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

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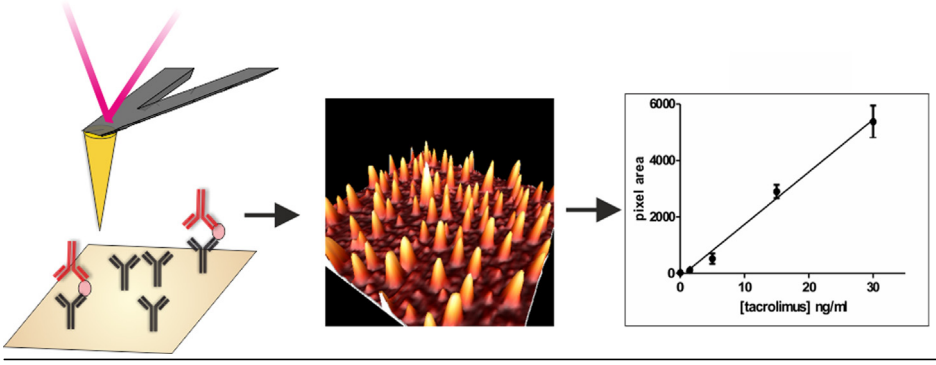
HIGHLIGHTS

- 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



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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

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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