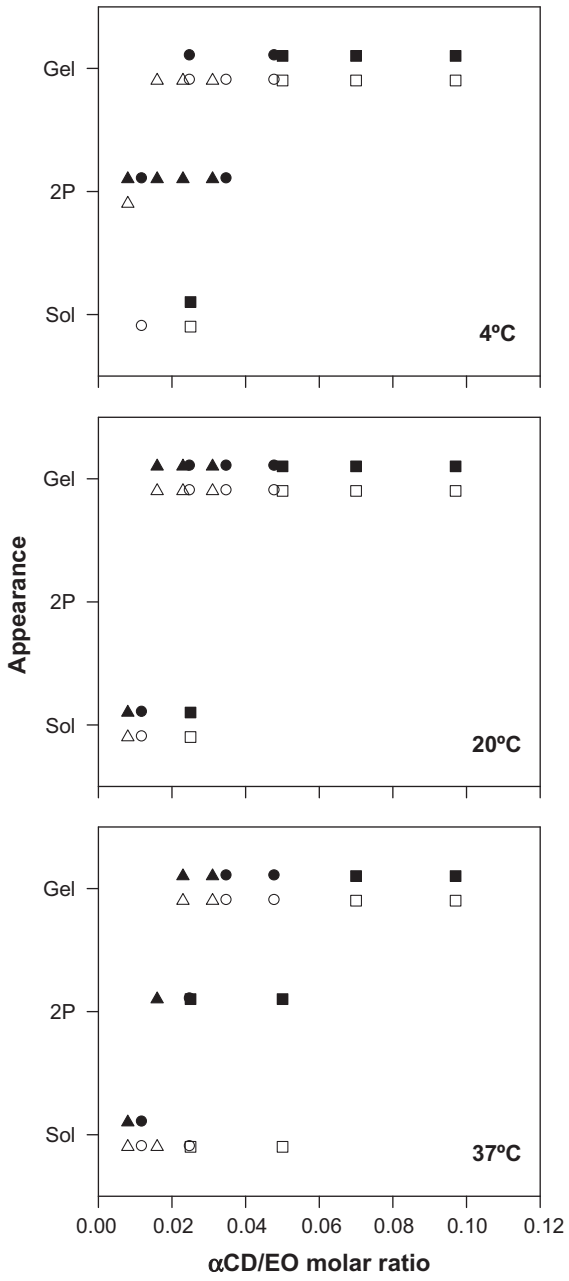

Fig. 5. Phase diagram of Pluronic:αCD formulations after being stored for 16 h (open symbols) or 8 days (solid symbols) at 4, 20 or 37 °C. Pluronic concentration was 6.5% (squares), 13% (circles) and 20% (upper triangles).

2.8. Antibacterial activity

Staphylococcus aureus ATCC 25923 was cultured on TSA plates and incubated at 37 °C for 24 h. Then, the microorganisms were removed from the isolation medium and suspended in PBS pH 7.4 to a final concentration of 7.8×10^8 CFU/ml. *S. aureus* was then seeded on Muller–Hinton Agar plates. Aliquots of each formulation were directly placed in the centre of the plates, except in the case of those prepared without αCD, which were previously embedded in sterile paper discs (6 mm in diameter). Then the plates were incubated at 37 °C, and the growth of bacteria on the surface was visually inspected at 24 and 48 h. Formulations without vancomycin were similarly evaluated.

3. Results and discussion

3.1. π-A isotherms

Supramolecular complexes between Pluronic varieties and αCD have been largely studied by NMR techniques [30,42]. Thus, as an initial step, we wanted to elucidate if a simpler alternative technique, such as the record of π-A isotherms, which requires minimum amounts of components and can be completed in a few minutes, could also reveal the association of Pluronic F127 and αCD. The π-A isotherms of the block copolymer were firstly recorded on water and on αCD aqueous solutions. We have previously observed that, using this technique, strong changes in the isotherms occur during the interaction of Pluronic F127 with hydroxypropyl-β-CD and methyl-β-CD. These CDs are surface active by themselves [43], and we observed that they can indeed form monolayers at the air–water interface [28]. The interactions with the βCD derivatives cause the drainage of some Pluronic F127 unimers towards the bulk (owing to an increase in the hydrophilicity of the macromolecule as the PPO block is threaded by CDs), a change in the CD concentration at the water surface, and the appearance of polypseudorotaxanes in the interface [28]. Although it has been previously reported that αCD is not surface active [43], we observed a small increase in the surface pressure when 0.001–0.1% αCD solutions were compressed, which indicate that they can move to the interface (Fig. 1). To the best of our knowledge, the π-A isotherm analysis of αCD interactions with poloxamers has been only carried out with Pluronic L61 (PEO_3–PPO_{30}–PEO_3) [44]. This copolymer did not show interaction with αCD, differently to what was observed with βCD derivatives. That finding can be attributed to the short PEO blocks, which have a length below that found as the minimum (200 Da) to form stable inclusion complexes [25]. The length should be not a problem in the case of Pluronic F127, which possesses PEO blocks of roughly 4200 Da. In fact we observed that, compared with Pluronic F127 isotherm recorded on water, the presence of αCD (either in the subfase or in the deposition solution) leads to a small increase in the pressure when the area is large and the copolymer is expanded. Under these conditions, the PO and EO units of the copolymer are lying flat on the interface and the increase in pressure can be attributed to the presence of some αCD also on the surface; some may be forming complexes with the PEO blocks, slightly increasing the area occupied per copolymer molecule. As the pressure increases, the EO units are pushed into the aqueous phase. This behavior is seen as a pseudoplateau, which finishes when all EO units are in the subphase [45–47]. In the presence of αCD, lower pressures were recorded in this pseudoplateau region (Fig. 1), which suggests that this cyclodextrin makes the immersion of EO units in the subphase more favorable. Further compression caused a rapid increase in pressure since the movement of PPO block becomes restricted. It has been reported that, in the absence of cyclodextrin, PEO chains in the subphase are in helicoidal conformation and entangle with those of neighbour copolymer molecules [45]. In the presence of αCD, the PEO chains may be partially inserted in

Table 2
Mean values of work (N·mm) required to draw the formulations from 1-ml syringes and the respective standard deviations, indicated in parenthesis.

αCD (% w/v)	Pluronic (% w/v)		
	6.5	13	20
0	11.61 (1.14)	13.27 (1.86)	11.31 (1.78)
2.5	13.95 (0.89)	11.12 (0.58)	8.74 (2.14)
5	9.96 (1.43)	16.00 (1.06)	11.08 (1.03)
7	15.74 (0.22)	10.80 (0.66)	7.71 (0.77)
9.7	22.73 (0.01)	9.93 (1.50)	9.95 (0.58)

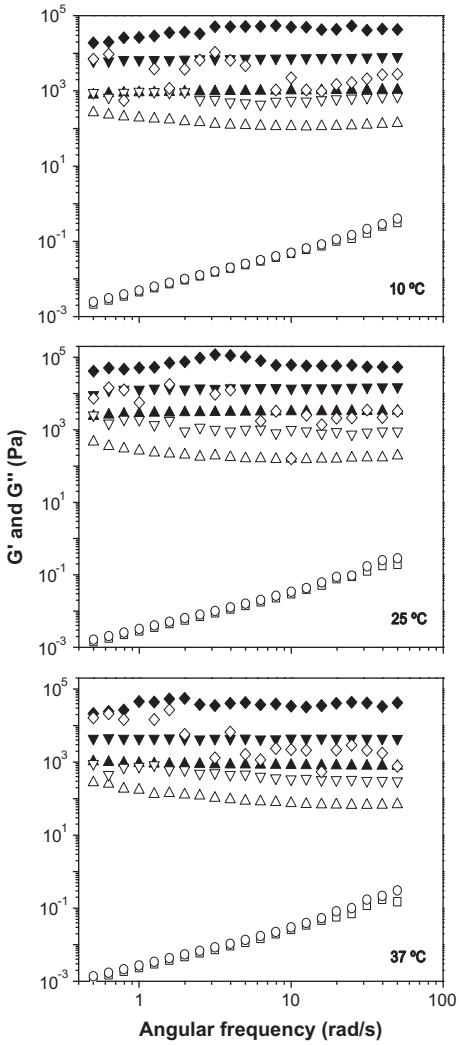

Fig. 7. Storage (full symbols) and loss (open symbols) moduli of the Pluronic–αCD formulations containing 6.5% copolymer without αCD (squares) or containing 2.5% (circles), 5% (up triangles), 7% (down triangles) or 9.7% (diamonds) αCD, recorded at 10, 25 and 37 °C. The systems without αCD (squares) or containing 2.5% (circles) had superimposable (small) loss modulus values and did not show perceptible storage modulus.

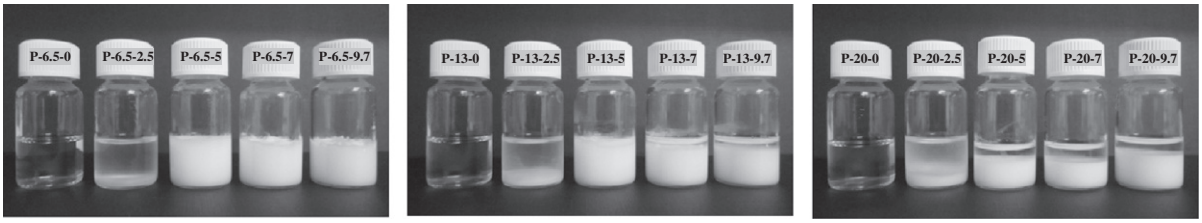

Fig. 6. Gel appearance of Pluronic–αCD formulations after two months of storage at 4 °C.

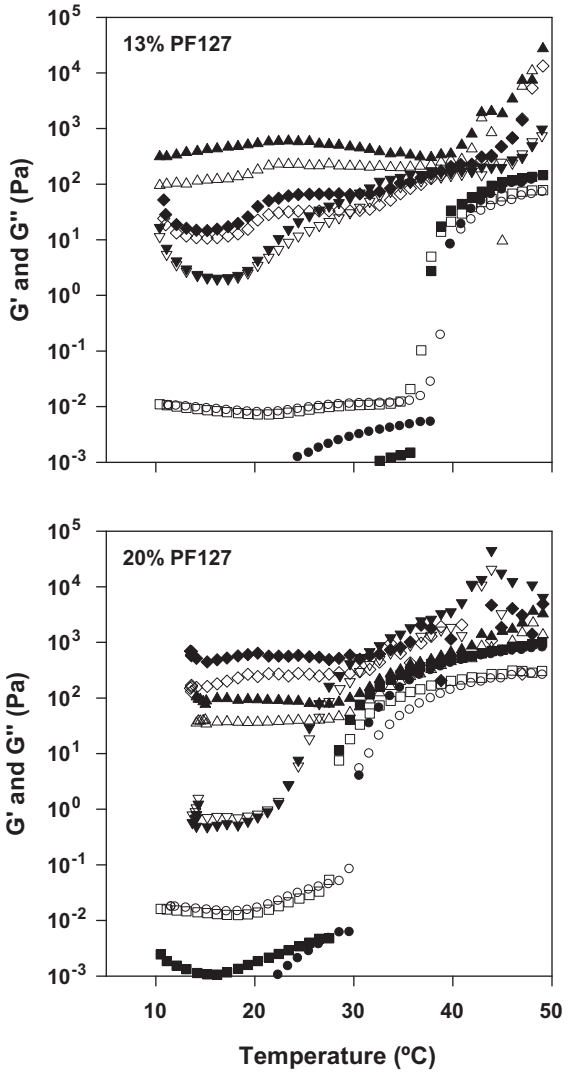

Fig. 8. Dependence of the storage (G′, solid symbols) and loss (G″, open symbols) moduli of 13% and 20% Pluronic F127 formulations without αCD (squares) or containing 2.5% (circles), 5% (up triangles), 7% (down triangles) or 9.7% (diamonds) αCD.

the CD cavities, adopting a more linear conformation. The αCD units of adjacent PEO chains are responsible for the interaction in the subphase. Similar profiles were recorded for the two αCD concentrations tested in the subphase, which indicates that in both cases the interface and the subphase contains sufficient αCD molecules for interacting with Pluronic F127. Furthermore, the profiles were also similar to those recorded when the Pluronic F127–αCD mixture was deposited on water surface. By contrast, they were quite different from those recorded using the same concentrations of βCD derivatives in the subphase. Hydroxypropyl-β-CD and methyl-β-CD caused more marked changes in the isotherms, probably because they interact with the PPO block, which is mainly responsible for the changes observed when the area is stretched [28]. To gain an insight into the thermodynamics of the Pluronic F127–αCD interactions, the differences between the pressure recorded for Pluronic F127 on αCD subphase and the sum of the pressures recoded for Pluronic F127 on water and for αCD alone were estimated as follows:

$$\Delta\pi = \pi_{\exp,A} - \pi_{ideal,A} = \pi_{\exp,A} - (\pi_{PF127,A} + \pi_{\alpha CD,A})$$

The negative values (−2.5 mN/m) recorded for areas below 100 mm^2 indicate favourable interaction between both components; that is, the area occupied by both components when they

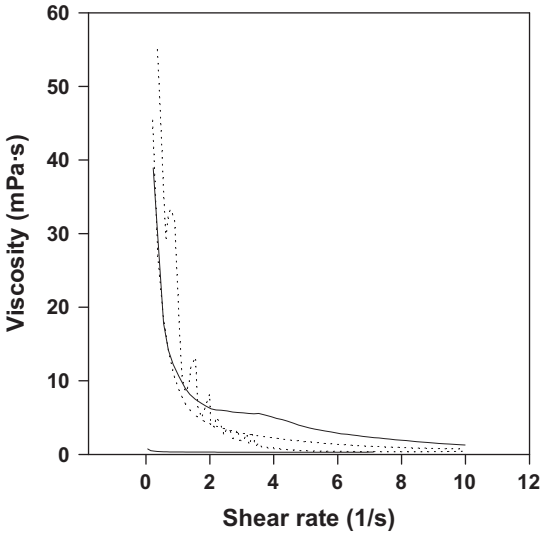

Fig. 9. Changes in viscosity of 6.5% (continuous lines) and 13% (dotted lines) Pluronic F127 formulations containing 9.7% αCD. For each formulation, the upper line corresponds to the increasing shear rate values.

are together is smaller than the sum of the areas occupied by each component alone [28]. Therefore, despite the changes in pressure are smaller than those previously observed for hydroxypropyl-β-CD and methyl-β-CD (up to −8 mN/m at 100 cm^2), αCD also causes an area-condensing effect which suggests a net attractive interaction among the PEO blocks immersed in the subphase. This finding is in agreement with the observation that in the bulk the polypseudorotaxanes aggregate to reduce the contact with the aqueous medium [25].

3.2. Gel appearance and stability

As soon as the Pluronic and αCD solutions were mixed, the systems became progressively turbid dispersions or white gels. Systems containing the lowest αCD concentration tested (2.5%) did not render gels at any temperature. Addition of greater proportions of αCD (although still far from saturation of EO groups) led to gels or phase separation depending on the time and the temperature at which the systems were stored (Table 1). The time required for strong gel formation at room temperature, as estimated using the inverted-tube test, is shown in Fig. 2. Those values were also corroborated by oscillatory rheometry analysis (Fig. 3). The greater the αCD concentration was, the faster the gel formation. Interestingly, the copolymer concentration only affected to the gelling rate of systems containing 5% αCD. Above this αCD concentration, the time required for gel formation was the same disregarding the copolymer concentration. It has been previously observed that the rate of complex formation of PEG depends on its molecular weight and that for PEG chains as large as each PEO block of Pluronic (roughly 4200 Da) the process requires several minutes [25]. In the case of Pluronic F127–5% αCD systems, the effect of the copolymer concentration on the gel time could be related to the fact that, after the threading of αCD onto PEO blocks, the polypseudorotaxanes (namely αCD-based nanocylinders with a channel type structure [25]) self-aggregate to minimize the contact with the aqueous medium. The concentration of polypseudorotaxanes should increase as the copolymer concentration raises, facilitating the formation of precipitated domains as observed for PEG-αCD systems [42]. In the particular case of Pluronic F127, because of the presence of the PPO block which does not form inclusion complexes with αCD, the association of the nanocylinders of αCD may act as the tie-junctions for gel formation. It should be noticed that, as previously found also for PEG

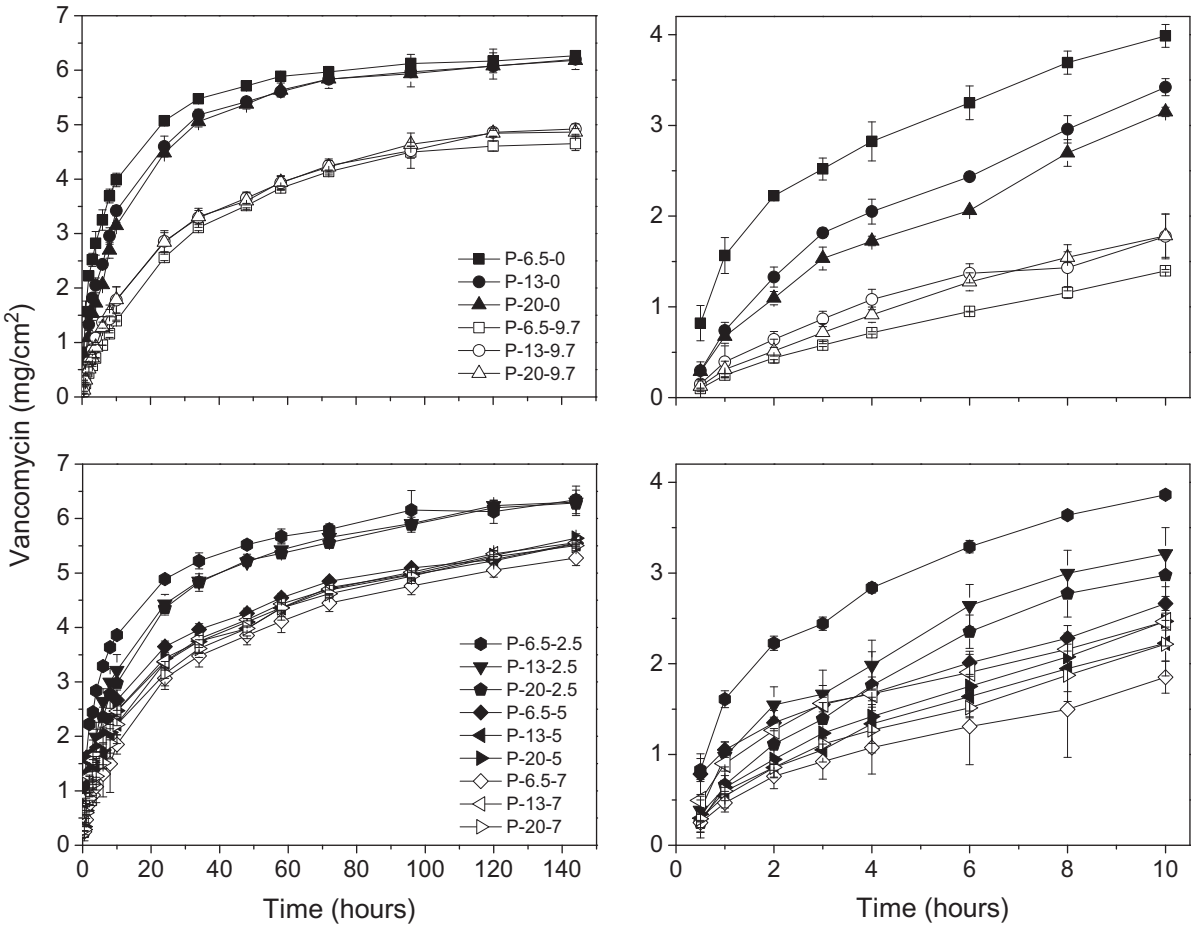

Fig. 10. Vancomycin released at 37 °C from Pluronic formulations with and without αCD. The data corresponding to the first 10 h are expanded in the plot on the right.

Table 3
Mean diffusion coefficients and the respective standard deviations, indicated in parenthesis, (n = 3). The goodness of the fit to the Higuchi equation (obtained with a confidence interval of 98%) can be assessed by the excellent correlation coefficients, R^2.

Formulations	D ($\times 10^6$ cm^2/s)	R^2
P-6.5-0	10.70 (0.28)	0.972
P-6.5-2.5	12.99 (0.66)	0.987
P-6.5-5	3.78 (0.66)	0.984
P-6.5-7	2.65 (1.59)	0.990
P-6.5-9.7	1.92 (0.09)	0.994
P-13-0	10.98 (0.16)	0.991
P-13-2.5	12.47 (1.08)	0.986
P-13-5	4.46 (1.62)	0.997
P-13-7	3.96 (0.48)	0.986
P-13-9.7	2.95 (0.45)	0.972
P-20-0	7.36 (0.58)	0.989
P-20-2.5	13.28 (1.29)	0.983
P-20-5	4.99 (2.92)	0.989
P-20-7	3.94 (0.45)	0.981
P-20-9.7	3.43 (0.98)	0.984

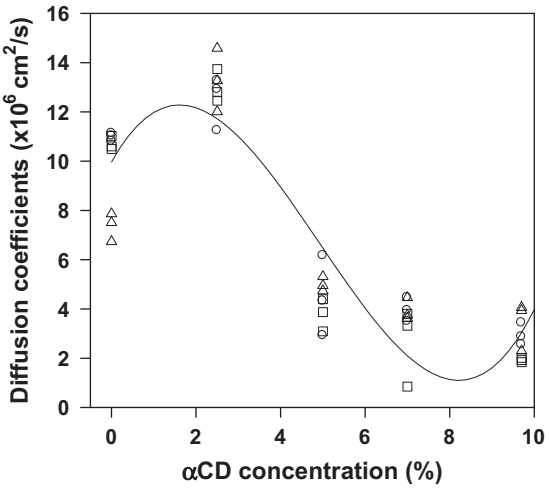

Fig. 11. Dependence of the diffusion coefficient on the αCD concentration for all Pluronic systems tested (6.5%, squares; 13%, circles; 20%, upper triangles). $D = 0.00000996712 + 0.00000307249$ αCD $- 0.00000114176$ αCD$^2 + 7.74594 \times 10^{-8}$ αCD^3 $R^2 = 0.833$; $F_{3,41\ \text{d.f.}} = 68.44$; $\alpha < 0.01$.

systems [42], turbidity of the systems occurred more rapidly than gel formation, indicating that polypseudorotaxane formation is faster than the association of the threaded αCD to lead to phase separation or to a three-dimensional network.

The inclusion complex formation between PEO block of the Pluronic and αCD in the gels was confirmed with wide-angle X-ray diffraction studies (Fig. 4). The major peaks for αCD were observed at 9.78°, 12.08°, 13.7°, 14.48°, 16.02°, 18.26° and 21.84° 2θ, while the main reflection of Pluronic F127 appeared at 19.45° and 23.62° 2θ. The X-ray pattern of the gel P-13-5 showed peaks at ca. 7.71°, 12.1° and 13.29° 2θ, which are coincident with those resulting of the hexagonal unit cell found for αCD inclusion complex with EO [48]. In particular, the sharp reflection at 19.35° strongly support

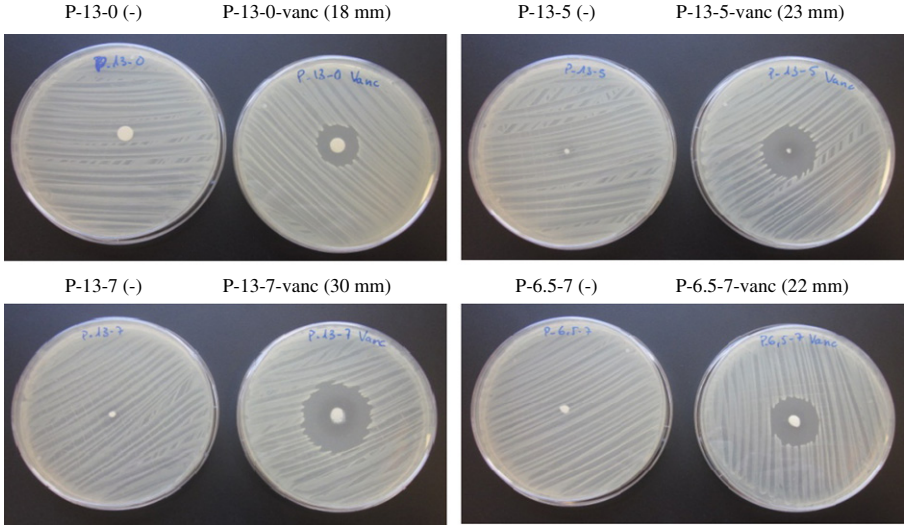

Fig. 12. Culture plates after incubation for 24 h at 37 °C, showing zones of inhibition for *Staphylococcus aureus*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the channel-type crystalline structure of the obtained polyrotaxanes [4,25]. The changes in the FTIR spectra (data not shown) were less significant although a new band appeared at 1343 cm^{-1} in the FTIR spectra of the dried gels, which was absent in the αCD spectrum, and may be assigned to the stretching band for the EO block included inside the αCD cavity.

The αCD:EO molar ratio played a relevant role in the physical stability of the systems (Fig. 5). The lower the ratio, the faster the trend to phase separation was. Particularly, 16 h after being prepared, systems containing 20% Pluronic F127 stored at 4 °C led to phase separation when the αCD:EO molar ratio was 0.008 and to gel formation for αCD:EO molar ratios equals to or above 0.016. Eight days latter phase separation was observed in all 20% Pluronic F127 systems stored at 4 °C (Fig. 6). By contrast, when stored at 37 °C, such rapid phase separation was only observed for systems containing 5% αCD and Pluronic at any concentration. Gels stability was maintained for more time at 20 °C than at 4 °C or 37 °C; after two months, formulations prepared with 6.5% Pluronic and 5–9.7% αCD did not evidence phase separation (Fig. 5). The effect of temperature on the evolution of the phase-separation process may be related to the fact that the interaction (threading) of αCD and PEO is more favorable at low temperature and also the stacking of the αCD nanotubes [42]. In the case of the PEG-αCD systems, water at 5 °C is not a good solvent for the polypseudorotaxanes and large precipitated domains are formed, while at 70 °C, the system is in good solvent conditions and the formation of polypseudorotaxanes is hindered [42]. The behavior of Pluronic–αCD systems against temperature is expected to be more complex, since self-association of PPO blocks as temperature increases may also play a role in the formation of aggregated domains. It should be noticed that Pluronic F127 concentrations tested are well above the critical micellar concentration in water (0.02–0.50%) [49]. Therefore, it seems plausible that at 20 °C, the rate of polypseudorotaxane formation and subsequent aggregation is lower than at 4 °C (driven by the stacking of the αCD nanotubes) or at 37 °C (driven by the self-aggregation of PPO blocks). If fast gelling is wanted, storage at 4 or 37 °C is beneficial. Conversely, if stability of the gels is preferred, 20 °C is a more adequate temperature for storage.

3.3. Syringeability and viscoelasticity

Pluronic–αCD formulations stored for 8 days at 4 °C were easily drawn from 1-ml syringes (Table 2). The work required for moving the plunger at a constant rate was quite similar to that required in the case of an empty syringe 14.20 (s.d. 1.59) N·mm. Only 6.5% Pluronic F127–9.7% αCD system, i.e., the one with the largest αCD:EO molar ratio (0.097), offered higher resistance to flow, although the value was still low and in the range that can be considered easily syringeable.

Viscoelastic behavior of the Pluronic–αCD formulations containing vancomycin was evaluated after being stored at 4 °C for 5 days (Fig. 7). 6.5% Pluronic F127 aqueous dispersions behaved as Newtonian fluids; the storage modulus (G′) being negligible at any temperature tested. Incorporation of 2.5% αCD (αCD:EO molar ratio 0.025) did not alter the rheological profile. By contrast, addition of αCD at 5% (αCD:EO molar ratio 0.050) caused the formulations to become structured gels with storage modulus above the loss modulus; the values of both moduli being practically independent of the angular frequency at any temperature tested. Further increase in αCD concentration (αCD:EO molar ratios 0.070 and 0.097) led to almost one order of magnitude increase in G′ and G″. No noticeable effect of the temperature on the viscoelastic parameters was recorded, which can be related to the fact that 6.5% Pluronic concentration is far from the concentration required for temperature-induced gelling.

Viscoelastic parameters of formulations containing 13% Pluronic showed a dependence on αCD similar to that exhibited by 6.5% copolymer formulations, although the effect of temperature was more evident, as also occurred in the case of 20% Pluronic systems (Fig. 8). 13% Pluronic F127 solely aqueous dispersion exhibited a sol to gel transition at ca. 39 °C rendering G′ and G″ values in the 10^2 Pa range. Formulations containing 2.5% αCD (αCD:EO molar ratio 0.012) showed a similar rheological profile. A sharp increase in G′ and G″ values was observed for 5% αCD systems (αCD:EO molar ratio 0.025) which resulted in moduli values close to 10^2 Pa already at room temperature, while at 39 °C, a further increase in G′ and G″ up to 10^4 Pa was recorded. Further increase in αCD concentration did not result in greater G′ and G″ values, but a decrease was observed. The gel temperature of 20% Pluronic F127 systems was 28.5 °C. Incorporation of 2.5% αCD slightly increased the gel temperature up to 30 °C. Further increase in αCD resulted in networks with greater G′ and G″, particularly at temperature below the gel transition. It is interesting to note that the greatest moduli values were achieved for 13% Pluronic–5% αCD and 20% Pluronic–9.7% αCD; i.e., for αCD:EO molar ratio 0.031–0.035.

When subjected to flow experiments, the Pluronic–αCD formulations were found to be thixotropic and reversible (Fig. 9). The

experiments were carried out at 20 °C applying increasing shear rates and at 37 °C applying decreasing shear rates in order to mimic the conditions of *in vivo* administration through a syringe. The viscosity of the formulations greatly diminished as the stirring increased. Therefore, even the networks that under oscillatory rheometry behaved as the most viscoelastic, easily disassembly under low shear rate values, which is in agreement with the good syringeability observed (Table 2). This property makes the formulations to be syringeable even through a fine needle. The drop in viscosity was restored to different extent as the shearing decreased at 37 °C. 13% Pluronic systems recovered more rapidly than 6.5% Pluronic ones (Fig. 9). The thixotropic behavior is explained by the physical nature of gelation.

3.4. Drug release

Vancomycin hydrochloride was incorporated to the formulations before Pluronic and αCD solutions were mixed. This drug was physically entrapped in the supramolecular gels and did not interfere in the Pluronic–αCD interaction. From the point of view of the application, Pluronic solution and vancomycin/αCD solution could be separately sterilized by filtration through 0.22-μm membranes, owing to their low viscosity at room temperature, and then mixed by transfer of one of the solutions to a syringe preloaded with the other solution. The gel could be then formed inside the syringe used for the administration. Drug diffusion profiles obtained using the Franz-Chien vertical diffusion cells setup are shown in Fig. 10. All formulations were able to sustain vancomycin release for several days. Nevertheless, those prepared without αCD released vancomycin quite fast disregarding Pluronic concentration. Small differences in drug diffusion coefficients were obtained when the release profiles from 6.5%, 13% and 20% Pluronic were fitted to the Higuchi equation (Table 3). The diffusion values were of the same order of magnitude as those previously recorded for 25% Pluronic gels loaded with 20 mg/ml vancomycin (21×10^{-6} cm^2/s) [39]. Although no diffusion values were reported, Talasaz et al. (2008) observed that similar vancomycin release rates can be achieved from gels in which Pluronic F127 was partially replaced by hydroxypropyl cellulose or hydroxypropyl methylcellulose, decreasing the copolymer concentration from 18% to 10% [50]. The goodness of the fitting to the Higuchi equation should be noted, which is foreseeable for gels exposed to minimal dilution with the release medium as one may expect to occur when the formulations are delivered into minimally irrigated body sites (as the bones).

Vancomycin release rate became even faster in the presence of 2.5% αCD, which is in agreement with the phase separation that occurs without increase in viscosity, as observed in the rheological experiments. By contrast, a brusque decrease in release rate was observed for systems containing 5% or more αCD (Fig. 10). The greater the αCD concentration, the smaller the diffusion coefficient was (Table 3). The effect of αCD was more marked in the case of 6.5% Pluronic F127 systems, as expected from the changes in viscoelasticity. Gel formulation without αCD released 80% drug during the first 24 h, while that prepared with 9.7% αCD released less than 45% in the same period of time. In this last case, 100% release was achieved at day 7.

Multiple regression of the diffusion coefficient values on the proportions of Pluronic and αCD confirmed that, in the evaluated intervals, Pluronic concentration does not significantly affect vancomycin release rate. By contrast, the increase in the diffusion coefficient values at 2.5% αCD and the subsequent decrease at 5% or higher αCD concentrations was shown as a polynomial dependence involving linear, quadratic and cubic effects (Fig. 11).

3.5. Antibacterial activity

The antibacterial activity of the vancomycin-loaded supramolecular gels was tested against *Staphylococcus aureus* cultures *in vitro*. The inhibition zone diameters ranged from 18 to 30 mm (Fig. 12). The antibacterial activity indicates that the release of vancomycin is above the MIC. As expected, the gels without antibiotic did not inhibit bacterial growth. Therefore, the possibility of incorporating quite large amounts of antimicrobial drug and to sustain its release may result in suitable formulations for the management of localized infections.

4. Conclusions

Combination of Pluronic F127 with αCD results in supramolecular systems that behave as viscoelastic but syringeable gels when the concentration of αCD is equal to or above 5%. This enables the decrease of Pluronic concentration up to 6.5% while keeping high storage and loss moduli. A strong dependence of the gel rate formation and physical stability on the Pluronic:αCD molar ratio and storage temperature was observed. Pluronic F127–αCD systems containing 6.5% copolymer and 5% or more αCD at 20 °C were the most physically stable, while the minimum Pluronic F127 concentration that provides thixotropy and temperature responsiveness was 13%. All gels sustained vancomycin release for several days being active against *S. aureus* in *in vitro* cultures. Therefore, 6.5–13% Pluronic F127 and 5–7% αCD appear as the most adequate concentrations for preparing injectable drug depots for the local treatment of infections. Among them, the formulation containing 13% Pluronic F127 and 5% αCD appears to be particularly promising since it combines fast gel formation, stability for one month, easy syringeability, high viscoelastic behaviour and control of drug release for several days.

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