Analytica Chimica Acta 884 (2015) 90–96

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Label-free quantification of Tacrolimus in biological samples by atomic force microscopy

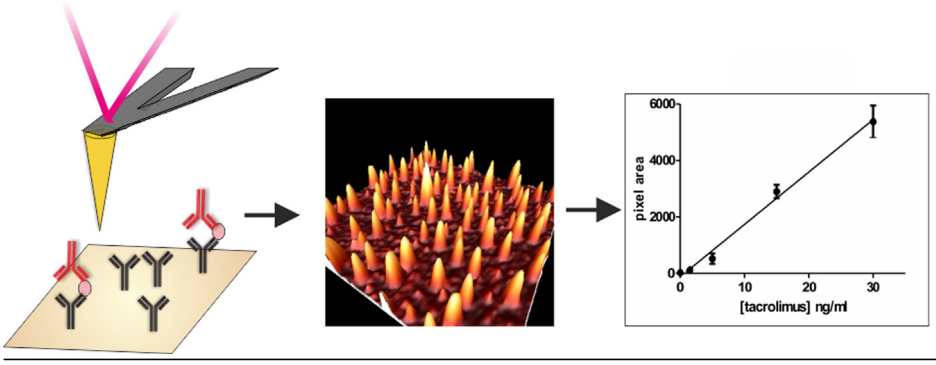
Michele Menotta [a,*], Sara Biagiotti [a], Laura Streppa [b,c], Luigia Rossi [a], Mauro Magnani [a]

[a] Department of Biomolecular Sciences, University of Urbino "Carlo Bo" via Saffi 2, Urbino, Italy
[b] Physics Laboratory, CNRS-ENS, UMR 5672, Lyon, France
[c] Cell and Molecular Biology Laboratory, CNRS-ENS Lyon, UMR 5239, IFR128, Lyon, France

HIGHLIGHTS

- Tacrolimus is a potent immunosuppressant drug that has to be continually monitored.
- We present an atomic force microscope approach for quantification of Tacrolimus in blood samples.
- Detection and quantification have been successfully achieved.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 18 February 2015
Received in revised form 5 May 2015
Accepted 7 May 2015
Available online 12 May 2015

Keywords:
Atomic force microscopy
Tacrolimus
FK506
Label free

ABSTRACT

In the present paper we describe an atomic force microscopy (AFM)-based method for the quantitative analysis of FK506 (Tacrolimus) in whole blood (WB) samples. Current reference methods used to quantify this immunosuppressive drug are based on mass spectrometry. In addition, an immunoenzymatic assay (ELISA) has been developed and is widely used in clinic, even though it shows a small but consistent overestimation of the actual drug concentration when compared with the mass spectrometry method. The AFM biosensor presented herein utilises the endogen drug receptor, FKBP12, to quantify Tacrolimus levels. The biosensor was first assayed to detect the free drug in solution, and subsequently used for the detection of Tacrolimus in blood samples. The sensor was suitable to generate a dose–response curve in the full range of clinical drug monitoring. A comparison with the clinically tested ELISA assay is also reported.

1. Introduction

Tacrolimus (also known as FK506) is a natural compound [1] with immunosuppressive activity that has improved the outcome of organ transplantation. Unfortunately, like other immunosuppressants, it has high pharmacokinetic variability, poor bioavailability and high toxicity. Thus, routine monitoring of Tacrolimus levels in the blood is necessary to minimize adverse side effects and to ensure effective immunosuppression [2,3]. Several assays have been developed for the quantification of the drug in biological samples. The most common methods for routine FK506 monitoring are monoclonal antibody-based immunoassays, while liquid chromatography-tandem mass spectrometry (LC–MS) is currently considered as the reference

* Corresponding author at: Department of Biomolecular Sciences, University of Urbino "Carlo Bo" via Saffi 2, 61029 Urbino (PU), Italy. Tel.: +39 722 305232; fax: +39 722 305324.
E-mail address: michele.menotta@uniurb.it (M. Menotta).

http://dx.doi.org/10.1016/j.aca.2015.05.014
0003-2670/

method [4–7]. LC–MS provides the most specific routine drug measurement, whereas immunoassays show a notable cross-reactivity with inactive metabolites [4,8], especially MIII and MV [9]. Nevertheless, immunoassays are widely and routinely used in the clinic because of their speed and the lack of mass spectrometric equipment in the clinic.

The molecular mechanism of the drug is mediated by its binding to FKBP12, a small ubiquitous protein belonging to the immunophilin superfamily. *In situ*, the drug binds immunophilin and the resulting drug–protein duplex forms a multi-protein complex, composed of FKBP12, Tacrolimus, calcineurine A and B subunits, and calmodulin, which inhibits the phosphatase activity of calcineurine. Enzyme inhibition suppresses de-phosphorylation of NF-AT, thus impeding its translocation into the nucleus and the transcription of the most characterized IL-2 target gene [10]. In this paper, we propose an immuno-AFM method for the quantification of Tacrolimus involving the capture of the drug on the mica surface using monoclonal antibodies. Subsequently, the detection and quantification of the captured molecule is carried out by its binding with the immunophilin FKBP12 (endogenous receptor of FK506). As reported by Murthy et al., assays using naturally occurring binding to immunophilins appear to provide a whole blood Tacrolimus concentration closer to the immunosuppressive activity found in the patient's blood [9]. To the best of our knowledge, the present work is the first of its kind to use AFM to quantify the amount of a drug in biological samples.

2. Experimental

2.1. Chemicals and reagents

FK506 (Tacrolimus) powder was obtained from LC Labs (MA, USA). A stock solution was prepared in 5 mg/ml acetonitrile and stored at −70 °C. Anti-Tacrolimus (anti-FK506) IgG from rabbit was purchased from Santa Cruz Biotechnology (California, USA), while Anti-FKBP12 from rabbit was purchased from Abnova GmbH (Germany). Mica was purchased from Novascan Technologies, Inc. (Ames, Iowa, USA). The recombinant protein FKBP12 was already available in our lab where it had been characterized [11]. All other reagents and chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA). An ultrapure water (18 MΩ cm, Millipore, MA USA) source was used in all of the experiments.

2.2. Dot immunoblotting

Recombinant FKBP12 was spotted in duplicate in two strips of nitrocellulose membrane (Bio-Rad CA USA) by dot-blot apparatus (Bio-Rad), in increasing quantities (0.2, 0.5, 1, and 2 μg/well). Subsequently, the nitrocellulose was blocked in a TBS (Tris-buffered saline) pH 7.4 solution with 5% non-fat dry milk for 1 h. One strip was then incubated with a solution of 25 μg/ml Tacrolimus in TBS for 30 min at 37 °C, while the control strip was incubated in TBS alone in the same conditions. After several washes in TBS, the two membranes were incubated with 1 μg/ml of the primary antibody anti-FK506 in 5% non-fat dry milk/TBS for 12 h at 4 °C and then with the secondary antibody (horseradish-peroxidase-conjugate goat anti-mouse; BioRad) 1/5000 in 5% non-fat dry milk/TTBS (Tween 20 0.1% in TBS) for 1 h at room temperature. After three additional washes in TTBS, the immunoreactive signals were detected by ECL kit (GE healthcare UK) following the manufacturer's instructions.

2.3. AFM surface preparation

Freshly cleaved mica surfaces, 0.5 × 0.5 cm in size, were incubated in a water solution containing 2 μg/ml of anti-FK506 for 30 s and then immediately washed and incubated in PBS solution for 30 min in order to block the charged surface. The surfaces were then used for drug capture from samples.

2.4. FK506 capture and detection

The drug capture was performed incubating the modified micas with solutions containing the drug. In a preliminary test, PBS spiked with 20 ng/ml of FK506 was tested.

A negative control was prepared in the same manner as a sample tester, with the exception of the drug capture step, which was substituted by incubation of the mica with PBS only.

Samples used to achieve dose–response curves of Tacrolimus from a WB matrix were obtained from the calibration standards contained in the ELISA kit Pro-Trac™ II (Tacrolimus ELISA kit, Diasorin). The obtained concentrations were 0, 1.5, 5, 15, and 30 ng of FK506 per ml of blood. The drug was extracted by a solid phase extraction (SPE) procedure. In brief, 1 ml of blood for each sample was subjected to lysis and de-proteinization with an aqueous $ZnSO_4$ solution and acetone as reported by Baldelli et al. [12]. The cleared supernatants were then loaded onto Isolute Cl8 cartridges (International Sorbent Technology, Tucson, Arizona, USA) and washed once with 50% methanol aqueous solution containing 0.1% formic acid and three times with 30% acetonitrile aqueous solution. The elution of FK506 was performed with pure acetonitrile. After acetonitrile evaporation, the pellets were dissolved in 5 μl of acetonitrile and the volume (1 ml) was then reconstituted to the original sample concentration by the addition of TBS. After incubation with the samples (1 h at 37 °C), all micas were washed twice with TBS containing 0.05% tween 20, and then 3 times with TBS. Drug detection was performed by adding FKBP12, previously immunocomplexed with anti-FKBP12 antibodies in a solution containing 100 mM 4-(2-hydroxyethyl)-1)-piperazineethanesulfonic acid (HEPES) pH 7.4 in a molar ratio of 2–1. By the last step FKBP12 (less than one nm height) had been derivatized to a 3–5 nm high molecule. The final concentration of the FKBP12/anti-FKBP12 complex was 1 μg/ml of FKBP12, and the incubation with micas was performed in TBS for 1 h at RT. The substrates were subsequently washed in TBS and then dried by nitrogen flow and imaged.

2.5. Atomic force microscopy

The XE-100 Atomic Force Microscope (AFM; PARK Systems Inc., Suwon, South Korea) was used in this study. The AFM was equipped with a 50 μm scanner controlled by the XEP 1.8 software. The instrument was set in true non-contact mode, with X–Y stage in a closed loop, while the Zscanner was set in closed loop and low voltage mode. The speed scan was set between 0.5 μm/s and 1.5 μm/s. The Zscanner resolution was set to 0.9 Å. The cantilevers used in this study were NCHR tips with a nominal spring constant of 42 N/m and a typical resonant frequency between 200 and 300 kHz.

2.6. Data analysis

The topographic images of all the experiments were processed in the same manner. Briefly, each image was flattened by a first order regression procedure to revise the slope of the sample. No other flattening was necessary since the instrumentation is capable of generating accurate images. The lower pixel intensity in the image was then set to 0 nm and

used as a reference for the relative height of the molecules layered in the mica surface. The average height of each imaged surface was calculated by XEI software (PARK Systems Inc.), and all the other topographic parameters were calculated by the region function. Images obtained collecting the phase signals were processed by filtering (mean method, kernel 3×3) in order to reduce the signal noise. The phase parameters were obtained by XEI, using the region analysis function. The processed images were further analysed to characterize the layered particles. For this purpose, a mask corresponding to a cut-off of 2 nm was generated by Gwyddion [13] and used as the input file for the imageJ [14] particle analysis task, allowing us to count and calculate the surface (in pixels) of the masked particles for each topographic image. All the generated data were used to make the calibration curve graphs.

2.7. ELISA assay

The ELISA assay was performed by using the Pro-Trac™ II Tacrolimus kit (Diasorin) following the manufacturer's instructions. The calibration curve was generated from sample standards enclosed in the kit and subsequently applied to indirectly quantify the samples tested by the AFM biosensor.

3. Results and discussion

Our newly developed detection method is based on simultaneous FKBP12/Tacrolimus/anti-FK506 interaction. The likelihood of the above-mentioned event was verified by dot-blot immune-assay. The amount of detected drug (Fig. 1) on the membrane was directly proportional to the amount of deposited FKBP12; thus, the concurrent binding among the three molecules was actually possible. This finding suggests that the portion of the drug bound in the hydrophobic pocket [15,16] of FKBP12 was different from the part recognized by the anti-FK506 antibody. The aforementioned occurrence ideally led to increasing nanosensor specificity, since both events have to take place in order to properly detect the drug. Metabolites lacking one of the two binding domains should not be detected by the proposed detection system; therefore, the overestimation problem associated with other immune-based assays should be avoided or, at least, notably reduced as described by Wei et al. [17].

The detection methodology proposed herein consists in the capture of the anti-FK506 drug, which was subsequently recognized by a pre-assembled FKBP12/antiFKBP12complex (Fig. 2). The last step allowed us to obtain a large molecular complex, easily recognizable by AFM.

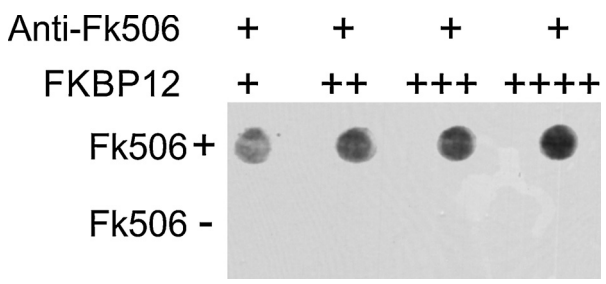


Fig. 1. Dot-blot assay performed to ascertain the simultaneous interaction among FKBP12, Tacrolimus and anti-FK506. In the dots, increasing amounts of FKBP12 were bound to nitrocellulose. After incubation with FK506 or no incubation, the detection was performed by anti-FK506. The immune reactive signal allowed us to positively verify that the concurrent binding among the three molecules was actually possible and unambiguous.

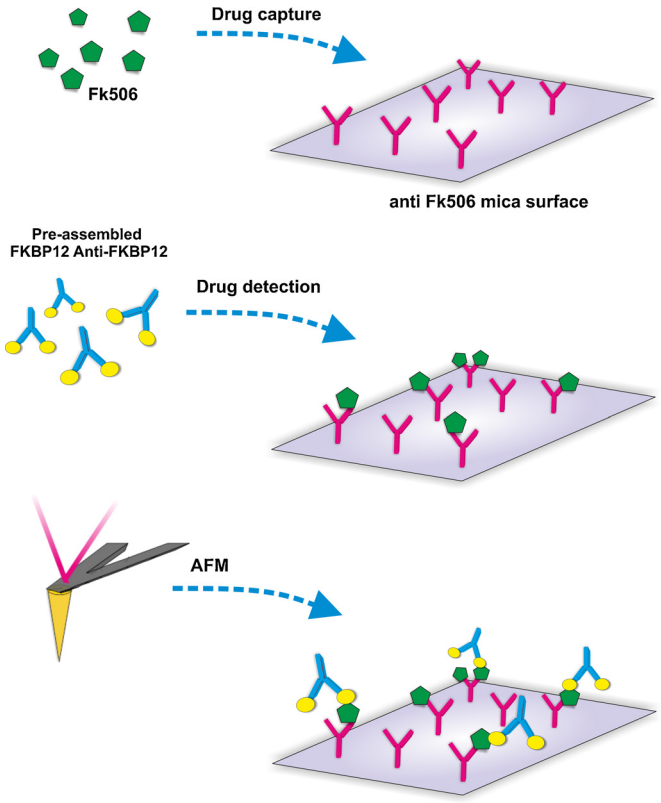


Fig. 2. Schematic of the adopted strategy for the Tacrolimus assay. The first step consisted in mica functionalization with the anti-FK506 antibody. The modified micas were subsequently incubated with the drug extracted from biological samples. The detection was performed by adding FKBP12, previously complex with anti-FKBP12 (the last step increases the FKBP12 magnitude).

Fig. 3a shows a typical surface image of the layered antibody obtained from a preliminary test of the adopted strategy. Over the surface, the particles ranged in height between 1 and 2 nm, like the free-deposited antibody. The mean roughness Ra value of the various analysed surfaces was 0.17 nm and RMS was 0.30 nm. In Fig. 3b the same surface after incubation with Tacrolimus (20 ng/ml) is shown. The drug mass is irrelevant to allow a detectable variation of the surface, and the addition of the only FKBP12 (Fig. 3c) did not enable us to generate a variance easily recordable by AFM. Fig. 3d shows a 20 ng/ml treated mica surface after incubation with anti-FKBP12 alone. No particle magnitude variation was observable. On the contrary, Fig. 3e shows the 20 ng/ml treated mica surface after incubation with the complex anti-FKBP12/FKBP12. Higher particles were easily recognizable and measurable.

The heights of the particles ranged from 1.5 (free antibody on the surface) to 6.3 nm (highest likely generable complex); roughness Ra and RMS were 0.52 nm and 0.83 nm respectively.

Because of the above-mentioned results, the descriptors used to quantify the recognition events for all subsequent tests consisted in the average heights of the analysed surfaces. In addition, the roughness was considered as a potential descriptor for the quantification of the drug, since it changed in the same manner as the average height factor. A further adopted quantification strategy was the estimation of the number of particles representing the anti-FKBP12/FKBP12 complex (expressed as counts per 4 square micrometres [18]), and the area (expressed as pixels) of the counted particles.

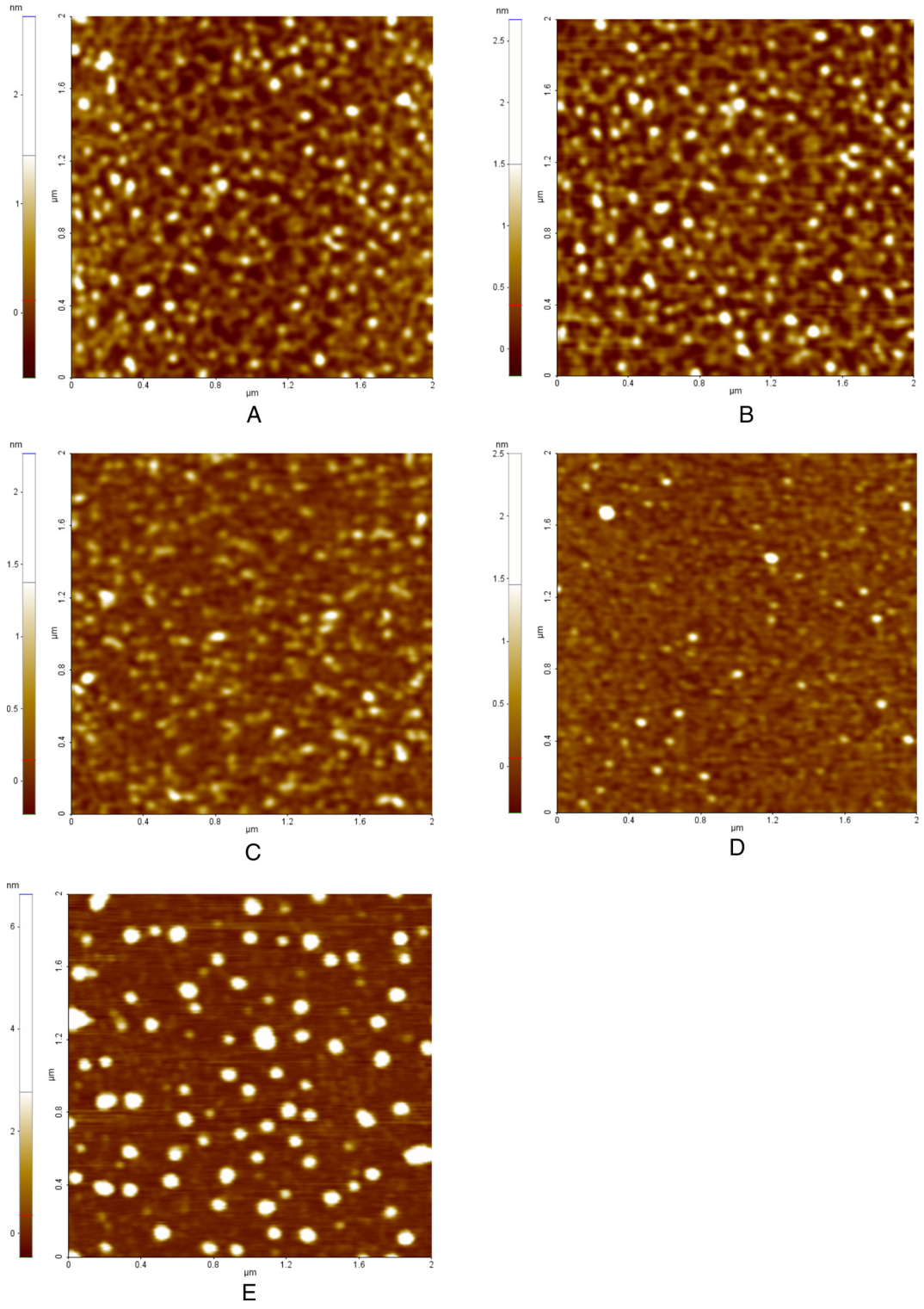


Fig. 3. Tacrolimus capturing from PBS spiked samples. Representative images of the layered antibodies anti-FK-506 before (A) and after Tacrolimus addition (B). In (C) a similar surface after the incubation with FKBP12, or with anti-FKBP12 (D) or with the complex FKBP12/anti-FKBP12 (E). The average height and roughness values of the topography signal of the surfaces increased consistently when performing the last step of the assay.

The quantification capability, the linearity and sensitivity of the developed methodology was tested by setting up calibration curves obtained by drug extraction from WB matrix samples spiked with increasing amounts of Tacrolimus (see material and methods). The narrow therapeutic range of the drug is comprised between 5 and 20 ng/ml [19,20] of blood, the tested Tacrolimus concentrations in blood were: 0 ng/ml (negative control of the assay), 1.5 ng/ml, 5 ng/ml, 15 ng/ml and 30 ng/ml. After drug extraction, the resulting suspensions were immediately incubated with the modified mica surfaces. All inferred data were obtained

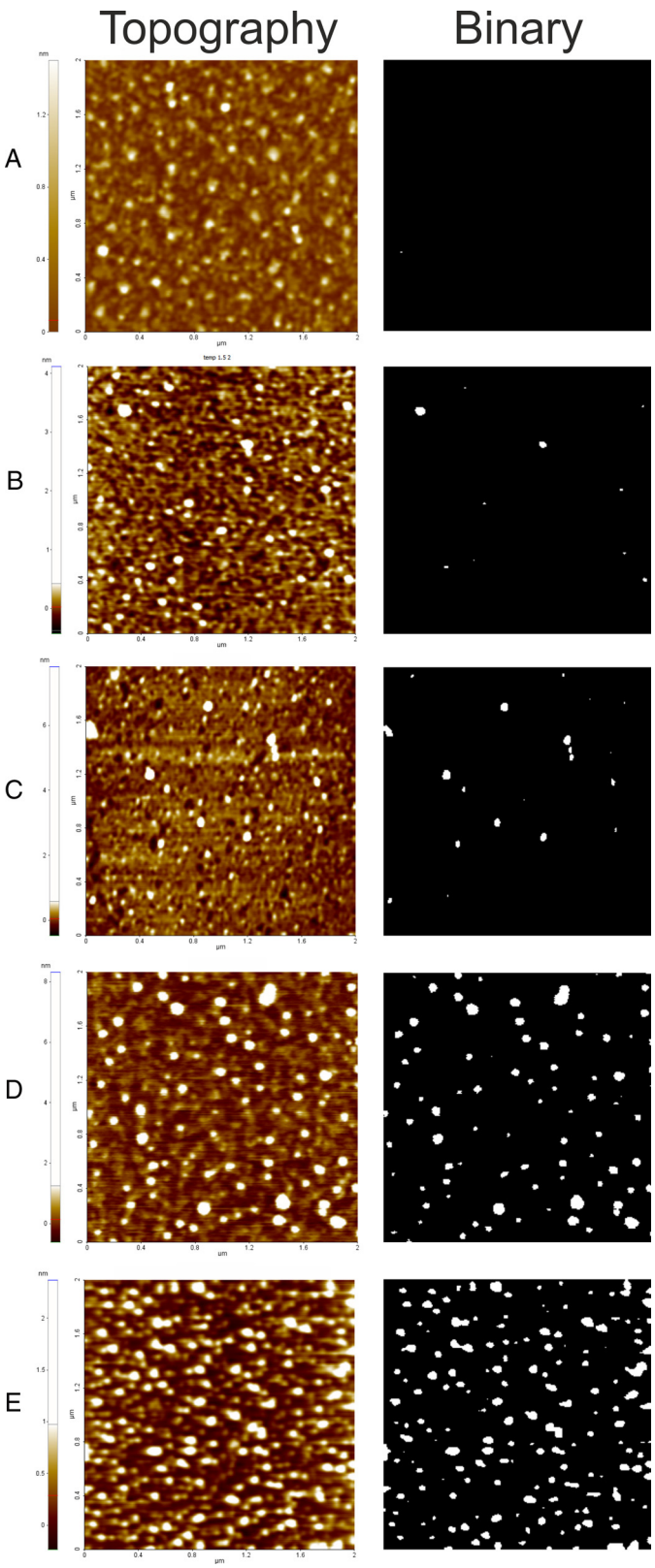


Fig. 4. Typical surface images obtained performing the assay on blood samples, in order to obtain a standard curve. In (A) the negative control of the test (blood), from (B) to (E), images referred to increasing quantities of Tacrolimus spiked blood samples are reported. In the binary column, the masks obtained by software, applying a height cut-off of 2 nm, subsequently employed in the ImageJ particle analysis procedure.

from experiments performed in triplicate and independently repeated three times ($n = 9$). In addition, independent and repeated tests were performed in order to ascertain the reproducibility of the method.

The representative images of the analysed surfaces are reported in Fig. 4a–e. In addition to the topographic pics, the masks used as input for particle analysis are also shown (cut-off 2 nm). Fig. 4a shows the negative control of the assay (unspiked blood) and Fig. 4b–e show the micas corresponding to 1.5 ng/ml, 5 ng/ml, 15 ng/ml and 30 ng/ml, respectively.

The first descriptor used was the average height of the topographic images, previously processed as described in material and methods. For the average height dataset, the values were 0.92 ± 0.02 nm for the control samples and 1.11 ± 0.05 nm, 1.21 ± 0.03 nm, and 1.43 ± 0.06 nm for the assayed drug concentration of 1.5 ng/ml, 5 ng/ml, and 15 ng/ml, respectively. Remarkably, the micas incubated with 30 ng/ml sample had a mean value which was significantly lower than all the previously reported values. In fact, the average height for the above-mentioned sample was equal to 0.82 ± 0.05 nm (Fig. 5a). This event was probably due to surface saturation that might occur at this drug concentration. By setting the lowest pixel as zero, the calculation was probably not related to the actual mica surface, but it was assigned to the lowest intensity pixel of the image belonging to some of the layered molecules. As a consequence, the relative calculated heights were incorrect. In addition, analyzing the topographic roughness Ra, the same dose/response curve was obtained, and the same problem emerged (data not shown). Taken together, these two results pointed to the occurrence of a crowding circumstance.

In order to avoid this saturation problem, and to confirm that saturation actually took place, we simply diluted the last concentration tested to a theoretical value of 15 ng/ml. After the image analysis and values update, the entire dataset was used to fit a non-linear regression ligand/receptor one-site binding curve (Fig. 5b). The fitting resulted in a ligand-receptor binding curve with a Kd of 1.29 nm (no biological significant is to be considered) and the R^2 resulted equal to 0.996. To ascertain that saturation did not occur at the upper limit of the therapeutic range (i.e., 20 ng/ml), samples at that concentration were tested, and the quantification, merged to the previously constructed dose–response graph, is shown in Fig. 5b (see the asterisk). It lies on the obtained standard curve, showing that the nanosensor is suitable for the whole range of drug concentration measurements.

The topographical roughness of Ra sample surfaces was also considered. Once again, the Ra increased in a concentration dependent manner in the range of 1.5–15 ng/ml, but suddenly decreased in the 30 ng/ml sample. However, as previously described, by diluting this last sample we were able to obtain a dose–response curve (Fig. 5c). Once again, the concentration equal to 20 ng/ml (upper limit of the therapeutic range) was tested in order to verify the saturation problem. As regards average height values, the Ra variable was not affected by overload at the 20 ng/ml sample concentration (Fig. 5c, asterisk).

The proposed biosensor was found to be suitable for the detection of Tacrolimus concentrations falling in the therapeutic range (5–20 ng/ml). However, over the 20 ng/ml limit, the sensor showed surface soaking. Hence, other surface descriptors were tested to overcome this limit and improve the quantification range to at least 30 ng/ml of Tacrolimus concentration.

The phase signal of the images was suitable for this purpose. In fact, the phase signal was not used as a Z feedback driving source, thus rendering it almost completely dependent on the physical properties of the scanned surface, and virtually, topographically independent. Even if its behaviour is tip dependent, using the phase roughness information (both Ra and RMS) extrapolated using the phase images, two linear dose–response charts were

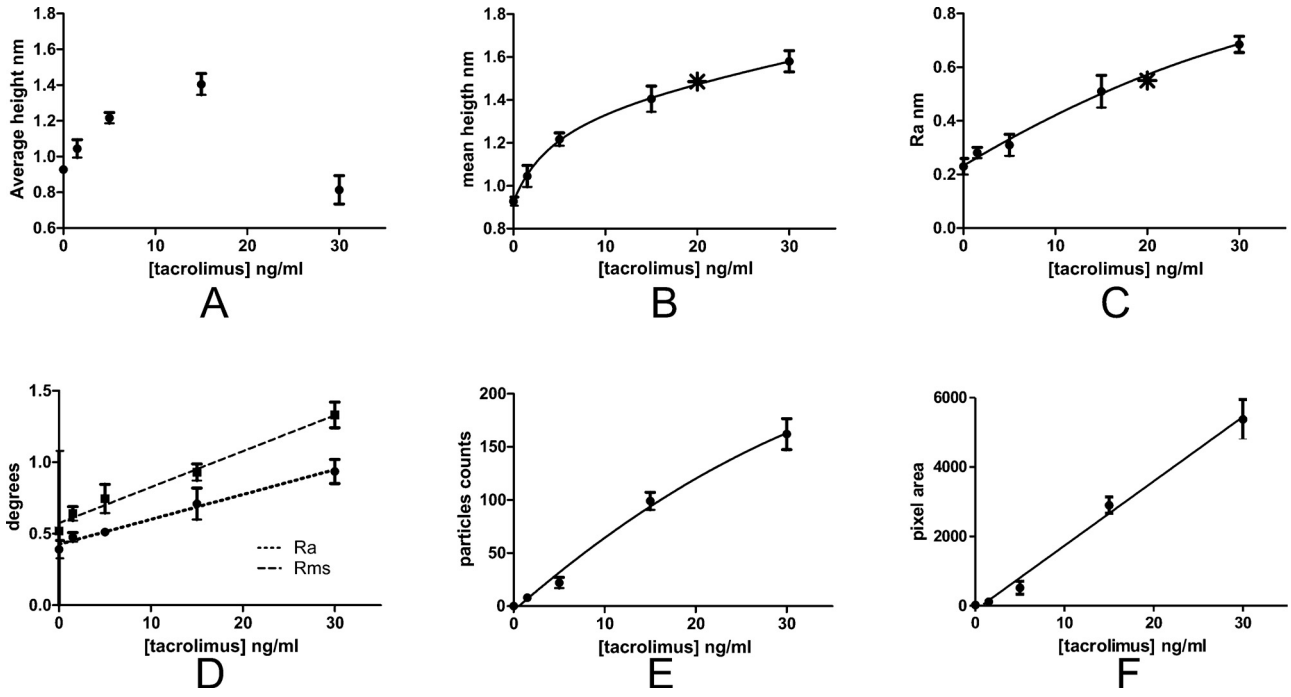


Fig. 5. Tacrolimus quantification charts. In (A) the plot of average height of the imaged surfaces at different Tacrolimus concentrations recovered from blood samples. The point corresponding to 30 ng/ml of Tacrolimus concentration could not be correctly quantified. In (B) the sample corresponding to 30 ng/ml of Tacrolimus was diluted (see text). After resampling and data adjustment, it was possible to fit a non-linear regression ligand/receptor one-site binding curve ($R^2 = 0.996$). The asterisk represents the quantification of a sample corresponding to the upper limit of the therapeutic window. In (C) the topography Ra roughness values of the signals used in (B), were used to draw a non-linear quantification curve ($R^2 = 0.98$). Also in this image, the asterisk shows the quantification of a sample corresponding to the upper limit of the therapeutic window. In (D) the phase signal was employed as a descriptor for the Tacrolimus quantification. The parameters used were the Ra and the RMS signal phase roughness. The regression fit was also appropriate for the 30 ng/ml sample bypassing the overload issue (see text). The coefficient of determination R^2 was 0.99 for Ra regression and 0.98 for the RMS fitted curve respectively. In (E) the regression analysis of the standard curve achieved by the particle count analysis of the images, the obtained non-linear curve showed a coefficient of determination R^2 of 0.99. Calculating the area of the particles as a function of the concentration of Tacrolimus, it was possible to linearize the standard curve (F) obtaining an R^2 of 0.99. Both the analyses carried out on particles made it possible to cover the full therapeutic range.

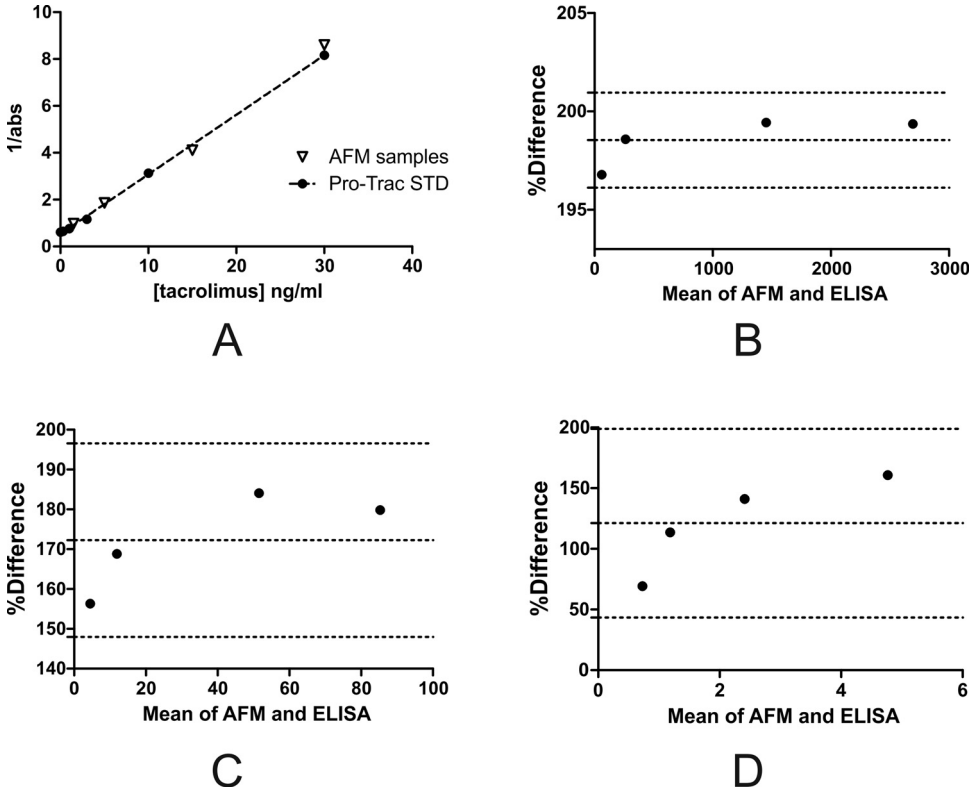


Fig. 6. Comparison between the ELISA assay and AFM method. In (A) dose– response curve of the ELISA standards and the indirect quantification of the AFM tested samples. In (B–D) the Bland–Altman plots from the comparison of the AFM assay (pixel area curve, counts curve and phase roughness curve, respectively) to the ELISA assay. A bias line and 95% limits of agreement are shown.

obtained (Fig. 5d). The regression fit also proved suitable for the 30 ng/ml sample bypassing the overload issue. The determination coefficient R^2 was 0.99 for Ra regression and 0.98 for the RMS fitted curve respectively.

Even though the roughness of the phase signal proved to be suitable for the linear quantification of Tacrolimus from blood samples, we proposed an additional quantification approach based on particle counting and analysis. We observed that even though the average height data of the 30 ng/ml micas were anomalous, the number of particles larger than the antibody alone behaved properly. Through automatic particle counting, expressed as particles per surface unit, we were able to draw a second-order calibration curve that performed correctly without the overload complications, and the R^2 index resulted equal to 0.9929 (Fig. 5e). Furthermore, the pixel area per surface unit of the drawn particles was used to trace a second linear regression curve that fitted appropriately (R^2 index equal to 99.24) for the estimation of Tacrolimus quantity (Fig. 5f).

Finally, the samples that had been prepared to evaluate the AFM biosensor were assayed by ELISA (Fig. 6a), and they were correctly quantified. The ELISA method was compared with the AFM method using the obtained data to compute a Bland–Altman plot for each linear dose–response curve previously obtained by the AFM assay (i.e., the pixel area curve, the particle counts curve and the phase roughness curve). The plots are reported in Fig. 6b–d, and the bias line and the points dispersion are in the 95% limits of agreement. Hence, our results show that the AFM assay yielded Tacrolimus drug concentrations similar to the ELISA values in the WB matrix across an assay range of 0–30 ng/mL.

4. Conclusion

This newly developed AFM biosensor is a proof of concept for Tacrolimus detection by AFM. The improvement production of this laboratory research platform by large-scale manufacturing, devices and samples scale down, together with the next generation-automated bench AFM might permit a rapid, highly sensitive quantification of Tacrolimus, with no need for large equipment or facilities. The simultaneous binding of two molecular species is necessary to perform the detection of Tacrolimus, and it should make it possible to improve the specificity of the proposed nanosensor and may limit or avoid the overestimation problem of the other immune-based assays.

The comparison of our method with a commercial ELISA kit showed the validity of the AFM assay for the full therapeutic range.

In the setup of the nanosensor, we used an “in home” antibody to improve the detection of the reporter-protein FKBP12, since it is only a few nanometres higher than the bait-antibody antiFK506 layered on the mica and therefore difficult reveal by topographic differences. The use of a secondary antibody will fine-tune the detection of the complexes. Indeed, FKBP12 could be coupled with several other molecules or different kinds of particles (i.e., recombinant proteins, nanoparticles or magnetic particles, suitable to perform the detection also using a different setup).

We have shown that various AFM generated data can be used to perform accurate Tacrolimus detection and quantification. The proposed method can obviously be extended to other kind of drugs whose biological receptors are known and can be used as a reporter system for the detection by AFM or other stand-alone instruments. Finally, our investigation showed, for the first time, the use of AFM to quantify the amount of a drug in biological samples.

References

[1] T. Kino, H. Hatanaka, S. Miyata, N. Inamura, M. Nishiyama, T. Yajima, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, FK-506, a novel immunosuppressant isolated from a Streptomyces. II. Immunosuppressive effect of FK-506 in vitro, J. Antibiot. (Tokyo) 40 (1987) 1256–1265.
[2] B.D. Kahan, P. Keown, G.A. Levy, A. Johnston, Therapeutic drug monitoring of immunosuppressant drugs in clinical practice, Clin. Ther. 24 (2002) 330–350.
[3] W.J. Jusko, A.W. Thomson, J. Fung, P. McMaster, S.H. Wong, E. Zylber-Katz, U. Christians, M. Winkler, W.E. Fitzsimmons, R. Lieberman, Consensus document: therapeutic monitoring of tacrolimus (FK-506), Ther. Drug Monit. 17 (1995) 606–614.
[4] E. Ventura, A. Bonardet, G.P. Pageaux, G. Mourad, J.P. Cristol, Calcineurin inhibitor determination in whole blood with the RXL dimension analyzer: a useful tool for immunosuppressive drug monitoring, Transplant. Proc. 41 (2009) 707–709.
[5] M. Karapirli, M. Kizilgun, O. Yesilyurt, H. Gul, Z.I. Kunak, E.O. Akgul, E. Macit, T. Cayci, K.Y. Gulcan, I. Aydin, H. Yaren, M. Seyrek, E. Cakir, H. Yaman, Simultaneous determination of cyclosporine A, tacrolimus, sirolimus, and everolimus in whole-blood samples by LC-MS/MS, ScientificWorldJournal 2012 (2012) 571201.
[6] Q. Li, D. Cao, Y. Huang, H. Xu, C. Yu, Z. Li, Development and validation of a sensitive LC-MS/MS method for determination of tacrolimus on dried blood spots, Biomed. Chromatogr. 27 (2013) 327–334.
[7] B.D. Noll, J.K. Coller, A.A. Somogyi, R.G. Morris, G.R. Russ, D.A. Hesselink, G.T. Van, B.C. Sallustio, Validation of an LC-MS/MS method to measure tacrolimus in rat kidney and liver tissue and its application to human kidney biopsies, Ther. Drug Monit. 35 (2013) 617–623.
[8] Y.W. Lee, Comparison between ultra-performance liquid chromatography with tandem mass spectrometry and a chemiluminescence immunoassay in the determination of cyclosporin A and tacrolimus levels in whole blood, Exp. Ther. Med. 6 (2013) 1535–1539.
[9] J.N. Murthy, D.L. Davis, R.W. Yatscoff, S.J. Soldin, Tacrolimus metabolite cross-reactivity in different tacrolimus assays, Clin. Biochem. 31 (1998) 613–617.
[10] J.P. Griffith, J.L. Kim, E.E. Kim, M.D. Sintchak, J.A. Thomson, M.J. Fitzgibbon, M.A. Fleming, P.R. Caron, K. Hsiao, M.A. Navia, X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex, Cell 82 (1995) 507–522.
[11] S. Biagiotti, L. Rossi, M. Bianchi, E. Giacomini, F. Pierige, G. Serafini, P.G. Conaldi, M. Magnani, Immunophilin-loaded erythrocytes as a new delivery strategy for immunosuppressive drugs, J. Control. Release 154 (2011) 306–313.
[12] S. Baldelli, S. Murgia, S. Merlini, S. Zenoni, N. Perico, G. Remuzzi, D. Cattaneo, High-performance liquid chromatography with ultraviolet detection for therapeutic drug monitoring of everolimus, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 816 (2005) 99–105.
[13] D. Nečas, P. Klapetek, Gwyddion: an open-source software for SPM data analysis, Cent. Eur. J. Phys. 10 (2012) 181–188.
[14] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, Nat. Methods 9 (2012) 671–675.
[15] G.D. Van Duyne, R.F. Standaert, P.A. Karplus, S.L. Schreiber, J. Clardy, Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin, J. Mol. Biol. 229 (1993) 105–124.
[16] R.E. Babine, S.L. Bender, Molecular recognition of proteinminus signligand complexes: applications to drug design, Chem. Rev. 97 (1997) 1359–1472.
[17] T.Q. Wei, Y.F. Zheng, M. Dubowy, M. Sharma, Sandwich assay for tacrolimus using 2 antitacrolimus antibodies, Clin. Chem. 60 (2014) 621–630.
[18] M. Menotta, R. Crinelli, E. Carloni, M. Bianchi, E. Giacomini, U. Valbusa, M. Magnani, Label-free quantification of activated NF-kappaB in biological samples by atomic force microscopy, Biosens. Bioelectron. 25 (2010) 2490–2496.
[19] S.M. Tsunoda, F.T. Aweeka, Drug concentration monitoring of immunosuppressive agents: focus on tacrolimus, mycophenolate mofetil and sirolimus, BioDrugs 14 (2000) 355–369.
[20] M. Behrend, Mycophenolate mofetil: suggested guidelines for use in kidney transplantation, BioDrugs 15 (2001) 37–53.