Electronic Supplementary Information

Antiadhesive and antibacterial properties of pillar[5]arene-based multilayers

Lucia Barbera, Laura M. De Plano, Domenico Franco, Giuseppe Gattuso, Salvatore P. P. Guglielmino, Gabriele Lando, Anna Notti,* Melchiorre F. Parisi and Ilenia Pisagatti*

Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali, Università di Messina, Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy. E-mail: anotti@unime.it, ipisagatti@unime.it

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Materials and Methods. Levofloxacin (1), Amikacin disulfate salt (2), poly(ethyleneimine) (PEI, Mw 750000, 50% in water) and poly(allylamine) hydrochloride (PAH, average Mw 50000) were purchased from Sigma-Aldrich. Other chemicals were reagent-grade and were used as received without any further purification. The strains of *Pseudomonas aeruginosa* ATCC27853 and *Staphylococcus aureus* ATCC29213 used in the microbiological studies were purchased from the American Type Culture Collection (LGC Promochem, Milan, Italy). Both microorganisms were stored at –80 °C, the former in a Luria-Bertani broth (LB) and the latter in a Trypti-case Soy broth (TSB) containing 20% (v/v) glycerol. Quartz slides were obtained from Alfa Aesar.



General Experimental. ¹H NMR spectra were recorded at 298 K in D₂O, at 300 or 500 MHz. The residual solvent peak (δ = 4.65 ppm) was used as an internal standard. Decacarboxylato-pillar[5]arene **WP5** was synthesized according to a literature procedure.¹ NOESY spectra were recorded on a spectrometer equipped with a pulse-field gradient probe, using a 350 ms mixing time, 16 transients for each increment (256 in total) and a relaxation time of 3 s. NMR titration studies were carried out at a fixed **WP5** concentration (1 mM) and samples were routinely prepared by dissolving solid **WP5** in D₂O or a D₂O phosphate buffer solution (50 mM Na₂HPO₄/NaH₂PO₄, pH 7.2). The stock solution of levofloxacin ([**1**] = 5 mM) was prepared by using the above-mentioned 1 mM **WP5** solutions as the solvent, so as to maintain a constant host concentration after addition of aliquots of the guest. The association constants were calculated by a nonlinear regression method using the WinEQNMR² program. UV/Vis absorption spectra were taken on a Varian

¹ T. Ogoshi, M. Hashizume, T.-A. Yamagishi and Y. Nakamoto, *Chem. Commun.*, 2010, 46, 3708.

² M. J. Hynes, J. Chem. Soc., Dalton Trans., 1993, 311.

Cary 50 spectrophotometer (additionally equipped with an optic fiber probe with a 1 cm fixed-length path). A Jasco FP-750 spectrofluorimeter equipped with an Hamamatsu R928 phototube was used to collect the emission spectrum of the (**WP5/PAH**)₈/levofloxacin films (λ_{exc} = 330 nm).

 $(WP5/PAH)_8$ layer-by-layer assembly. Glass and quartz slides (1×1 cm) were first ultrasonicated in EtOH for 15 min, washed with concentrated nitric acid (69%) for 30 min, copiously rinsed with water and then dried for 24 h at 60 °C. The initial amination step was carried out by soaking the pretreated slides in an aqueous solution of poly(ethyleneimine) (3 mg/mL) for 15 min. Subsequent layers were built by successive and alternate immersions (15 min each) of the PEI-coated slides into aqueous solutions of WP5 (1.3 mg/mL) or PAH (3 mg/mL), with an intermediate rinsing step in ultrapure water in between, until the number of desired bilayers (n = 8) was reached. Antibiotic loading was accomplished by dipping the substrates coated with the (WP5/PAH)₈ film into an aqueous amikacin or levofloxacin solution (10 mM) for 1 h. Samples were washed three times with ultrapure water and then dried under a nitrogen stream.

Determination of the minimum inhibitory concentration (MIC). The MIC is the lowest concentration of an antimicrobial agent able to prevent the growth of a bacterial strain. In order to determine the MIC of amikacin and levofloxacin, cultures of *P. aeruginosa* or *S. aureus*, at a final inoculum of approximately 10^4 – 10^5 bacteria per mL, were cultured with increasing aliquots of amikacin or levofloxacin ranging between 0.25 and 64 µg/mL. The MIC₉₀ –*i.e.* the lowest concentration of a drug required to prevent 90% of microbial growth– of amikacin was found to be 4 µg/mL for both strains, while that of levofloxacin was found to be 2 and 1 µg/mL for *P. aeruginosa* and *S. aureus*, respectively.

Antibacterial activity assessment. *P. aeruginosa* ATCC27853 and *S. aureus* ATCC29213 were grown at 37 °C in a Mueller Hinton broth (MHB) prior to use. For the antimicrobial activity test in solution, bacterial counts were evaluated using standard plating methods. To this end, an overnight culture of *P. aeruginosa* or *S. aureus* was transferred into a fresh MHB and incubated at 37 °C under vigorous shaking (250 rpm) for 3 h. The bacterial culture in exponential growth was diluted at a final inoculum of approximately 10^4 – 10^5 bacteria per mL. Substrates coated with the (**WP5/PAH**)₈/antibiotic film and the unloaded (**WP5/PAH**)₈ film, were placed into a sterile 6-well microplate and

each well was then filled with 4 mL of the bacterial culture in the MHB medium (*P. aeuriginosa* 3.5×10^4 bacteria/mL, *S. aureus* 4.5×10^4 bacteria/mL). 100 µL-aliquots of the bacterial suspension were collected at specific time intervals (2, 4, 6 and 8 h) and then diluted with 900 µL aliquots of a sterile physiological saline solution. 100 µL aliquots of the resulting diluted suspensions were spread on a Mueller Hinton Agar (MHA) plate and incubated overnight at 37 °C.

Microbial growth was evaluated according to the colony-forming unit (CFU) assay, assuming that each colony has originated from a single bacterium. After incubation, the CFU count was calculated on colonies in the 30–300 range, using the following equation:

$$CFU = \frac{number \ of \ colonies}{volume \ (0.1) \ \times \ dilution \ factor} \tag{eq.}$$

CFU values derive from an average of three independent measurements (error within ± 5%).

Bacterial adhesion and proliferation on the surface of the substrates coated either with the (**WP5/PAH**)₈/antibiotic films or the unloaded (**WP5/PAH**)₈ film was assessed using a live/dead BacLight bacterial viability kit as follows: after 8 h of cell culturing, the substrates were treated with a mixture of SYTO® 9 green-fluorescence nucleic acid stain and red-fluorescence dye (propidium iodide), so that bacteria with intact cellular membranes would be green-stained, whereas bacteria with damaged cellular membranes would be red-stained. After 15 min, the samples were visualized under fluorescence microscopy using a Leica DMRE epifluorescence microscope with a Leica C Plan 63× lens, using a BP 515–560 nm excitation filter in combination with a LP 590 nm suppression filter. A quantitative evaluation of the cell adhesion was estimated by using the Scion Image Software (Windows version of the NIH ImageJ Software) in the automated counting (single colour) image mode in terms of percentage of covered area (% A).

Kinetics of levofloxacin release from the (WP5/PAH)₈ **film**. The release of levofloxacin from (**WP5/PAH**)₈ films was assessed spectrophotometrically in a 50 mM phosphate buffer (PBS) using an optic fiber probe. The apparatus consisted of a glass cell containing 20 mL of PBS solution, in which the optic fiber was immersed together with a combined glass electrode (Metrohm, 6.023.100) and the substrate coated with a (**WP5/PAH**)₈/levofloxacin

film. The cell was thermostatted at 25.0±0.1 °C and the solution present inside was magnetically stirred and kept under a nitrogen atmosphere to prevent unwanted redox and acid-base processes caused by the presence of oxygen and carbon dioxide. The glass electrode was calibrated at the same temperature using two buffers at pH = 4.0 and 7.0. The uncertainties associated with the measured quantities (±0.03 for pH, ±0.002 for Abs and ± 0.7 µg for the amount of antibiotic released) were calculated by propagation of the error from the calibration curves.

The concentration of levofloxacin released from the (**WP5/PAH**)₈ film into the PBS solution was monitored by looking at the increase of antibiotic absorbance (λ_{max} = 289 nm) over a period of 8 h. To this end, a previously-determined 4-point calibration curve was used.



Fig. S1 ¹H NMR spectra (300 MHz, 298 K, D₂O) of: a) [**WP5**] = 1.0 mM; b) [**WP5**] = 1.0 mM and [**1**] = 0.5 mM; c) [**WP5**] = [**1**] = 1 mM; d) [**WP5**] = 1 mM and [**1**] = 3 mM and e) [**1**] = 4.0 mM.



Fig. S2 Section of the 2D NOESY spectrum (500 MHz, 298 K, D_2O) of a [WP5] = [1] = 16 mM solution.



Fig. S3 The non-linear curve-fitting of the ¹H NMR titration experiment (300 MHz, 298 K, D_2O) of a 1 mM solution of **WP5** upon varying the concentration of **1** from 0.099 to 3.33 mM.



Fig. S4 The non-linear curve-fitting of the ¹H NMR titration experiment (300 MHz, 298 K, D_2O Na₂HPO₄/NaH₂PO₄ 50 mM, pH 7.2) of a 1 mM buffered solution of **WP5** upon varying the concentration of **1** from 0.099 to 4.12 mM.





Staphylococcus aureus ATCC29213



Fig. S5. Fluorescent images and related percentages of covered area (% A) of *P. aeuriginosa* (top) and *S. aureus* (bottom) cells adhering to: (A) (**WP5/PAH**)₈, (B) (**WP5/PAH**)₈/levofloxacin, (C) (**WP5/PAH**)₈/amikacin.



Fig. S6. Uv-vis spectra of a (**WP5/PAH**)₈ multilayer film before (blue trace) and after 8 h immersion in PBS (50 mM) followed by extensive rinsing in water (red trace).