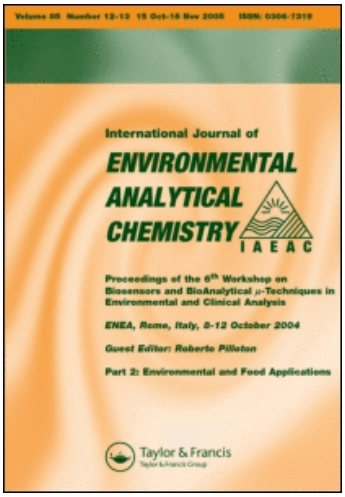

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Sara Morandi[a]; Marta Puggelli[a]; Gabriella Caminati[a]
[a] Dipartimento di Chimica, Universita' di Firenze via della Lastruccia 3, Firenze, Italy

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Phospholipid nanofilms for the detection of Rifaximin in solution

SARA MORANDI*, MARTA PUGGELLI and GABRIELLA CAMINATI

Dipartimento di Chimica, Universita' di Firenze via della Lastruccia 3,
50019 Sesto Fiorentino, Firenze, Italy

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A preliminary study for the realization of a nanofilm-based sensor for the detection of antibiotic residues is reported. The Langmuir–Blodgett (LB) technology is employed for the construction of an ultra-thin immobilization matrix for Rifaximin (Rfx), an antibiotic widely used in veterinary medicine. Dipalmitoyl phosphatidylglycerol is used as an organic building block of the monolayers constituting the nanofilm. Incorporation of Rfx in the film through specific interactions with the phospholipids is evidenced by UV-Vis spectroscopy and electrochemical measurements. The LB film modified surfaces were used with two different detection methods, i.e. spectrophotometry and cyclic voltammetry. Measurements as a function of Rfx concentration showed that it is possible to reveal the presence of Rfx in solution at a concentration as low as 1×10^{-8} M. Our results show that the presence of the LB nanofilm promotes migration of the Rfx molecule towards the detection device inducing a rapid response and a significant increase of the detection limit.

Keywords: Langmuir–Blodgett films; Antibiotic; Nanosensors; Cyclic voltammetry

1. Introduction

Rifaximin is a member of the Ansamycin group, an important family of antibiotic widely used for the treatment of infections caused by aerobic Gram-negative bacteria. These molecules are potent and specific inhibitors of bacterial DNA-dependent RNA polymerase and they have been tested recently as potential inhibitors of HIV RT [1]. The ubiquitous presence of antibiotics in food of animal origin can have serious health consequences on human kind ranging from allergic reactions to the development of antibiotic-resistant bacteria [2]. The demand for fast, reliable and continuous measurements of antibiotics in medicine, biotechnology and environmental science evolved the need for small, easy-to-handle and inexpensive sensor devices.

In particular, Rifaximin (Rfx) has been synthesized [3, 4] and used extensively in veterinary medicine for the treatment of clinical mastitis of lactating dairy cows [5, 6], where extensive use of this antibiotic inevitably causes milk contamination

*Corresponding author. Fax: +39-55-4573385. Email: morandi@csgi.unifi.it

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by Rfx residues. The European Union (EU) set the Maximum Residue Limit (MRL) in milk to 80 μg kg^{-1} ([Rfx] = 1 × 10^{-7} M). In this respect, residue screening tests are important for daily antibiotic control, as well as for preventing antibiotic-contaminated milk from entering the processing cycle of milk products. Current detection methods for rifamycins in pre-treated milk samples [7] include HPLC, thin-layer-chromatography, spectrophotometry and voltammetry [8], but only scarce literature can be found for the detection of Rfx in solution.

The long-term goal of this work is the realization of a specific low-cost, sensitive and easy-to-use screening method for rifaximin using nanofilm technology.

In this work we describe the preliminary steps in the realization of nanofilm-based sensors for the detection of Rfx residues in milk solutions after pre-treatment. The Langmuir–Blodgett (LB) method allows us to construct mono or multilayers of organic molecules on a variety of solid supports. LB technology has been considered as a convenient tool for designing artificial systems with biological functions such as biosensors [9–15]. In this work the LB procedure was used to produce an ultra-thin layer to be used as the immobilization matrix for the Rfx antibiotic. Since rifamycins are known to penetrate by passive diffusion the phospholipids bilayer of the bacterial membrane [16, 17], we selected phospholipid molecules as the immobilization matrix for Rfx. Several phospholipids were tested in a previous work [18], and dipalmitoyl phosphatidylglycerol was found to provide the highest interaction with Rfx molecules.

The resulting LB film was used directly as the sensing surface for coupling with the detection device: two detection methods were employed, i.e. UV-Vis spectroscopy and Cyclic Voltammetry (CV). The direct use of these analytical methods for Rfx in aqueous solution prevents the detection of low concentrations of Rfx as requested by the current MRL. The utilization of nanofilm-based methods appears promising in extending the detection limits of Rfx for these conventional and widespread analytical techniques.

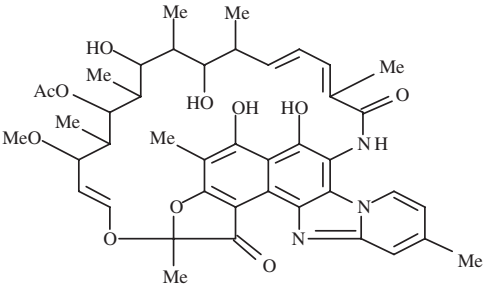
2. Experimental

2.1 *Materials*

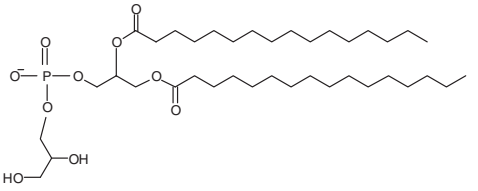
Dipalmitoyl phosphatidylglycerol sodium salt (DPPG-Na, purity > 99%) was supplied by Sigma, Rifaximin (Rfx, purity 99%) was a gift from FATRO-Pharmaceutical Veterinary Industry (Bologna, Italy). The chemical structures of Rfx and DPPG-Na are reported in figure 1.

Chloroform/methanol (10 : 1 v/v) mixtures were used for DPPG-Na spreading solutions, Aldrich (Italy) supplied both solvents. Aqueous solutions containing the antibiotic were prepared dissolving Rfx in water/dimethylsulfoxide (50 : 1 v/v) mixtures, the solubility limit for Rfx in this mixture is around 3 × 10^{-4} M. Dimethylsulfoxide (DMSO) was purchased from Merck (Germany). Water was obtained from a Milli-RO coupled with a Milli-Q set-up (Millipore, resistivity=18.2 MΩ cm^{-1}, pH = 5.6 at 20°C). Phosphate buffer 0.1 M (pH = 7.0,), used for cyclic voltammetry measurements, was prepared using the following products: di-potassium hydrogen phosphate (K_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4), purchased by Fluka.

Glass, chromium evaporated glass, QS quartz (Hellma, Germany) and glassy carbon (GC) slides (Goodfellow, Cambridge) were used as solid supports for LB films.



Rfx



DPPG-Na

Figure 1. Chemical structure of Rfx and DPPG-Na.

GC slides were polished with water dispersed diamond paste (16, 3, 1 μm diameter). All slides were cleaned by immersion in chromic acid and piraña solution and carefully rinsed in water. Rfx solutions for incubation experiments were stored under nitrogen and protected from light; the pH of the solutions was checked as a function of antibiotic concentration and time.

2.2 *Methods*

LB films were prepared using a KSV 3000 film balance apparatus (KSV, Finland). DPPG-Na was spread from chloroform/methanol solution on water subphase ($T = 21 \pm 0.5^{\circ}$C, pH: 5.6). Twenty minutes were allowed for solvent evaporation before monolayer compression. The compression rate was $6\,\text{mm}\,\text{min}^{-1}$. DPPG-Na monolayers were transferred from water–air interface to the solid slides at constant surface pressure, 20 min were allowed for monolayer equilibration at the selected target surface pressure. The dipper rate was $3\,\text{mm}\,\text{min}^{-1}$ for LB transfer onto all solid slides except for LB transfer on the GC slides, where a dipper rate of $4\,\text{mm}\,\text{min}^{-1}$ was used. Incorporation of Rfx in the transferred DPPG-Na layers was achieved incubating the LB films in aqueous solution of the antibiotic for 15 h. The ellipsometric thickness of the LB films of DPPG-Na deposited on chromium evaporated quartz slides was measured with a Nulling Ellipsometer (Rudolph Research Instruments, USA). Contact angles were measured with an *Automated Contact Angle Goniometer* (Ramé-Hart, USA) equipped with an *Auto Pipetting System*. A double beam UV/VIS spectrometer Lambda 900 (Perkin Elmer) was used for the recording of the UV/VIS spectra of Rfx solutions in the region between 200 and 600 nm (slit width = 1 nm, scan speed = $300\,\text{nm}\,\text{min}^{-1}$). UV-Vis spectra of solutions of the antibiotic compound in water were measured in a 1 cm quartz cell using the pure solvent as reference probes. The Rfx concentration range investigated was 5×10^{-7}–$1 \times 10^{-4}\,\text{mol}\,\text{L}^{-1}$.

Absorption spectra of LB films were recorded using 2 nm slits, each spectrum reported in this work was the average of at least 20 scans.

Cyclic voltammograms were recorded using an Autolab Potentiostat/Galvanostat PG STAT 30 (Ecochemie, Netherlands). All the cyclic voltammograms were collected using a scan speed of $10\,mV\,s^{-1}$. We used an electrochemical cell equipped with a platinum counter electrode and an Ag/AgCl reference electrode; the working electrode was either a GC slide coated by LB film (system a) or a LB film free GC slide (system b).

3. Results and discussion

Nano-layers of DPPG-Na were transferred onto a solid support by means of the Langmuir–Blodgett technique. Optimization of the experimental conditions for the transfer of DPPG-Na monolayer from pure water solution was performed on different solid support, i.e. glass, quartz, glassy carbon or chromium evaporated slides. We always selected a transfer surface pressure, $\pi_{tr} = 25\,mN\,m^{-1}$, that was shown in a previous paper to ensure optimal interactions between the phospholipid and Rfx [19]. The transfer ratio [20], which was always found to be close to unity, indicates the complete and homogeneous transfer of a single monolayer under the employed experimental conditions. Contact angle measurements [10] for water on the outer surface of the LB film were found to be in the range $40^\circ < \Theta < 50^\circ$ suggesting a partially hydrophilic and homogeneous coverage. The ellipsometric thickness for the LB films was found to be 11 ± 1 Å, this finding indicates that the DPPG molecules are loosely packed in the transferred monolayer with their aliphatic chains tilted with respect to the normal at the interface.

3.1 *Spectroscopic investigation of Rfx incorporated in Langmuir–Blodgett films*

The antibiotic was allowed to interact with DPPG-Na layers by immersion of the LB film of DPPG-Na in an aqueous solution of the Rfx as described in the experimental part. Incubated samples were prepared for Rfx concentration in the range 5×10^{-7} M to 1×10^{-4} M.

Rifaximin is known to absorb strongly in both the ultraviolet and visible spectral regions [21, 22] due to the presence of several chromophores in the molecule. The electronic absorption spectra of Rfx in solution is reported in figure 2: the UV-Visible spectrum of Rfx in water solution exhibits five main absorption bands located at $\lambda_1 = 260$ nm, $\lambda_2 = 292$ nm, $\lambda_3 = 320$ nm, $\lambda_4 = 370$ nm and a broad band in the range 430–450 nm in agreement with literature data [23].

LB films of DPPG-Na immersed in pure water subphase did not show any absorption bands and were used as background reference in order to subtract the alkyl chains scattering contribution to the absorption spectra. The electronic absorption spectra of 1 LB layer of DPPG-Na incubated in Rfx solution is also reported in figure 2. The spectrum exhibits the same absorption features observed for Rfx in solution: this behaviour evidences clearly that the drug is effectively incorporated in the LB film by means of the incubation procedure.

DPPG-Na LB samples were incubated in solutions containing different amounts of Rfx, the corresponding UV-Vis absorption spectra show the presence of Rfx in the LB system for Rfx concentrations as low as 5×10^{-7} M.

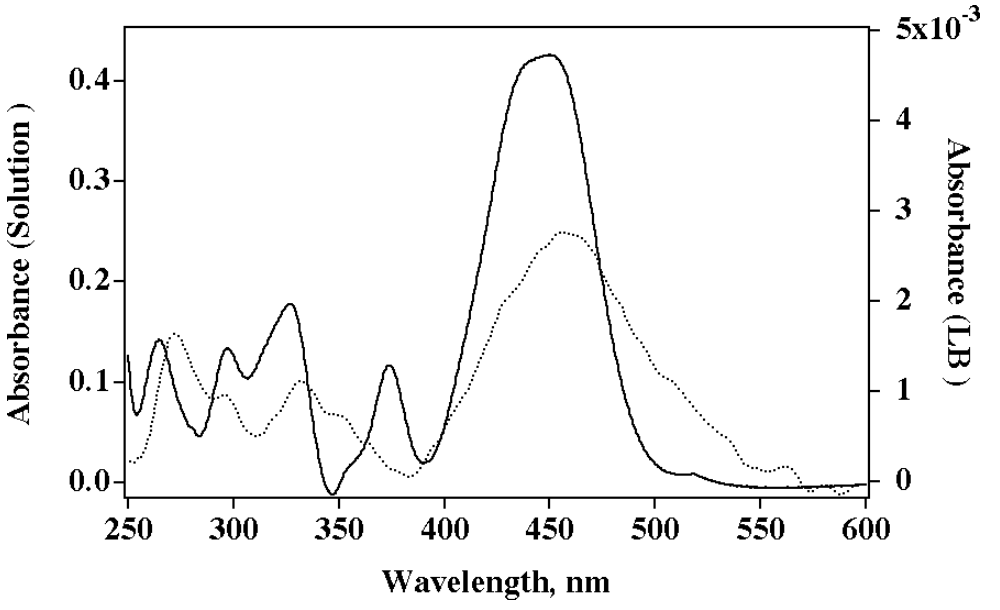


Figure 2. UV-Vis spectra of one LB layer of DPPG-Na (dashed line) incubated in Rfx aqueous solution and UV-Vis spectra of Rfx aqueous solution (solid line) $[\mathrm{Rfx}] = 5 \times 10^{-5}$ M.

The absorbance at $\lambda = 450$ nm as a function of [Rfx] in the incubation solution is reported in figure 3. We can observe a linear behaviour for $[\mathrm{Rfx}] \leq 5 \times 10^{-6}$ M whereas a saturation behaviour is detected at higher concentrations.

3.2 *Cyclic voltammetry of Rfx in solution: effect of the LB film*

Figure 4 shows the cyclic voltammograms obtained for Rfx solutions using LB film covered GC electrodes (system a) as well as LB film free GC electrodes (system b). In the same figure we also report the results obtained for bare GC electrodes in buffer solution. A similar background contribution was also found for LB covered electrodes in the absence of Rfx in the same potential range.

CV measurements in figure 4 evidence oxidation and reduction peaks at $E = 271$ mV and $E = 205$ mV, respectively. We ascribed these peaks to the presence of Rfx in solution. Previous literature [24] on CV characterization of another member of the same ansamycin family, i.e. Rifamycin SV, showed a similar voltammogram with an oxidation potential of 260 mV that the authors ascribed to the naphthohydroquinone–quinone moiety.

The shape of the voltammogram remains unaltered in the whole Rfx concetration range whereas the position of the peaks change for $[\mathrm{Rfx}] > 5 \times 10^{-6}$ M towards higher potential. The same behaviour is found for bare GC electrodes and LB covered electrodes.

The presence of the LB film on the GC electrode induces striking differences in the low Rfx concentration regime: the detection limit of the drug is drastically decreased and higher currents are measured. These results are summarized in figure 5 where the oxidation current is reported as a function of Rfx concentration for both systems.

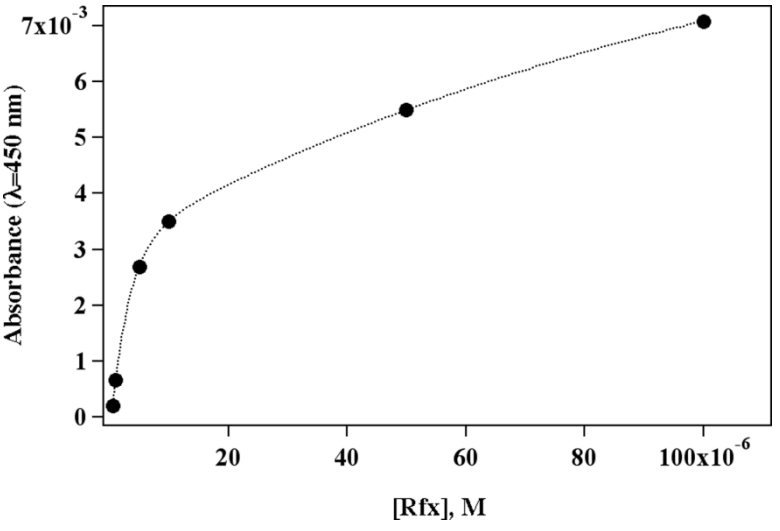


Figure 3. Absorbance at $\lambda = 450$ nm for 1 LB of DPPG-Na as a function of the Rfx concentration in the incubation solution.

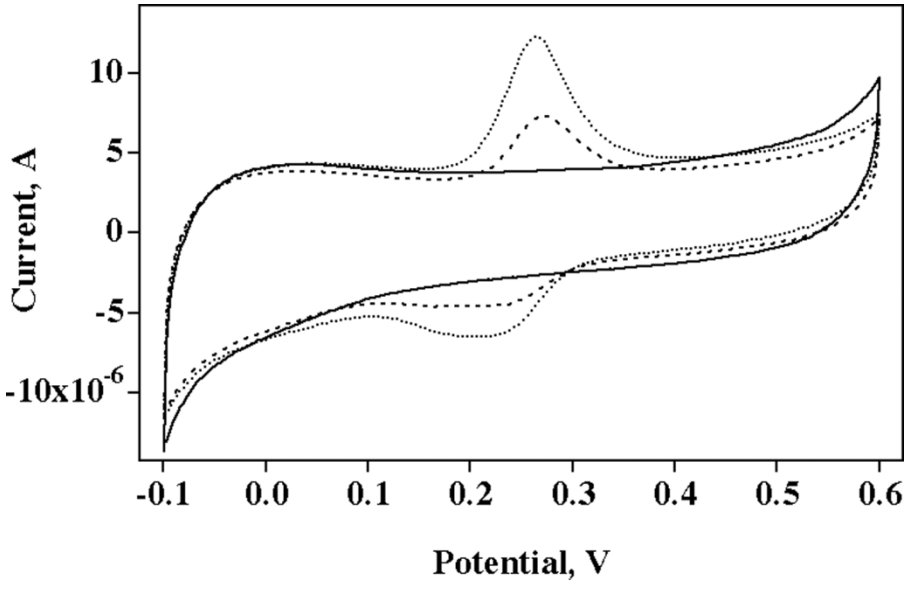


Figure 4. Cyclic voltammograms of system a (dotted line) and system b (dashed line) in $[\mathrm{Rfx}] = 5 \times 10^{-7}$ M buffer solution, the solid line corresponds to LB film covered GC electrodes in buffer solution without Rfx.

For $[\mathrm{Rfx}] = 1 \times 10^{-8}$ M we could not detect any current in the absence of the LB film on the GC electrode. Moreover, the current increases almost one order of magnitude for system a. This result is particularly relevant for sensors application and in fact we recall that the MRL for Rfx in milk is 1×10^{-7} M.

Differences between the two systems vanish in the high concentration regime at $[\mathrm{Rfx}] = 1 \times 10^{-6}$ M, where the two plots almost coincide. This behaviour may be

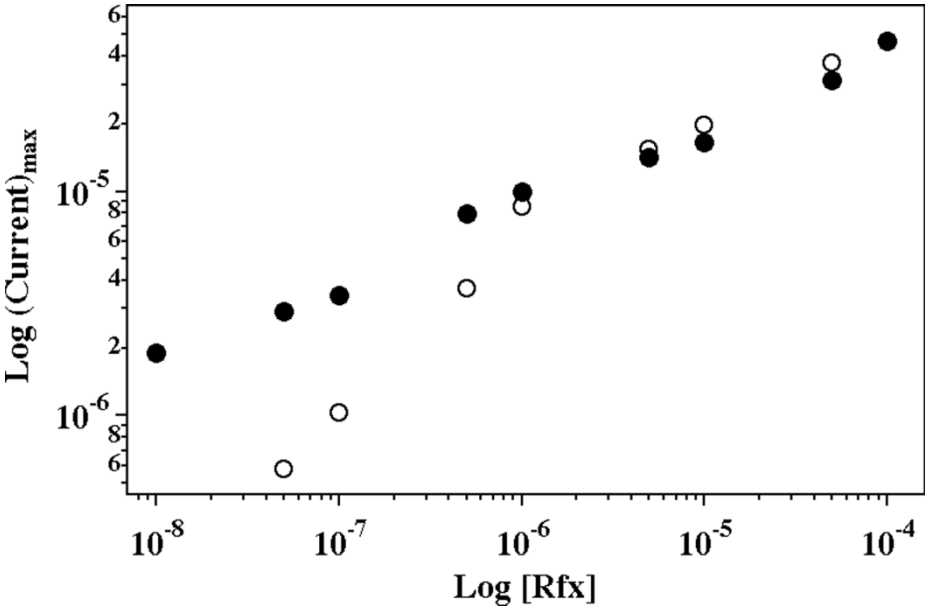


Figure 5. Peak oxidation current as a function of Rfx concentration system a (closed circles) and system b (empty circles).

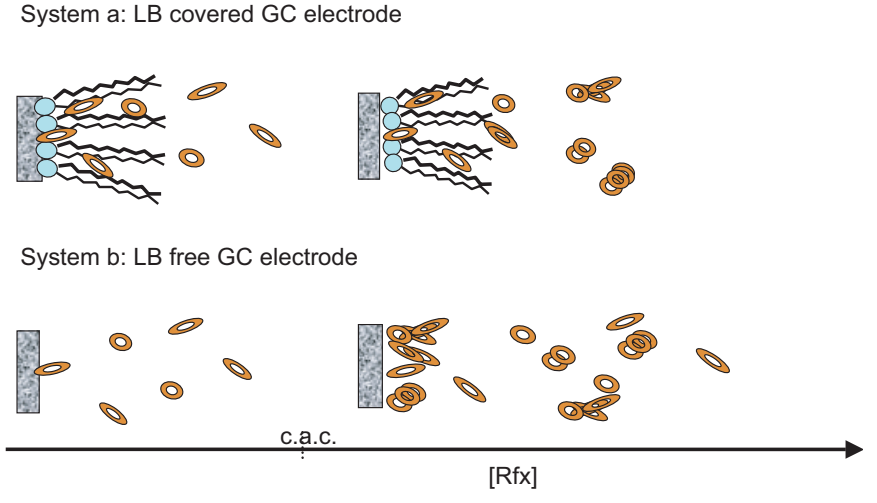


Figure 6. Cartoon depicting the possible mechanism occurring at the electrodes in system a and in system b as a function of Rfx in solution.

explained by the formation of Rfx aggregates in solution already found in previous works by our group [25] and other authors [26]. Concentrations higher than 1×10^{-4} M could not be used due to solubility limits (see section 2).

The rationale for this behaviour is described in the cartoon in figure 6 where we report two limiting cases in the low and high concentration regime for system a and system b.

Rifaximin is incorporated in the LB film that covers the electrodes due to favourable interactions with DPPG-Na matrix [19]. The increased amount of antibiotic near the electrode surface is responsible for the higher current observed. Moreover, the enhanced localization of Rfx in the LB film allows us to detect the presence of Rfx in solution even at very low concentrations.

As the Rfx concentration increases, the molecules start to aggregate due to stacking interactions between the aromatic moieties [26], the critical aggregation concentration (c.a.c) was previously found to be $[Rfx] = 8 \times 10^{-6}$ M by means of surface tension measurements [25]. Whereas the aggregates freely diffuse to the bare GC electrode surface, the presence of the LB film hinders such diffusion.

4. Conclusions

We successfully realized nanofilms consisting of a single monolayer of DPPG-Na on solid support that can be coupled to a detection device for the determination of Rfx, a representative member of the large Rifamycin family.

UV-Vis spectroscopy showed that Rfx interacts with the phopsholipid nanofilm and is included in the LB film upon a simple incubation procedure.

The migration of Rfx towards the LB film of DPPG-Na is further demonstrated by electrochemical experiments as a function of antibiotic concentration.

Whereas UV-Vis spectroscopy cannot be used as a detection method for Rfx concentration lower than 5×10^{-7} M, we found that the use of LB film covered glassy carbon electrodes in CV allowed us to obtain a higher current signal and therefore to easily detect the extremely low concentration required by current legislation.

These results can be explained by considering that the presence of 1 LB layer on the GC electrode enhances the migration of the antibiotic at the electrode surface allowing to reach the MRL detection limit for Rfx.

Acknowledgements

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