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Research paper

Polypropylene grafted with smart polymers (PNIPAAm/PAAc) for loading and controlled release of vancomycin

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ABSTRACT

New smart surface-modified polypropylene (PP) was prepared for improving the loading and the sustained delivery of vancomycin and, thus, reducing the risk of biofilm formation when used as component of biomedical devices. Isothermal titration calorimetry (ITC) served for screening the most suitable monomers for grafting; the drug preferentially bonding to ionized acrylic acid (AAc). A *net*-PP-g-PNIPAAm-*inter*-*net*-PAAc was synthesized by first grafting and cross-linking of *N*-isopropylacrylamide onto PP films and then interpenetrating a second network by redox polymerization and cross-linking of AAc. PP-g-PAAc slabs were prepared by grafting AAc and, optionally, cross-linking. The amount and composition of grafted polymer (FTIR-ATR), morphology (SEM), temperature- and pH-responsiveness (swelling measurements), thermal behavior (DSC), friction coefficient (rheometry), drug loading and release rate, and effect against methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms (modified robbins device) were evaluated. Grafting of AAc notably decreased the friction coefficient from 0.28 ± 0.03 to 0.05 ± 0.02 and enhanced the vancomycin loading (up to 2.5 mg/cm^2). Drug-loaded films showed a pH-dependent release rate, sustaining the release in pH 7.4 aqueous media at 37 °C for several hours. All drug-loaded films reduced biofilm formation by MRSA; the anti-biofilm effect being statistically significant (91.7% reduction, $\alpha < 0.05$) for PP-g-PAAc with the thinnest grafting layer.

1. Introduction

The use of implants and medical devices has become a leading cause of health-care-related bloodstream infections, which are associated with considerable morbidity and mortality [1–4]. Vascular-catheter related septicemias have enormous economic and sanitary repercussions, the mortality rates being as high as 25% in critically ill patients [1–4]. The main source of infection is contamination from the skin at the moment of the insertion, mainly with opportunistic bacteria such as *Staphylococcus* spp. If the inocula exceed threshold levels or if the host defenses are impaired, the bacteria can attach to the surface of the medical device and subsequently form a biofilm [5]. Cells in a biofilm (sessile cells) are phenotypically and physiologically different from non-adhered (planktonic) cells. One of the typical properties of sessile cells is their increased resistance to antimicrobial agents [5–7] which is reflected by the much higher minimal inhibitory concentrations (MIC) for sessile cells compared to planktonic cells. The incorporation of antimicrobial agents in the device may be an effective way of preventing the development of these biofilm-related infections, avoiding the systemic collateral effects of high doses of antibiotics, and overcoming concerns on bacterial resistance [4,8,9].

Antiseptics and antibiotics can be incorporated into the material of the device or covalently bound or adsorbed onto its surface to achieve sustained delivery of sufficient amounts of drug in the microenvironment of the medical device [10,11]. Despite the progress in this field, the development of materials suitable for the production of catheters and implants, able to sorb a sufficient amount of drug and to provide a local delivery at the appropriate rate is still a challenging task [12]. The aim of this work was to explore the benefits of modifying polypropylene (PP) surfaces with stimuli-responsive polymers to improve the loading and controlled release of vancomycin without compromising the frictional properties. Vancomycin is one of the most frequently chosen antibiotics for the treatment of methicillin-resistant *Staphylococcus*

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aureus (MRSA) infections associated with the use of catheters [4]. PP is widely used as component of meshes for abdominal hernias, sutures and catheters, and it can easily become colonised with bacteria [13–15]. PP surfaces have been modified with ions, grafted antibacterial polymers or immobilized enzymes in order to improve biocompatibility or to prevent bacterial attachment and growth [16–18]. Gamma irradiation of PP has been shown to be an efficient procedure for the grafting of pH- and temperature-responsive polymers [19]. Three-dimensional networks (hydrogels) or interpenetrated networks (IPNs) covalently attached to the PP surface can undergo phase transitions as a function of the microenvironmental conditions [20]. Although not tested yet, cross-linked smart brushes may be useful to load a drug by diffusion and non-covalent interactions when the swollen state is achieved and to control its release when collapsed. For a rational design, isothermal titration microcalorimetry (ITC) was first applied to identify monomers able to interact with the drug. Afterwards, we prepared three sets of grafted PP films, one having cross-linked poly(acrylic acid) (PAAc), another with cross-linked poly(*N*-isopropyl acrylamide) (PNIPAAm) and the third one having interpenetrating networks of both. Finally, the responsiveness towards pH and temperature, the friction coefficient, the ability to load and to control the release of vancomycin, and the effect against MRSA biofilms were evaluated.

2. Materials and methods

2.1. Materials

Isotactic PP films (60 μm thickness) or slabs (1.9 mm thickness) of 71% crystallinity were supplied by PEMEX (Mexico DF, Mexico) and BGSPAZIO IBI, S.L. (Alicante, Spain), respectively; *N*-isopropylacrylamide (NIPAAm) was obtained from Aldrich Co., USA and further purified by dissolving and recrystallizing in hexane/toluene (50/50 vol.%). Acrylic acid (AAc), sodium acrylate (NaAc), *N*,*N*′-methylenebis(acrylamide) (MBAAm), *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (TEMED) and ammonium persulfate (APS) were supplied by Sigma–Aldrich Corp. (St. Louis, MO, USA). AAc was vacuum-distilled before use. Hexane, toluene and dimethylformamide came from Baker (Mexico DF, Mexico) and vancomycin HCl from Roig Farma (Barcelona, Spain). Distilled water was used for all experiments.

2.2. Isothermal titration microcalorimetry (ITC) of vancomycin with AAc, NaAc and NIPAAm

The energetics of the interactions between vancomycin and AAc, NaAc or NIPAAm were evaluated by ITC (VP-ITC MicroCal Inc., Northampton, MA). The experiments were carried out in duplicate (variability <5%) in water at 25 °C, titrating the monomer solution (0.5 M) in the drug solution (0.01 M). A degassed aliquot (1.436 ml) of the drug solution was added to the reaction cell, an identical volume of medium without vancomycin was placed in the reference cell, and 300 μl of degassed AAc, NaAc or NIPAAm solution were loaded in the titration syringe. The binding experiment involved 58 sequential additions of small aliquots (5 μl) of the monomer solution to the reaction cell under continuous stirring (280 rpm). After each addition, the heat effect was recorded. Control experiments were carried out under identical conditions to obtain the heats of dilution and mixing involved in (i) injection of monomer solution into water and (ii) addition of water to the vancomycin solution. The injection schedule (number of injections, volume of injection, and time between injections) was set up using interactive software (Origin© 7.0; OriginLab Corporation, Northampton, MA), all data being stored in a computer. In order to estimate the net reaction enthalpy, the dilution enthalpies were subtracted from the apparent titration enthalpies. Best fit values of the stoichiometry number (n_i), enthalpy change (ΔH_i) and binding constant (K_i) were obtained by fitting to a suitable model of the calorimetric data, using a non-linear least-squares Marquardt algorithm routine provided by the ITC equipment manufacturer (Origin™ software, Microcal Inc.). ΔG_i was calculated as follows:

$$\Delta G_i = -RT \ln K_i \quad (1)$$

in which R is the gas constant and T the absolute temperature. The entropy change (ΔS_i) was calculated using the expression:

$$T\Delta S_i = \Delta H_i - \Delta G_i \quad (2)$$

2.3. PP films grafted with PNIPAAm (PP-g-PNIPAAm)

The following pre-irradiation method was applied [20]. PP films (1.2 cm × 4 cm × 0.06 mm) were irradiated in air with a ^{60}Co gamma source (Gamma Beam 651 PT, Nordion Co., Canada) at a dose rate of 2.6 kGy/h and a total radiation dose of 30 kGy. The irradiated samples were placed in glass ampoules containing an aqueous solution of NIPAAm 1 M, which were saturated with argon for 20 min, sealed and heated to 70 °C for 1.5–5 h. Then, the grafted films were immersed in water for 24 h (replacing the medium several times) and dried. The grafted percentage was estimated as

$$g\ (\%) = [(W_f - W_i)/W_i] \times 100 \quad (3)$$

W_f and W_i being the weight of PP film after and before grafting, respectively.

The PP-g-PNIPAAm films were placed in glass ampoules with distilled water (7 ml) and cross-linked using two different methods: (i) saturation with argon, sealing the ampoules and irradiation at a dose rate of 2.6 kGy/h with a total radiation dose of 40 kGy; or (ii) addition of the cross-linker MBAAm (1.5% w/w) to the ampoules and then proceeding as described in (i). The resultant *net*-PP-g-PNIPAAm films were immersed in water for 24 h and dried under vacuum.

2.4. PP films grafted with PAAc (PP-g-PAAc)

PP films or slabs (0.06 or 1.9 mm thickness, respectively) were irradiated in air with a ^{60}Co gamma source (Gamma Beam 651 PT from Nordion Co., Canada) at a dose rate of 2.6 kGy/h and a total radiation dose of 10 kGy. The irradiated samples were placed in glass ampoules containing aqueous solutions of AAc 40% (v/v). The ampoules were saturated with argon for 20 min, sealed and heated at 50 °C for 20–75 min. The grafted films and slabs were immersed in water for 24 h and then dried. The grafted percentage was estimated applying Eq. (3). Some PP-g-PAAcs were cross-linked to obtain *net*-PP-g-PAAc, as described in Section 2.3.

2.5. PP films grafted with cross-linked PNIPAAm and interpenetrated with PAAc (net-PP-g-PNIPAAm-inter-net-PAAc)

To form the second network of the IPN, a cross-linked PP-g-PNIPAAm film (with 379% PNIPAAm grafting, i.e. 71% content in PNIPAAm and 8% in cross-linker MBAAm) swollen for 24 h in AAc (30 mg/ml) and MBAAm (2.02 mg/ml) solutions in DMF:H_2O 63:37 (v/v). After this time period, APS (0.67 mg/ml) and TEMED (0.67 mg/ml) were added to start the polymerization and the ampoules were sealed. After another 24 h, the films were extracted with water to separate unreacted additives and the free polymer. Every 24 h the medium was replaced and its absorbance from 190 to 800 nm was recorded for several days.

2.6. Characterization of the grafted films

2.6.1. IR spectroscopy

Infrared spectra were recorded from 700 to 4400 cm^{-1} in a Perkin-Elmer Paragon 500 FTIR-ATR spectrometer (Mexico DF, Mexico) equipped with a SeZn glass.

2.6.2. Swelling studies

Dried films were immersed in water at 20 °C for 24 h and the swelling was calculated as relative weight gain, the sample being weighed after careful wiping of its surfaces with a soft tissue, as follows:

$$Sw\ (\%) = 100[(W_t - W_i)/W_i] \quad (4)$$

where W_t and W_i are the weights of the swollen at time t and of the dry film, respectively. Once the equilibrium was reached, the maximum swelling was estimated.

The ratio of the swelling percentage of samples at 20 and 40 °C was used to quantify the temperature-responsiveness, as follows:

$$S_T = S_{ST}/S_{CT} \quad (5)$$

where S_{ST} is the highest swelling (swollen state) and S_{CT} is the lowest swelling (collapsed state) in the temperature range studied. The LCST was estimated as the inflection point of the plot *Sw* (%) as a function of temperature.

The sensitiveness to pH was evaluated in Na_2HPO_4/citric acid buffer solutions, in the 2.2–8.0 pH range (HI 4212 potentiometer, Hanna Instruments, CA, USA) at 37 °C. The critical pH was identified as the inflection point of the plot *Sw* (%) as a function of pH. The pH sensitivity was estimated as the ratio of the highest swelling (S_{SpH}) to the lowest swelling (S_{CpH}) in the pH range evaluated:

$$S_{pH} = S_{SpH}/S_{CpH} \quad (6)$$

2.6.3. Thermal analysis

Differential scanning calorimetry (DSC) scans were recorded using a TA Instruments Model 2010 (TA Instruments, New Castle, DE, USA) under nitrogen atmosphere (flow rate of 60 ml/min), after calibration with indium. Glass transition temperature (T_g) determinations were carried out at a heating rate of 10 °C/min from 25 to 220 °C. The LCST of PP-g-PNIPAAm and *net*-PP-g-PNIPAAm-*inter*-*net*-PAAc swollen in water (at 5 °C for 24 h) was estimated at a heating rate of 1 °C/min from 20 to 45 °C.

2.6.4. Scanning electron microscopy

The grafted PP films were swollen in water for 48 h at 4 °C, frozen by immersion in liquid nitrogen, fractured to an adequate size, freeze-dried for 24 h and covered with Au. The micrographs of the surface and the lateral edge were obtained using a SEM at 20 kV (LEICA S440 Microsystems, Cambridge, UK).

2.6.5. Friction coefficient

The friction force of films hydrated in pH 7.4 buffer was measured, in duplicate, at 25 °C using a Rheolyst AR1000N rheometer (TA Instruments, Crawley, UK) equipped with an AR2500 data analyzer and a Peltier plate. The films were cut as disks (10 mm diameter). The surface of the disk was blotted with filter paper and immediately glued (Loctite® Super Glue-3, Henkel, Barcelona, Spain) to a 4-cm steel plate geometry. One milliliter of pH 7.4 buffer was put on the surface of the Peltier plate and the geometry was moved towards the plate to an initial gap of 0.3 mm. The experiment consisted of a conditioning step applying 5 ± 0.01 N normal force (W) for 15 min and a peak hold step with an angular velocity of 0.05 rad/s for another 15 min [21]. Since the velocity changes with the distance from the center of the axis, the obtained torque, T, is a total value over the velocity range from 0 to ωR, where R is the radius of the gel disk. Thus, the total friction, F, and the coefficient of friction, μ, were determined as follows [22]:

$$F = \frac{4T}{3R} \quad (7)$$

$$\mu = \frac{F}{W} \quad (8)$$

2.6.6. Vancomycin-loading studies

Dried pieces of films (30–40 mg) or slabs (700–900 mg) were placed in 0.4 mg/ml drug aqueous solutions (4 or 100 ml, respectively). Samples were allowed to equilibrate at 20 °C protected from light. The amount of vancomycin loaded in each film was calculated as the difference between the initial and final concentrations in the surrounding solution, determined by UV spectrophotometry at 281 nm (Agilent 8453, Böblingen, Germany). The changes in the concentration in the loading medium over time were recorded and the loading was considered to be finished when an equilibrium was reached (generally after 2–3 days). Then, the films were dried in an oven at 40 °C.

2.6.7. Vancomycin-release studies

Dried drug-loaded films (30–40 mg) were transferred to test tubes containing 4–10 ml of HCl 0.1 M (pH 1.2) or USP phosphate buffer pH 7.4 (ionic strength 0.27) and thermostatized at 37 °C. The experiments were carried out in triplicate (without stirring) under *sink* conditions (maximum vancomycin concentration below a tenth of the solubility coefficient). Samples of the release medium (1 ml) were withdrawn at regular intervals (after being gently hand-shaken to ensure a homogeneous concentration in the medium) and returned to the vial immediately after their drug concentration was measured spectrophotometrically at 281 nm. After 24 h in the release medium, the films were washed as follows: 24 h in HCl 0.1 M, 24 h in phosphate buffer pH 7.4, and 24 h more in distilled water at 4 °C. Finally, the films were dried at 40 °C and then reloaded as explained in Section 2.6.6 for carrying out again release experiments as described above.

The average vancomycin-release rate per surface area (both top and bottom surfaces) in a short time interval was determined as follows:

$$ARR = \frac{\Delta M_t/\Delta t}{A} \quad (9)$$

where ΔM_t represents the change in vancomycin cumulative mass in the Δt time interval and A the PP film surface area. The minimum required flux of vancomycin that must be delivered to the near-wall zone of PP films to kill bacteria, also known as minimum killing release rate (N_{kill}) required at the local interface, was estimated using the equation [23]:

$$\frac{C_s}{N_{kill} \cdot r_o/D} = G\left[\frac{x/r_0}{Re \cdot Sc}\right]^{1/3} \quad (10)$$

where C_s is the wall surface concentration (0.5 μg/ml); r_0 the effective hydraulic radius (0.3 cm) and x the axial distance from the entrance ($x/r_0 = 0.1$); D the diffusivity of vancomycin in fluid (2.73×10^{-6} cm^2/s); G a constant for tube geometry, which equals to 1.22; *Re* the Reynolds number, typically 200 for laminar flow; and *Sc* the Schmidt number (kinematic viscosity/diffusivity = 4.0×10^5).

2.6.8. Microbiological tests

PP-g-PAAc and *net*-PP-g-PAAc were prepared as described in Section 2.4 but using PP slabs of 1.9-mm thickness. The grafted slabs and a non-grafted PP slab (to be used as control) were immersed in pH 7.4 buffer phosphate for one day, rinsed with water, dried and immersed in 0.4 mg/ml vancomycin solution (100 ml)

for 2 days. The amount of drug loaded was calculated as mentioned above. A clinical methicillin-resistant *S. aureus* (MRSA) isolate (isolated from a patient at the Ghent University Hospital, Ghent, Belgium) was grown on Tryptic Soy Agar (TSA) (Oxoid, Drongen, Belgium) at 37 °C. From larger pieces of the various materials (Table 4), small disks (diameter 6.8 mm) were punched. Biofilms were formed using six homemade stainless steel modified robbins devices (MRD) [24]. One MRD contains six individual ports in a linear array along a channel of rectangular cross-section, with dimensions of 10.0 mm (width), 145.0 mm (length), and 3.5 mm (depth), respectively. Each port accepts a press-fit plug holding a disk. The six MRDs, containing the disks (36 in total) were placed in a custom-made aluminium heating block. Feedback from a Pt electrode placed into the heating block ensured a constant temperature of 37 °C in the MRDs. The tubing, valves and MRDs were washed, assembled and autoclaved prior to each run. Assembly was done in a laminar air-flow cabinet to prevent contamination. The tubing was connected to a peristaltic pump (Watson-Marlow 505S, Bredel, Wilmington, MA) and bottles either with growth media or inocula. The setup included a bypass to allow rinsing of the tubing at the inlet side of the devices and the removal of air bubbles. At the outlet side, a clamp was placed on the tubing to prevent the drainage of the liquid when the pump was off. Four sterile centrifugation tubes with 10 ml TSA were inoculated with the organism and incubated for 17 h at 37 °C. The cells were harvested and washed three times with 5-ml phosphate buffer (0.003 M $Na_2HPO_4{\cdot}12H_2O$ and 0.0015 M KH_2PO_4, pH 7.4), the pellets were combined and finally resuspended in 10-ml phosphate buffer. Bottles containing 99 ml of phosphate buffer were inoculated with 1 ml of the prepared suspension and were connected to the MRD. After introduction of the inoculated solutions into the MRDs, the latter were flipped over to improve the adhesion of the cells to the disks. Once the devices were filled with the suspensions, the tubing at the inlet and outlet side was clamped off and the remaining cell suspension in the tubing at the inlet side was flushed out through the bypass. After 1 h, the devices were flipped back, the clamps were loosened and the pump was started to allow a continuous flow of the growth medium, TSA diluted (1:500) in phosphate buffer (flow rate 150 ml/24 h). The biofilms were allowed to develop on the disks for 24 h, after which the pump was stopped and the plugs were removed. Each disk was transferred to test tubes with 10 ml 0.9% (w/v) NaCl and the tubes were subjected three times to 30 s of sonication (Branson 3510, 42 kHz, 100 W, Branson Ultrasonics Corp.) and 30 s of vortex-mixing to remove the biofilm cells from the disks. Using this procedure all cells are removed from the disks and clumps of cells are broken apart. The number of sessile *S. aureus* cells was quantified by plating on TSA. All plates were incubated at 37 °C for 48 h and the number of colony-forming units (CFU) per disk was calculated by counting colonies on the plates. All experiments were carried out on at least 3 disks for each composition. Statistical analysis on the log-transformed data (ANOVA with the Scheffé post hoc test) was performed using SPSS 15.0 software (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Selection of the composition of the grafted polymers

Since PP does not sorb vancomycin, surface modifications with polymers that promote the sorption and retention of this drug are required. To achieve this purpose, two different approaches were considered. A first approach was the grafting of polymers able to directly interact with the drug through ionic and/or hydrophobic bonds; in this respect chains containing acidic groups could be adequate due to the weakly basic character of vancomycin. A second approach was the grafting of a stimuli-responsive cross-linked network that swells under the loading conditions facilitating drug diffusion into the network and that shrinks at physiological pH or temperature enabling the control of drug release. Cross-linked PAAc and PNIPAAm could be suitable as pH- and temperature-responsive candidates, respectively.

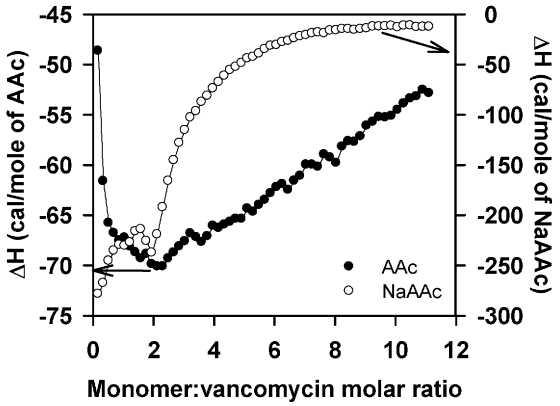

Fig. 1. ITC titrations of vancomycin with AAc (solid symbols) or NaAc (open symbols) in water.

First, the analysis of the ability of different monomers to interact with the drug was evaluated using isothermal titration calorimetry (ITC), a very sensitive technique which was previously used to quantify weak interactions between drugs and cyclodextrins or monomers [25,26]. In the case of acid monomers, their degree of ionization can have a notable repercussion on the nature and intensity of the interactions with the drug. Therefore, AAc, its sodium salt NaAc, and NIPAAm were evaluated using ITC. Fig. 1 shows the titration profiles of vancomycin with AAc (solution of pH 2.3) and NaAc (solution of pH 8.8). An exothermic interaction was observed for both monomers, with a maximum in enthalpy for a monomer:vancomycin 2:1 molar ratio. However, the interaction with NaAc was four times more energetic than with AAc, which reveals a preferential binding of the drug with the monomer when ionized. Similar differences have been found when changing the ionization state of cell wall fragments upon vancomycin binding, which were assigned to entropic contributions due to differ-

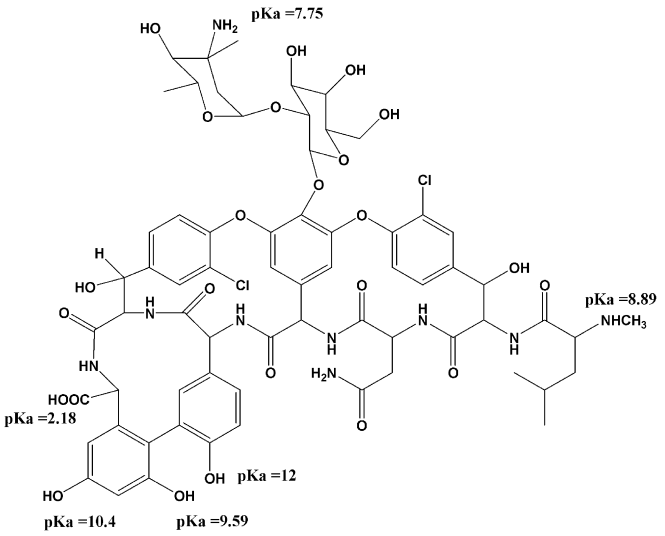

Fig. 2. The structure and pK_a values of vancomycin.

Table 1
Thermodynamic parameters of vancomycin:monomer interaction obtained by ITC analysis after fitting to three different sequential sites

Monomer	K_1 (M^{-1})	ΔH_1 (kJ mol^{-1})	$T\Delta S_1$ (kJ mol^{-1})	K_2 (M^{-1})	ΔH_2 (kJ mol^{-1})	$T\Delta S_2$ (kJ mol^{-1})	K_3 (M^{-1})	ΔH_3 (kJ mol^{-1})	$T\Delta S_3$ (kJ mol^{-1})
AAc	7.3	−0.4	13.9	3.6	−0.1	2.7	3.6	−0.4	2.9
NaAc	637	−1.6	14.4	9338	−0.5	22.2	68	−2.1	8.3

K_i, affinity constant; ΔH_i, complexation enthalpy; $T\Delta S_i$, complexation entropy.
Mean of estimated uncertainties from experimental values ±10%.

ences in the solvation state of the involved atomic groups [27]. The interaction with PNIPAAm was much weaker and practically negligible, as reflected in the low value of the binding constant estimated using a one-site model (K_1 = 0.788 M^{-1}).

Vancomycin is a large molecule (M_w 1449.3 g/mol) with 6 pK_a values corresponding to one primary amino and one secondary amino groups, one carboxylic acid group and three phenol groups ([28], Fig. 2). Vancomycin hydrochloride solution (0.01 M) in water has a pH of 6.3, which means that the molecule is positively charged at the primary and secondary amine groups and its carboxylic acid group bears a negative charge [28]. The small volume of AAc or NaAc might slightly change the pH towards a more acidic or more alkaline values, respectively, without a significant change in the net charge of the drug. The best fitting of the calorimetric data for the titration with AAc or NaAc was obtained when a model consisting of three different sequential sites with different probabilities was adopted (Table 1), which might be related to the interaction of the two amine groups via electrostatic forces and of the phenolic groups via hydrogen bonding with the acrylic acid groups. For the present model, the binding constants K_1, K_2 and K_3 must be defined relative to the progress of saturation, so that

$$K_1 = [MX]/[M][X]; \quad K_2 = [MX_2]/[MX][X];$$
$$K_3 = [MX_3]/[MX_2][X] \qquad (11)$$

In this model, there is no distinction as to which sites are saturated, but only as to the total number of sites are saturated. On the other hand, at the vancomycin concentration used, this antibiotic is preferably in its dimeric form and, thus, dimerization can also affect the interaction [29]. In fact, vancomycin dimers have shown an enhanced binding affinity compared to unimers. Moreover, ligand binding promotes the dimerization of the drug. The cooperative mechanism between both processes might also be reflected in the thermodynamic quantities derived from the fitting model [30]. The dramatically higher binding constants recorded for NaAc, compared to AAc, would support the latter statements. Finally, the vancomycin binding seems to be entropy-driven, as reflected by the positive $T\Delta S$ values. The decrease observed for the third type of binding site might be related to an entropic penalty due to restriction of molecular movements within the complexes.

Taking into account the ITC results, PP films were grafted with poly(acrylic acid) to try to achieve specific binding of the drug. Since drug-loading may also depend on the swelling degree and the mesh size of the polymer network, PAAc-grafted films were cross-linked applying gamma irradiation in the presence and absence of MBAAm. Similarly, temperature-responsive grafted PP films were prepared with PNIPAAm, some being cross-linked to enable the hosting of the drug in the aqueous phase of the network. Finally, the grafted PNIPAAm network was interpenetrated with a PAAc network to form an IPN.

Table 2
Composition of the grafted films prepared from NIPAAm or AAc

Code	Monomer	PP-g-PNIPAAm or PP-g-PAAc		*net*-PP-g-PNIPAAm or *net*-PP-g-PAAc	
		% g	Grafted polymer per unit surface (mg/cm^2)	Conditions	Polymer weight (%)
J42	NIPAAm	521	14.40	–	–
J18x	NIPAAm	282	7.79	γ 40 kGy + MBAAm	74
J17x	NIPAAm	293	8.18	γ 40 kGy	74
C156	AAc	84	2.56	–	–
C147	AAc	250	7.12	–	–
C168	AAc	315	9.15	–	–
C169	AAc	432	12.30	–	–
C148x	AAc	334	9.57	γ 30 kGy + MBAAm	73
C150x	AAc	324	9.58	γ 30 kGy	72
J19IPN*	NIPAAm and PAAc	379	16.14	γ 40 kGy + MBAAm	79

Several grafted polymers were cross-linked to obtain *net*-PP-g-PNIPAAm or *net*-PP-g-PAAc. The *net*-PP-g-PNIPAAm-*inter*-*net*-PAAc (J19IPN*) contained 29.4 wt% PAAc, 55.8 wt% PNIPAAm, and 14.8 wt% PP and its PAAc/PNIPAAm molar ratio was 45/55 mol%.

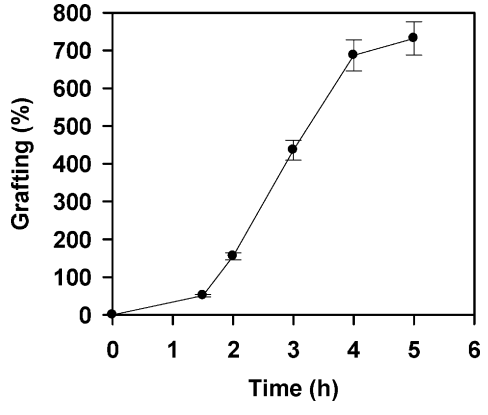

Fig. 3. Evolution of grafting yield of PNIPAAm onto pre-irradiated PP films as a function of time. Dose rate 2.6 kGy/h; pre-irradiation dose 30 kGy; 1 M NIPAAm concentration in aqueous solution.

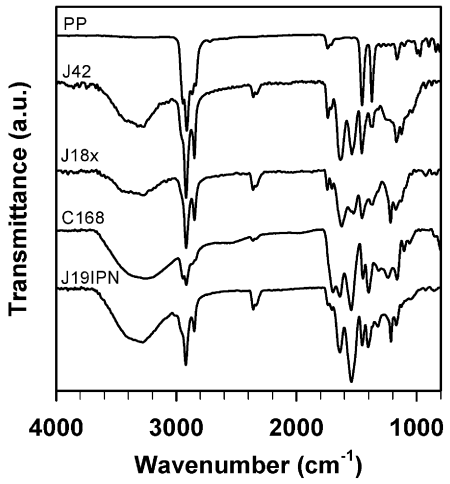

Fig. 4. FTIR spectra of the grafted PP films (codes as in Table 2).

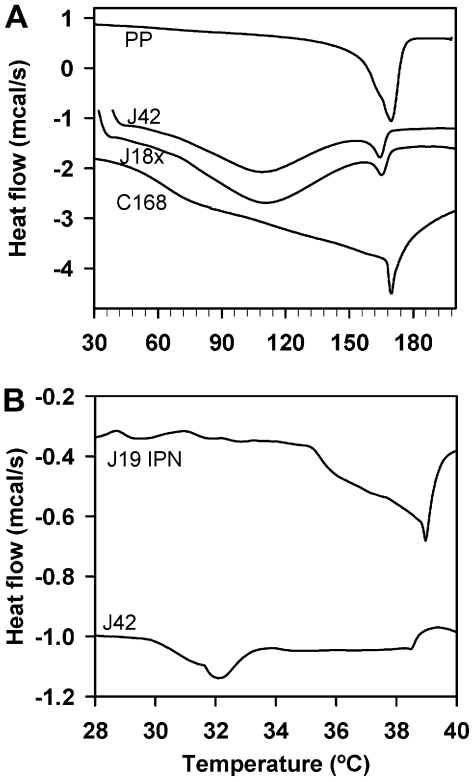

Fig. 5. DSC scans of (A) dried grafted films and (B) water-swollen PP films grafted with cross-linked PNIPAAm and interpenetrated with PAAc (*net*-PP-g-PNIPAAm-*inter*-*net*-PAAc). Codes as in Table 2.

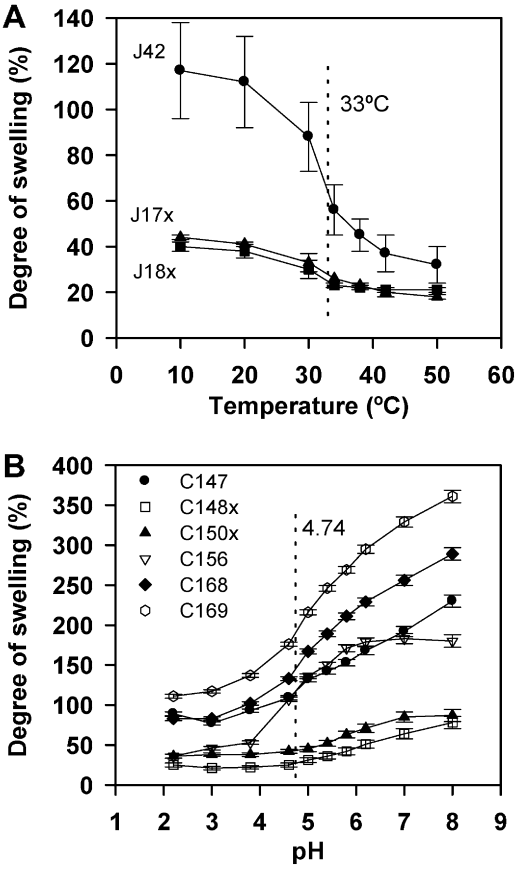

Fig. 6. Dependence of the degree of swelling of (A) PP-g-PNIPAAm (J42) and *net*-PP-g-PNIPAAm (J17x and J18x) on temperature and (B) PP-g-PAAc (C147, C156, C168 and C169) and *net*-PP-g-PAAc (C148x and C150x) on pH. The dotted lines represent the LCST of PNIPAAm and the pK_a value of AAc.

3.2. Preparation and characterization of the grafted PP films

The gain in PNIPAAm or PAAc of PP films was calculated as the increase in weight after the grafting procedure. Fig. 3 shows the kinetics of grafting of PNIPAAm onto PP films by the oxidative pre-irradiation method (variability below 6%). The amount of grafted PNIPAAm increased with the reaction time, as expected for radiation-induced grafting. Under the experimental conditions chosen, grafts from 50% to 730% were obtained. By using this grafting method, different grafting percentages can be achieved in an easy and controlled way.

Table 2 summarizes the compositions of all grafted PP films, i.e. those with brushes of PNIPAAm (PP-g-PNIPAAm) or PAAc (PP-g-PAAc) and those that were subjected to the cross-linking of PNIPAAm or PAAc (*net*-PP-g-PNIPAAm or *net*-PP-g-PAAc). Additionally, an IPN of PNIPAAm and PAAc was formed on the PP film (*net*-PP-g-PNIPAAm-*inter*-*net*-PAAc) having a 45:55 PAAc:PNIPAAm molar ratio. The low UV-absorbance values of the washing solutions indicated that the yield of the polymerization process was high and that immersion in water for 24 h was sufficient to remove all unreacted substances and the homopolymers.

The chemical composition of the films was confirmed by FTIR-ATR (Fig. 4). All films showed the characteristic bands of unmodified isotactic PP film at 2914–2846, 1453 and 1373 cm^{-1}, which indicates that a relatively thin layer of grafted polymer had been formed on the PP surface and that the PP structure was not altered during the process [31]. Additionally, the grafting of PNIPAAm was evidenced by the appearance of new bands at 3314, 1638 and 1540 cm^{-1}, which confirmed the presence of amide groups and a doublet at 1388 and 1370 cm^{-1} assigned to the isopropyl group [32]. Those films grafted with PAAc showed the coexistence of bands at 1715 cm^{-1} and at 1646–1540 cm^{-1} assigned to non-ionized and ionized carboxylic acid groups, respectively [33].

The DSC scans of PP-g-PNIPAAm films showed, in addition to the characteristic melting peak of PP at 165–169 °C, a broad endotherm in the 80–130 °C range that would be due to evaporation of residual water and also to the concomitance of the glass transition of the PNIPAAm chains (Fig. 5A). Since pure PNIPAAm has a T_g around 142–144 °C [34], the residual solvent would have shifted the T_g to lower temperatures. In the case of PP-g-PAAc films, a clear T_g was observed around 60 °C. The temperature-responsiveness of water-swollen PP-g-PNIPAAm and *net*-PP-g-PNIPAAm-*inter*-*net*-PAAc was clearly seen as the endotherm associated to the low critical solubility temperature (LCST) of PNIPAAm (Fig. 5B). The pres-

Table 3
Stimuli-sensitiveness and loading ability (mean values and standard deviations) of the grafted PP films

Code	Critical value of the triggering stimuli	Sensitiveness to the stimuli	Loading		
			(mg/g film)	(mg/cm^2)	(mg/g PAAc)
J42	32 °C	S_T = 3.7	1.1 (0.4)	–	–
J18x	28 °C	S_T = 2.0	1.8 (0.4)	–	–
J17x	28 °C	S_T = 2.4	1.9 (0.9)	–	–
C147	pH 5.4	S_{pH} = 2.6	47.1 (15.0)	0.124 (0.040)	65.9 (21.0)
C168	pH 5.4	S_{pH} = 3.5	51.6 (11.7)	0.136 (0.031)	68.0 (15.4)
C169	pH 5.4	S_{pH} = 3.2	74.9 (9.2)	0.198 (0.024)	92.2 (11.3)
C148x	pH 5.4	S_{pH} = 3.1	24.8 (4.0)	0.065 (0.010)	34.1 (5.5)
C150x	pH 5.4	S_{pH} = 2.5	26.6 (10.4)	0.070 (0.027)	36.7 (14.4)
J19IPN	33 °C; pH 5.4	S_T = 1.2; S_{pH} = 3.0	40.2 (3.9)	0.106 (0.010)	136.7 (13.6)

In all cases the variability of the parameter used to quantify the sensitiveness was below 5%.

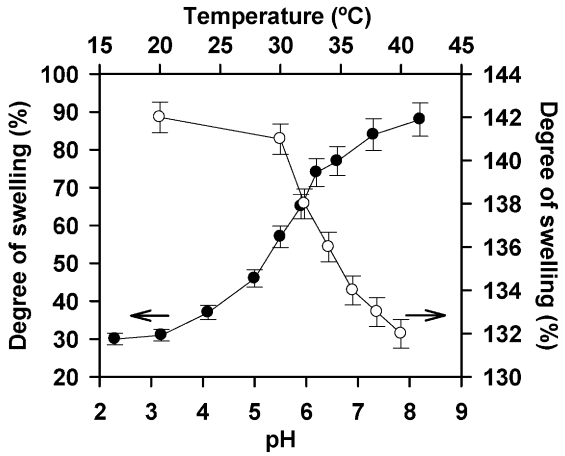

Fig. 7. Dependence of the degree of swelling of *net*-PP-g-PNIPAAm-*inter*-*net*-PAAc on the temperature (water, open symbols) and on the pH (buffer at 37 °C, full symbols).

ence of the *net*-PAAc shifted the transition to a higher temperature, the collapse beginning at 35 °C.

All films quickly swelled once immersed in aqueous medium reaching the equilibrium in less than 60 min. As expected, PP-g-PNIPAAm films showed important differences in the swelling degree as a function of temperature (Fig. 6A) and PP-g-PAAc films showed pH-responsiveness (Fig. 6B). The sensitiveness to each variable increased with the grafting percentage and decreased as the degree of cross-linking of the grafted polymer increased (Table 3). Small changes in the critical value of the triggering stimuli were also observed. The inflexion point of the swelling of PP-g-PAAc did not occur at the pK_a value of isolated AAc monomers (4.74) but at pH 5.4 due to the influence of other AAcs in the vicinity [35,36]. The *net*-PP-g-PNIPAAm-*inter*-*net*-PAAc was dually responsive, being swollen at low temperature or alkaline pH, and collapsed above 35 °C or below pH 4 (Fig. 7). This confirms that a true interpenetrating system was created in which the PNIPAAm and PAAc networks can fully display their responsiveness. Since the LCST of PNIPAAm copolymers is highly dependent on the polarity of the comonomers, the advantages of having an IPN compared to a sim-

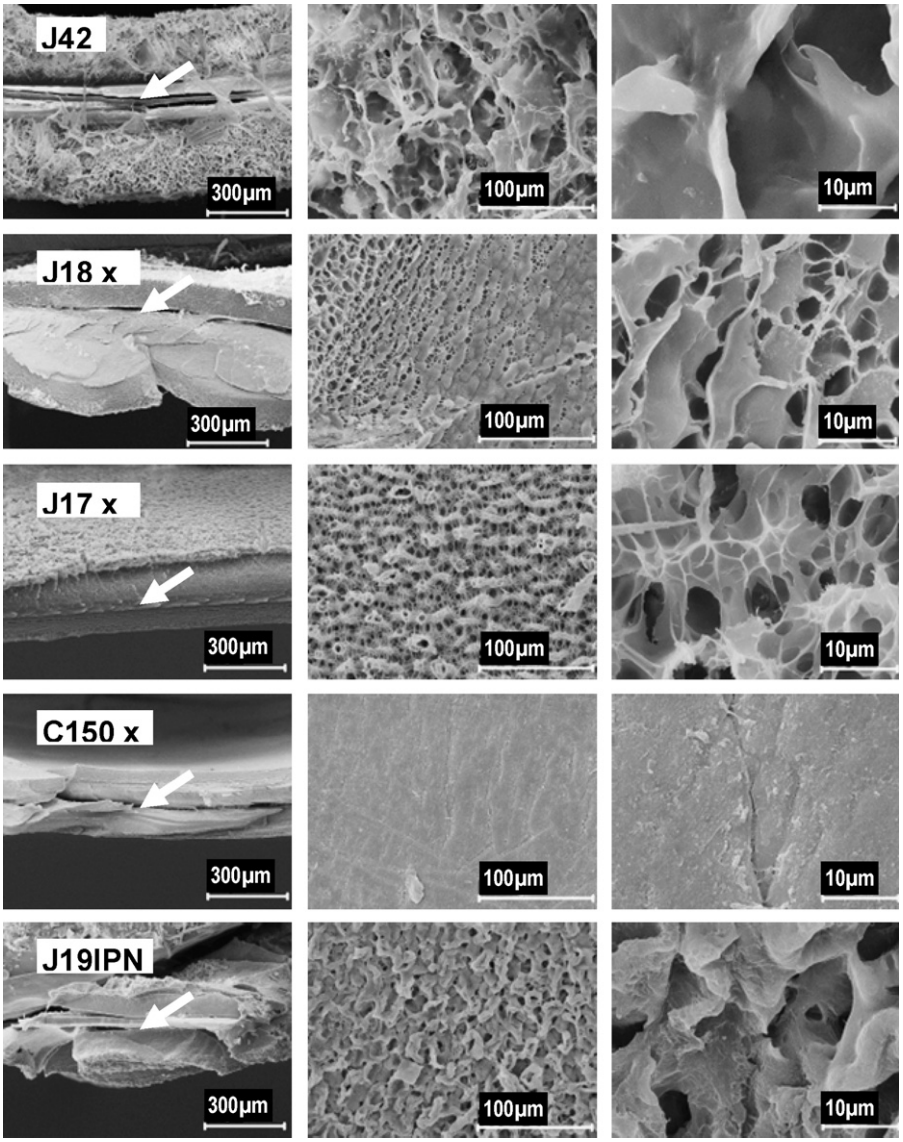

Fig. 8. SEM micrographs of PP-g-PNIPAAm (J42), *net*-PP-g-PNIPAAm (J17x and J18x), *net*-PP-g-PAAc (C150x) and *net*-PP-g-PNIPAAm-*inter*-*net*-PAAc (J19 IPN). The first column shows the transversal cut (PP film is indicated by an arrow) and the second and third columns are views from the top.

ple copolymerized network are obvious [37]. Nevertheless, the temperature-sensitiveness of the swelling was somewhat restricted due to the PAAc chains in the IPN causing the subdivision of the PNIPA network in microdomains, some regions of which cannot be involved in the transition [38].

The microstructure of the grafted layers is shown in Fig. 8. The grafted polymer covers the PP film forming a layer of about 100 μm thickness. In the case of non-cross-linked PNIPAAm (J42), the layer was highly porous. The porosity and the size of the pores decreased in the cross-linked *net*-PP-g-PNIPAAm. The less porous layers were those containing PAAc which may be due to the shrinking of the polymer and the consequent hydrogen-bonding formation among the acrylic acid groups during drying. The interpenetration of the PNIPAAm network with PAAc resulted in medium-porosity structure, which indicates that the presence of PNIPAAm hinders the collapse of the PAAc network.

3.3. Friction coefficient

The effect of the grafting on the sliding of the films was also taken into account, bearing in mind the influence of this property on the easiness of insertion and removal of catheters and implants. The friction should be as low as possible in order to prevent damage of the tissue or vessel upon application [39]. Unmodified PP films showed values of friction coefficient, μ, against the Peltier plate (surface of chrome-plated copper) of 0.28 ± 0.03, which is in range of the values previously reported for this material when assayed against polyethylene [40]. The presence of the smart polymers at the surface of PP significantly decreased the μ values of the hydrated films (pH 7.4 buffer). The friction coefficient of *net*-PP-g-PNIPAAm was 0.19 ± 0.02. Grafting with the more hydrophilic PAAc made the films significantly more lubricious with μ values of 0.05 ± 0.02. The beneficial effect of grafting PAAc hydrogels on the sliding of solid substrates has been previously reported [41]. In our case, the thickness of the PAAc layer grafted on the PP films largely exceeds the minimum of 3 μm required for effectiveness on polyamide-12 and poly(etherester-b-amide) films [42]. The friction coefficient values obtained for the PP-g-PAAc and *net*-PP-g-PAAc are in the range of slippery materials [39], such as acrylic hydrogels intended for comfortable soft contact lenses [21].

3.4. Vancomycin loading and release from the grafted PP films

As expected, unmodified PP films did not sorb vancomycin. Grafting with cross-linked PNIPAAm slightly increased the amount sorbed mainly because the PNIPAAm network can host dissolved drug in its aqueous phase (Table 3). By contrast, the drug-loading was remarkably increased when PAAc was grafted. It is important to note that the grafted films were previously swollen in pH 7.4 phosphate buffer, then dried and finally immersed in the drug solution in water. The pretreatment in pH 7.4 buffer was critical for the access of the drug to the acrylic network, as it enabled the ionization of the AAc groups and the subsequent swelling of the grafted layer in the loading solution. The loading was then carried out in water to avoid possible adverse effects of the ionic strength of the medium on the drug–polymer interactions.

As can be seen in Table 3, the higher the AAc content, the higher the loading. PP-g-PAAc films were able to take up almost all the drug present in the loading solution, avoiding any waste of non-sorbed drug. Thus, the values shown in Table 3 are not the saturation levels. The relatively lower drug uptake of *net*-PP-g-PAAc can be explained by steric hindrance when the PAAc brushes are cross-linked and, consequently, the mesh size is smaller.

The *net*-PP-g-PNIPAAm-*inter*-*net*-PAAc had a much higher loading capability than the sum of the *net*-PP-g-PNIPAAm and the *net*-PP-g-PAAc, which indicates that interpenetration synergistically improves the ability of the grafted polymers to sorb the drug. At the loading conditions (20 °C), the PNIPAAm network is completely swollen and forces the PAAc network to expand. This facilitates the contact of the drug with the acrylic acid groups and also enhances the volume of the aqueous phase entrapped into the IPN, i.e. more drug can diffuse from the loading solution to the microenvironment of the IPN.

One gram of each film immersed in 1 L of medium can easily provide enough vamcomycin to exceed the MIC_{90} (1 μg/ml) and MBC_{90} (minimal bactericidal concentration, 8 μg/ml) for the MRSA [42]. Thus, if these grafted PPs were used as material for catheters or hernia meshes, an efficient prevention of infections caused by *S. aureus* could be expected. Fig. 9 shows the drug release profiles from the grafted PP films in HCl 0.1 M and in pH 7.4 phosphate buffer. Although the PAAc network is swollen at pH 7.4, a slower delivery occurred at this pH compared with pH 1.2. This effect is again explained by the strength of the drug–PAAc interactions, which are at maximum when the acrylic acid groups are ionized. Vancomycin is a freely soluble drug (200 mg/ml in water and 11 mg/ml at pH 7.4 [43]) and the concentrations attained during the release tests were below 0.2 mg/ml. Thus, the contribution of the lower solubility at pH 7.4 to the differences in the release profiles should be irrelevant.

To evaluate the reloading capability of the films, several ones were subjected to four cycles of loading/release. In all cycles, the amount of vancomycin sorbed and its release rate were similar to those observed for the first loading/release experiment.

From the point of view of the efficiency of the vancomycin-loaded PP films to kill bacteria attempting to adhere to a catheter-type device surface, the "instantaneous" release rate per sur-

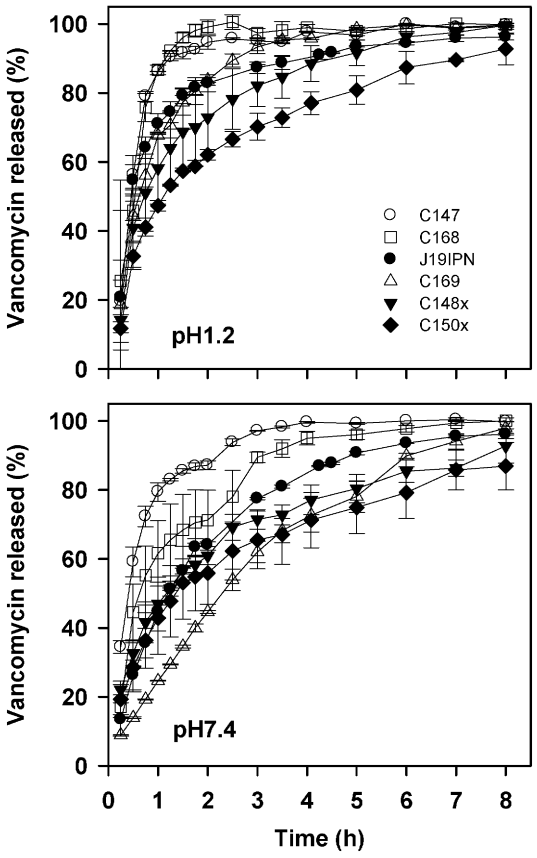

Fig. 9. Vancomycin release profiles from the grafted PP films (codes as in Table 2) in HCl 0.1 M (pH 1.2) and in pH 7.4 phosphate buffer.

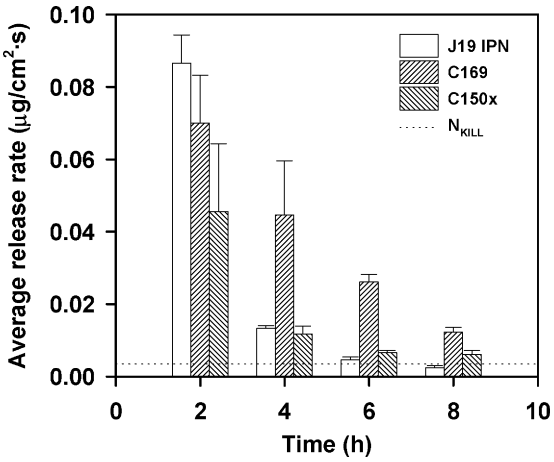

Fig. 10. Average release rates of vancomycin from some grafted PP films (codes as in Table 2) in pH 7.4 phosphate buffer. The N_{kill} value of 3.5×10^{-3} μg cm^{-2} s^{-1} is shown as a dotted line.

face area (ARR) is a critical parameter [23]. Fig. 10 shows the ARR of vancomycin at 2 h intervals. The theoretical N_{kill} value was calculated using Eq. (10) with the following data: $C_s = 0.5$ μg/ml (MIC_{50} for *Staphylococcus* spp. [41]); $D = 2.73 \times 10^{-6}$ cm^2/s estimated for vancomycin with a molecular weight of 1449.3 g/mol and molecular radius of 0.83 nm using the Stokes–Einstein equation with a viscosity of the medium equals to 0.01 Poise; $Sc = 4.0 \times 10^5$; $r_0 = 0.3$ cm for a tube of 0.6 cm diameter; and $x/r_0 = 0.1$ [23,44]. Therefore, this results in a N_{kill} value of 3.5×10^{-3} μg cm^{-2} s^{-1}). The PP films that combine a high loading with sufficient ability to sustain the release at pH 7.4 provided ARR values above the N_{kill} for at least 6 h and even longer than 8 h (PP-g-PAAc, code C169). The eradication of bacteria during the early period following implantation is critical to preventing the development of bacterial biofilms on catheters and implants [12,45]. The grafting of PP films with AAc or IPNs of PAAc and PNIPAAm enables the tuning of the amount of vancomycin loaded as well as the drug release rate. Therefore, this approach could be useful to modifying the surface of PP-based medical devices in order to reduce the likelihood of infections.

3.5. Microbiological tests

In order to gain an insight into the anti-biofilm effect of vancomycin-loaded PP-g-PAAc and *net*-PP-g-PAAc, biofilm experiments with a MRSA strain were carried out using the MRD (Fig. 11). Relatively thick (1.9 mm) PP slabs were used to fulfill the experimental requirements. Each slab was immersed in 100 ml of vancomycin solution (0.4 mg/ml) to reach the saturation of the loading. Table 4 shows the contents in vancomycin before the biofilm experiment.

The MRD system has been shown to be particularly useful to forming high-density microbial biofilms on various materials [24]

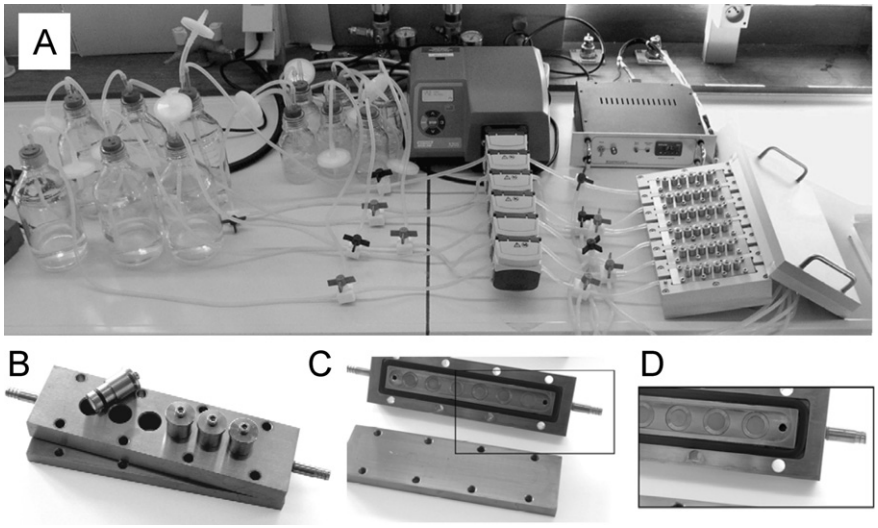

Fig. 11. Set-up of the entire modified robbins device (MRD) biofilm model system (A), close-up of a single MRD (B), close-up of the interior of a single MRD (C) holding six plugs (D).

Table 4
Composition of the grafted PP-g-PAAc slabs used for the microbiological tests, number of sessile MRSA cells (log CFU per disk, average and standard deviation) recovered from various disks following biofilm formation in the MRD, and reduction percentage compared to unmodified PP (control)

Code	g (%)	Cross-linking conditions	Grafted polymer per unit surface (mg/cm^2)	Loading		Biofilm test	
				(mg/cm^2)	(mg/g PAAc)	Number of sessile cells (log)	Reduction (%)
Control	0	–	0	0	0	5.11 (0.14)	–
C19	6.3	30 kGy	3.97	0.971	244.8	4.99 (0.13)	24.1
C20	5.2	–	3.33	0.369	110.7	4.03 (0.10)	91.7*
C21	6.3	30 kGy + MBAAm	4.07	0.918	225.7	4.84 (0.26)	46.3
C23	38.0	30 kGy	23.59	2.567	108.8	4.92 (0.27)	35.4
C25	35.6	30 kGy + MBAAm	21.71	2.451	112.9	5.02 (0.20)	18.7
C26	37.3	–	23.22	0.937	40.4	4.91 (0.29)	36.9

* Statistically significant, $\alpha < 0.05$.

and this was confirmed in this study. Approximately 1.3×10^5 CFU was present in biofilms formed on unmodified PP disks (Table 4). Following 1 h of adhesion and 24 h of biofilm formation, all vancomycin-loaded PP disks tested contained less MRSA biofilm biomass than the unmodified PP disks, with reductions ranging from 18.7% to 91.7%. The reduction in biofilm was statistically significant ($\alpha < 0.05$) for C20 (Table 4). Using this biofilm model system, disks are subjected to a continuous flow of fresh growth medium (150 ml in 24 h; pH 7.3 ± 0.2). This not only creates ideal conditions for microbial growth (as nutrients are constantly provided to the bacteria and waste products are removed) but also prevents accumulation of vancomycin in the reactor since any drug released from the surface of PP disks is immediately washed away, effectively reducing the contact time between the sessile bacteria and vancomycin. This is in contrast with static biofilm model systems (e.g. microtiter plates) in which the released vancomycin could accumulate. While in the microtiter plate tests anti-biofilm effects are often overrated, the MRD system used in the present study probably represents the worst case scenario and as such the reductions observed are still promising. The most efficient surface-modified PP was the slab covered with the non-cross-linked PAAc that was able to load more vancomycin (C20); the amount of vancomycin loaded per gram of PAAc being similar to that of PP-g-PAAc film able to provide vancomycin release rates above N_{kill} for the longest time (C169; Fig. 10).

4. Conclusions

ITC experiments revealed a preferential interaction of vancomycin with acrylic acid when ionized. Such information helped to conveniently prepare and condition grafted PP films with PAAc or IPNs of PAAc and PNIPAAm in order to achieve the maximum loading and also to explain the sustained delivery at pH 7.4. Sliding of the PP films was also improved by the grafting. Microbiological tests indicate that PP-g-PAAc are potentially useful as components of prostheses or catheters to be loaded with vancomycin in order to prevent the rapid development of microbial biofilms after their implantation or insertion.

Acknowledgements

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