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# Therapeutic Drug Monitoring of Amikacin Using a Surface Plasmon Resonance Biosensor

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## Abstract

The therapeutic drug monitoring (TDM) of pharmaceutical drugs with narrow therapeutic ranges is of great importance in the clinical setting. It provides useful information towards the enhancement of drug therapies, aiding in dosage control and toxicity risk management. Amikacin is an aminoglycoside antibiotic commonly used in neonatal therapies that is indicated for TDM due to the toxicity risks inherent in its use. Current techniques for TDM such as high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) are costly, time consuming, and cannot be performed at the site of action. Over the last decades, surface plasmon resonance (SPR) biosensors have become increasingly popular in clinical diagnostics due to their ability to detect biomolecular interactions in real-time.

We present an SPR-based competitive immunoassay for the detection of the antibiotic amikacin, suitable for TDM in both adults and neonates. We have obtained high specificity and sensitivity levels with an  $IC_{50}$  value of 1.3 ng/mL and a limit of detection of 0.1 ng/mL, which comfortably comply with the drug's therapeutic range. Simple dilution of serum can therefore be sufficient to analyze low-volume real samples from neonates, increasing the potential of the methodology for TDM. Compared to current TDM conventional methods, this SPR-based immunoassay can provide advantages such as simplicity, potential portability, and label-free measurements with the possibility of high throughput. This work is the foundation towards the development of an integrated, simple use, highly sensitive, fast, and point-of-care sensing platform for the opportune TDM of antibiotics and other drugs in a clinical setting.

**Keywords:** Therapeutic drug monitoring; Surface plasmon resonance; Amikacin; Immunoassay

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4 **1. Introduction**  
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8 In the last decade research related to immunosensors has been focused towards the development  
9 of point-of-care devices that can allow management of a particular issue whether it is food safety,  
10 security, environmental, or medical related [1, 2]. Therapeutic drug monitoring (TDM) is a branch of  
11 clinical chemistry and pharmacology that specializes in the measurement of therapeutic drug  
12 concentrations in blood or other body fluids. TDM is indicated for drugs that have narrow therapeutic  
13 ranges, i.e. drugs that present a high risk of toxicity when overdosed or ineffectiveness when underdosed  
14 [3]. Moreover, neonate (newborn) patients have unique drug dispositions that indicate the need of TDM  
15 in order to ensure a safe drug therapy [4]. This monitoring aims to enhance drug efficacy through toxicity  
16 risk management, dosage control, and assistance in clinical diagnosis [5, 6].  
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19 Antibiotics are drugs that are suitable for TDM and they are the most widely used drugs in  
20 neonatal therapy due to newborns' high susceptibility to infections [7]. Amikacin (AK) is an  
21 aminoglycoside antibiotic commonly used in neonatal therapies [8, 9], and is indicated for TDM due to  
22 the toxicity risks inherent to its use as well as its narrow therapeutic range (1-30 µg/mL) [10, 11]. TDM of  
23 Amikacin and other pharmaceutical drugs is mostly performed in blood serum or plasma [6, 12] and  
24 given the low blood volume in neonates of approximately 80 mL/kg, small sample sizes are most  
25 desirable in order to avoid detrimental impact to the patient due to blood loss [4]. Current conventional  
26 methods for TDM are high performance liquid chromatography (HPLC) [13, 14], gas chromatography-  
27 mass spectrometry (GC-MS) [15, 16], and other commercial immunoassays [17, 18] (if available for the  
28 desired analyte), which are costly and time consuming. Additionally, these methods are complex and  
29 cannot be performed at the site of action [6].  
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32 Surface plasmon resonance (SPR) is a widely known optical technique thoroughly studied in the  
33 last few decades, which uses evanescent waves to investigate surface phenomena and generate a signal  
34 related to a change in refractive index at an interface. It has become popular in a variety of fields  
35 including environmental protection, fundamental biological studies, food safety and clinical diagnosis  
36 applications [19]. Advantages of SPR biosensing include real-time and label-free monitoring, simplicity,  
37 high levels of sensitivity and fast-response measurements, making it a potential alternative to current  
38 clinical and laboratory measurement techniques such as enzyme immunoassays (ELISA, EMIT) and  
39 chromatographic techniques (LC-MS, HPLC) [19, 20]. While SPR biosensing has been used for the  
40 detection of antibiotics in milk samples for food safety applications [21, 22], their application for TDM  
41 purposes, especially in neonates, has not been approached. The antibiotic kanamycin has been monitored  
42 using a fluorometric technique based on plasmon resonance light-scattering (PRLS) of gold nanoparticles  
43 (AuNPs) [23], albeit in urine samples for monitoring of nephrotoxicity and ototoxicity.  
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4 In this work we present a SPR-based competitive immunoassay for the detection of AK, suitable  
5 for TDM in both adults and neonates. We have obtained high specificity levels and a high sensitivity  
6 which complies with the drug's therapeutic range, demonstrating the effectiveness of this approach to  
7 further point-of-care on-site TDM monitoring.  
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## 10 11 12 **2. Materials and Methods**

### 13 ***2.1 Chemicals and Reagents***

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17 Reagents for the self-assembled monolayer (SAM) formation (16-mercaptohexadecanoic acid  
18 (MHDA) and 11-mercaptoundecanol (MUOH)), common reagents (N-hydroxysuccinimide (NHS), 1-  
19 ethyl-3(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), ethanolamine), inorganic salts for  
20 buffer preparation (phosphate buffer saline (PBS), 2-(N-morpholino)ethanesulfonic acid (MES), Tween  
21 20), bovine serum albumin (BSA) and AK were purchased from Sigma Aldrich.  
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26 Polyclonal IgG anti-AK antibody was purchased from Abcam (England) and AK-BSA conjugate  
27 was synthesized as described in Section 2.4.  
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### 30 31 ***2.2 Instrumentation***

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34 SPR measurements were performed using a homemade SPR biosensor based on the Kretschmann  
35 configuration under a prism-coupling scheme. A polarized 3 mW laser beam with an operational  
36 wavelength of 670 nm is directed at a fixed angle towards the gold-coated sensing surface. The sensing  
37 chips are 10 x 10 x 0.3 mm glass coated with 2 nm of chromium and 45 nm of gold, from Ssens  
38 (Netherlands). SPR measurements are performed at a fixed angle of incidence. The optimum  
39 measurement angle is selected during calibration to ensure maximum sensitivity to changes in the  
40 refractive index, which in turn are caused by mass variations at the sensing surface. These variations are  
41 detected as changes in the reflected light intensity at a photodiode, and are subsequently amplified and  
42 converted to a digital signal, which we refer to as SPR signal (intensity variation of the reflected light at a  
43 fixed angle).  
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47 Sample sizes of 250  $\mu$ L are injected by a diaphragm pump to the flow delivery system, which  
48 incorporates a flow cell with two independent channels. The system incorporates all the optics, electronics  
49 and fluidic components necessary to function autonomously.  
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### 52 53 54 55 56 57 58 ***2.3 Synthesis of the AK-BSA conjugate***

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4 AK-BSA conjugate was prepared based on the conjugation of the amino groups of AK to the  
5 carboxylic groups present in BSA. Briefly, a solution of AK (11.11 mg/ml in MES buffer pH 5.0) and the  
6 carrier protein BSA (10 mg/ml in MilliQ water) was incubated for 3 hours with 10 mg of EDC as a linker.  
7 Next, purification of the conjugate was achieved by using PBS 10 mM (pH 7.4) as a purifying buffer and  
8 a 30 kDa centrifugal filter. The solution was mixed with 250  $\mu$ L of purifying buffer in the filter and was  
9 centrifuged at 12000 RPM during 120 s, after which the residues from the collection tube were discarded.  
10 This process was repeated three times, yielding the final conjugate solution. Conjugate concentration was  
11 determined by UV resulting in 14.3 mg/ml.  
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#### 19 ***2.4 Immobilization Procedure***

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22 Gold chips were prepared for immobilization by a wash with ethanol, followed by 20 minutes of  
23 surface cleansing with UV-ozone (Bioforce Nanosciences, Inc, model UV-TC.220) to eliminate organic  
24 contamination. A second rinse with ethanol was performed and finally the chips were dried with N<sub>2</sub>.  
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27 A covalent strategy was used for biofunctionalization of the surface. After chip cleaning, the  
28 formation of an alkanethiol self-assembled monolayer (SAM) was carried out ex-situ by coating the gold  
29 chip overnight with a mixed solution of MHDA:MUOH in ethanol, with a molar ratio of 1:1 and at a total  
30 concentration of 250  $\mu$ M. The chip was rinsed with ethanol and dried with N<sub>2</sub>, and mounted in the SPR  
31 platform. Next, an AK-BSA conjugate was covalently coupled to the carboxylic groups of the SAM  
32 through activation of the acid groups with a solution of 0.2 M EDC and 0.05M NHS in MES buffer (pH  
33 5.0). Subsequently, after the concentration of AK-BSA conjugate in PBS buffer was injected, a 1 M  
34 ethanolamine solution (pH 8.5) was used for the deactivation of unreacted carboxylic groups. This  
35 procedure was done at a flow-rate of 14  $\mu$ L/min.  
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#### 44 ***2.5 SPR immunoassay format***

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47 An indirect competitive immunoassay format was used for the detection of AK levels given the  
48 low molecular weight of the molecule (585.60 Da). Therefore, the AK-BSA conjugate was immobilized  
49 and used as the sensing bioactive surface. After assay optimization, samples consisting of mixtures (1:1  
50 v/v) of different analyte concentrations (ranging from 1 pg/mL to 5  $\mu$ g/mL in PBS or PBST (0.05%  
51 Tween 20)) and a fixed antibody concentration were flowed over the sensing layer at a speed of 22  
52  $\mu$ L/min. Samples were incubated for 10 min at room temperature before injection and monitoring was  
53 performed in real time. Since antibody binding to the immobilized conjugate is inhibited by the presence  
54 of analyte, lower analyte concentrations resulted in high SPR signals and vice versa. Standard calibration  
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4 curves were constructed with at least 7 points (7 different AK concentrations) with three replicate  
5 measurements each. Reutilization of the sensor surface was achieved using a solution of NaOH 45 mM as  
6 a regeneration solution, during 120 s at a speed of 22  $\mu\text{L}/\text{min}$  after each sample injection.  
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## 10 **2.6 Data analysis**

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14 Standard calibration curves were obtained by plotting the average SPR signal and standard  
15 deviation of three samples for each AK concentration, versus the logarithm of the AK concentration of  
16 the sample. These experimental points were fitted to the four-parameter logistic equation:  
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$$19 \quad y = \{D + (A - D) / [1 + (x/C)^B]\}$$

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24 where  $y$  is the SPR signal (variation of reflectance at a fixed angle, % Reflectance),  $A$  is the maximum  
25 signal (zero concentration of analyte),  $D$  is the asymptotic minimum (background signal),  $B$  is the slope of  
26 the sigmoidal curve at the inflection point, and  $C$  is the concentration which gives 50% of the maximum  
27 signal,  $\text{IC}_{50}$ .  
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## 32 **3. Results and Discussion**

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36 Molecular weight of the analyte is a key factor when selecting the immunoassay configuration in  
37 SPR biosensing. A direct assay configuration (which uses an antibody coated surface) may be the best  
38 option in order to simplify conditions, even more with views of developing point-of-care devices, but it  
39 becomes an unfeasible approach due to the low molecular weight of AK (585.60 Da), which inevitably  
40 would lead to low detectability. Therefore, we proceeded to the optimization of an indirect competitive  
41 inhibition assay (Fig. 1), in which samples consisting of a fixed concentration of antibodies incubated  
42 with different concentrations of the analyte are then flowed over an antigen-coated surface. Compared  
43 with direct assays, indirect approach avoids some drawbacks related to the immobilization of antibodies  
44 to the solid surface and their potential loss of affinity, either due to their attachment which can affect the  
45 native structure, or simply during assay development and optimization. This process usually requires  
46 working under conditions that allow regeneration of the bioactive surface (i.e. basic or acid conditions  
47 which disrupt the interactions between the antibody and the target molecule, therefore releasing it and  
48 leaving the receptor ready for the next sample). Subjecting the antibody coated surface to these conditions  
49 usually lead to an irreversible alteration of antibody structure, and therefore to its binding ability.  
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51 Moreover, the direct approach has to deal with the proper orientation of the antibodies on the surface,  
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4 which can also affect the overall assay performance. On the other hand, in an indirect assay the  
5 immobilization of the target, either directly or through a carrier molecule (typically a protein) which  
6 previously has been decorated with the analyte, provides a higher degree of robustness and reusability  
7 even under mild or harsh regeneration conditions.  
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12 In the indirect immunoassay, parameters such as antigen conjugate concentration, antibody  
13 concentration and interaction buffer become crucial to obtain the best features in terms of sensitivity,  
14 reproducibility and stability. These factors together with the regeneration solution were optimized to  
15 achieve the best sensor performance.  
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19 AK-BSA conjugate concentrations ranging from 5 to 30  $\mu\text{g/mL}$  of AK-BSA were tested over  
20 gold substrates coated with a mixed SAM (MHDA:MUOH 1:1). As can be seen in Table 1 a gradually  
21 increasing amount of conjugate was bound to the surface as the added antigen concentration is also  
22 higher, until approaching saturation of the surface at 30  $\mu\text{g/mL}$  of AK-BSA.  
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27 The concentration of fixed antibody to be used in the inhibition immunoassay influences  
28 immunoassay characteristics such as the limit of detection (LOD), sensitivity, and dynamic range.  
29 Additionally, in order to favor competition, a low concentration of free antibody will usually give better  
30 results. Therefore, there is a trade-off between using low concentrations for a better assay sensitivity, and  
31 using a concentration high enough (but well below saturation level of the antigens) to obtain discernible  
32 signals for a wider range of analyte concentrations.  
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37 Several antibody concentrations (up to 6  $\mu\text{g/mL}$ ) were tested and the signal obtained did not reach  
38 saturation in any case. The average signal response when injecting 6  $\mu\text{g/mL}$  of anti-AK antibody over  
39 chips immobilized with 5, 15 and 30  $\mu\text{g/mL}$  of AK-BSA was of 0.30, 0.53, and 0.78 respectively. As  
40 could be expected, results showed an overall higher response to the same antibody concentration for  
41 surfaces in which higher immobilization signals were obtained. Finally an AK-BSA concentration of 30  
42  $\mu\text{g/mL}$  and an antibody concentration of 6  $\mu\text{g/mL}$  were deemed optimal, guaranteeing a sufficient  
43 maximum SPR signal in absence of analyte while assuring a wide dynamic range for clear differentiation  
44 between different analyte concentrations.  
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50 In order to assess the stability of the assay and also to test the reusability and robustness of the  
51 bioactive surface, we studied several regeneration conditions in order to remove the bound antibody. The  
52 performance of several acid and basic solutions (i.e. HCl, glycine and NaOH at different concentrations)  
53 was compared. An effective regeneration is crucial to ensure the reusability of the sensing surface and the  
54 reproducibility of the measurements. Incomplete removal of antibody will produce disperse  
55 measurements between replicates and an excessively harsh conditions might compromise the stability of  
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4 the sensing surface reducing its life span. Regeneration with NaOH 45 mM solution injected for 120 s  
5 was selected as optimum conditions to achieve complete removal of the bound antibody. These conditions  
6 ensured the reusability of the surface over a span of 3 days and at least 40 measurement cycles, observing  
7 then a decrease in the SPR signal of 11% (taking as reference the zero analyte concentration). Stability in  
8 the day-to-day measurements was evidenced by consistence in the SPR signals, confirming the reliability  
9 and robustness of the immobilized chips.  
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14 With these initial conditions, calibration curves were performed, both in PBS and PBST (PBS  
15 containing 0.05% Tween 20) Figure 2 shows sensograms indicating the SPR response to the binding of  
16 different concentrations of AK, followed by their respective regeneration cycles. As expected, SPR  
17 signals decrease in response to increasing AK concentrations.  
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21 Significant better performance was obtained using PBST instead of PBS. When using PBS as  
22 running buffer, lower reproducibility was observed (relatively high standard deviation between  
23 measurements). On the other hand, the use of PBST resulted in a lower dispersion between replicates and,  
24 most importantly, in a much higher sensitivity (see Figure 3).  
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29 Table 2 summarizes immunoassay features for both buffers and as can be seen a LOD one order  
30 of magnitude better was reached using PBST instead of PBS, with a slope of the linear area close to 1,  
31 which may result in a more precise and accurate calibration curve.  
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35 Specificity of the sensing surface was evaluated with anti-vancomycin antibody (6 µg/mL)  
36 obtaining negligible SPR signals (data not shown). Vancomycin (Van) is an antibiotic also used in  
37 newborn therapies for the treatment of infections caused by Gram-positive bacteria, and can usually be  
38 coadministered with AK. Thus, the specificity of the current assay was also tested to assess any potential  
39 cross-reactivity with Van, by performing the inhibition assay with a fixed concentration of anti-AK  
40 antibody (6 µg/ml) and varying concentrations of vancomycin. As can be seen in Figure 3 no inhibition is  
41 observed even at high concentrations of vancomycin (9-18% of the maximum signal), which confirms the  
42 low specificity of the assay towards Van and its suitability for the detection of AK.  
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49 According to the obtained assay features, this SPR-based approach can be very useful for the  
50 determination of AK since the sensitivity levels obtained are far below the drug's therapeutic range (1-30  
51 µg/mL) and comparable or higher than current TDM methods [24–26]. This high sensitivity entails  
52 advantages for the detection of AK in real human samples by allowing sample dilution of approximately  
53 5000 times, resulting in sample sizes of few nanoliters. Non-specific binding of matrix components  
54 present in serum can sometimes produce analytical interference (positive signal) which masks the specific  
55 signal leading to wrong results. This extremely high dilution fold, (which represents less than 0.02% of  
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4 sample) becomes convenient in order to minimize the potential negative effects of the complex matrix on  
5 the sensing surface. Moreover, in intended uses where the clinical samples come from blood drawn from  
6 infants, the low detectability of the assay overcomes the problems associated with dilution of the sample,  
7 which sometimes, in assays that show moderate to fair sensitivities, implies to fall below the  
8 quantification or even the detection limit.  
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#### 14 **4. Conclusions**

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17 A highly sensitive, label-free SPR immunosensor for the therapeutic monitoring of AK has been  
18 developed. The sensor uses a sample size of 250  $\mu\text{L}$ , but according to the high sensitivity obtained, which  
19 allows high sample dilution to enter in the working range, very low patient's sample volume of few  
20 nanoliters will be presumably required, making the sensor favorable for TDM in neonates while  
21 maintaining quantification capability. The sensor is based on an indirect competitive immunoassay with  
22 AK immobilized on the gold surface. The immobilization process and other immunoassay parameters  
23 were individually optimized for the best performance. We have obtained a very good sensitivity with an  
24  $\text{IC}_{50}$  value of 1.3 ng/mL. A low LOD of 0.1 ng/mL was achieved, which comfortably complies with the  
25 drug's therapeutic range and is adequate for the assay's application in TDM. Reproducibility in the  
26 measurements has been also achieved, as well as reusability of the surface for at least 40 measurements  
27 over a span of 3 days, confirming the robustness of the method. Finally, non-specificity of the assay  
28 towards Van (a drug often coadministered with AK) was also demonstrated.  
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38 This SPR immunoassay method provides advantages over current TDM conventional methods  
39 (such as enzyme immunoassays and chromatographic techniques) allowing the fast, label-free and real-  
40 time determination of AK concentrations, while achieving comparable or even lower detection limits. To  
41 our knowledge this could be the first application of SPR in the therapeutic monitoring of antibiotics. This  
42 assay demonstrates the potential of SPR as a method for point-of-care TDM of antibiotics (and other  
43 drugs) in a clinical setting. Future work will involve the evaluation of the technique using real serum  
44 samples, as well as the detection of other drugs indicated for TDM.  
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4 **Figure Legends**  
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7 **Fig. 1** Indirect competitive assay. (a) formation of the alkanethiol mixed SAM (b) immobilization of the  
8 AK-BSA conjugate (c) Samples consisting of a mixture of a fixed antibody concentration and varying  
9 analyte concentrations are incubated for 10 minutes and flowed over the sensing surface. Reutilization of  
10 the surface is achieved by means of a regeneration solution  
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13 **Fig. 2** Sensograms showing the interaction of free anti-AK fraction at different AK concentrations (1) and  
14 the subsequent regeneration step with NaOH 45 mM (2) after signal stabilization  
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17 **Fig. 3** Standard calibration curve for AK in PBS (black) and PBST 0.05% Tween (blue). Each point  
18 corresponds to the mean value and standard deviation of triplicate measurements. In red, specificity study,  
19 performed with vancomycin (Van) as analyte instead of AK, using anti-AK antibody under optimized  
20 assay conditions  
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**Table 1:** Immobilization of AK-BSA

<b>[AK-BSA], (<math>\mu\text{g/mL}</math>)</b>	<b>Immobilization signal, (a.u)</b>
<b>5</b>	<b><math>0.33 \pm 0.03^a</math></b>
<b>10</b>	<b><math>0.85 \pm 0.03</math></b>
<b>15</b>	<b><math>0.97 \pm 0.07</math></b>
<b>30</b>	<b><math>1.11 \pm 0.05</math></b>

<sup>a</sup>: mean signal  $\pm$  standard deviation of at least 3 replicates

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**Table 2.** Amikacin immunoassay features

	<b>LOD (IC<sub>90</sub>)</b>	<b>IC<sub>50</sub></b>	<b>Dynamic range (IC<sub>80</sub>-IC<sub>20</sub>)</b>	<b>Slope</b>
<b>PBS</b>	0.5 ng/mL (0.85 nM)	43.2 ng/mL (73.8 nM)	2.3– 809 ng/mL (3.9-1383 nM)	-0.41
<b>PBST</b>	0.1 ng/mL (0.17 nM)	1.3 ng/mL (2.3 nM)	0.3 – 5.4 ng/mL (0.51-9.2 nM)	-1.02

Figure 1

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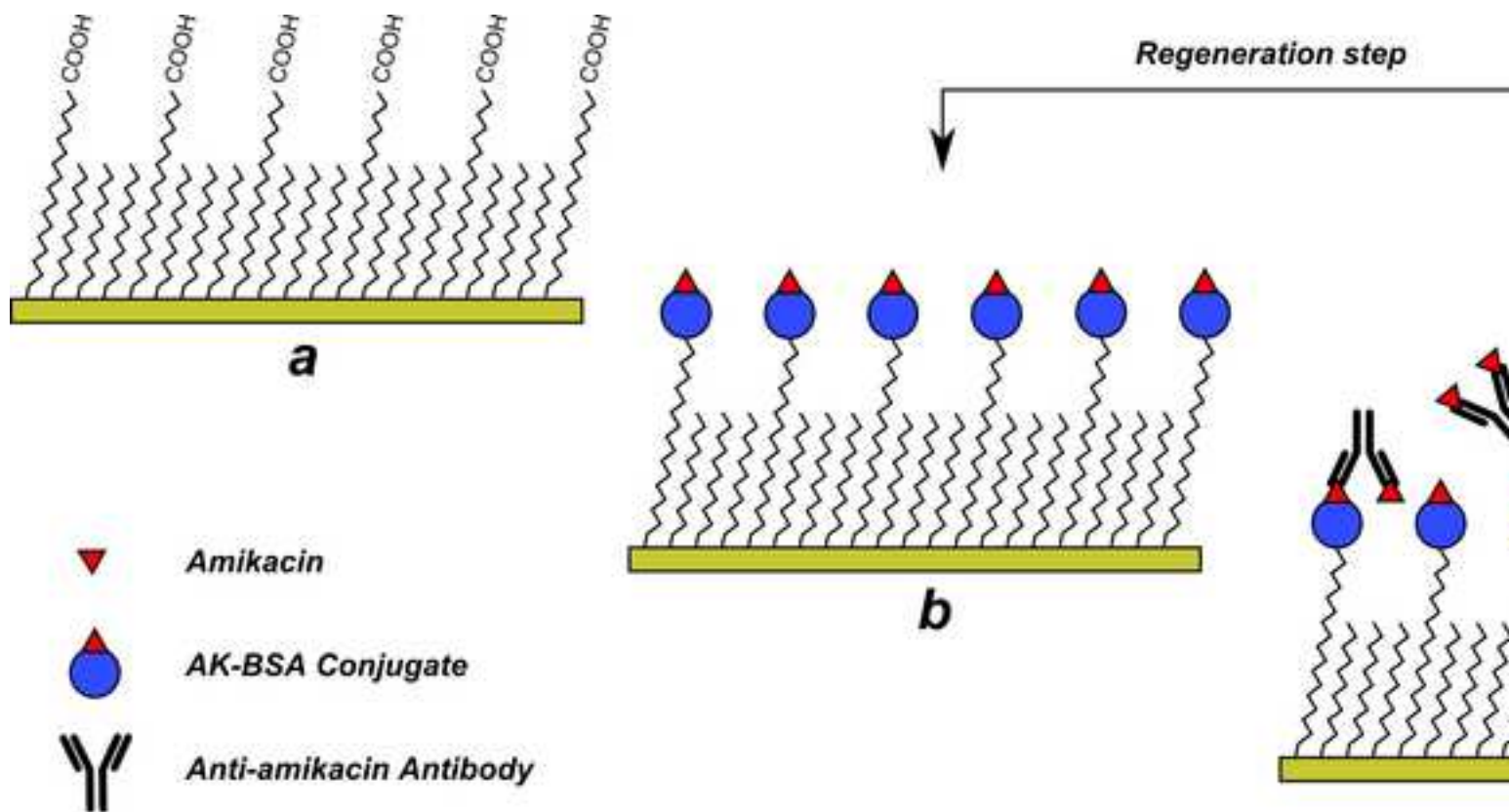


Figure 2

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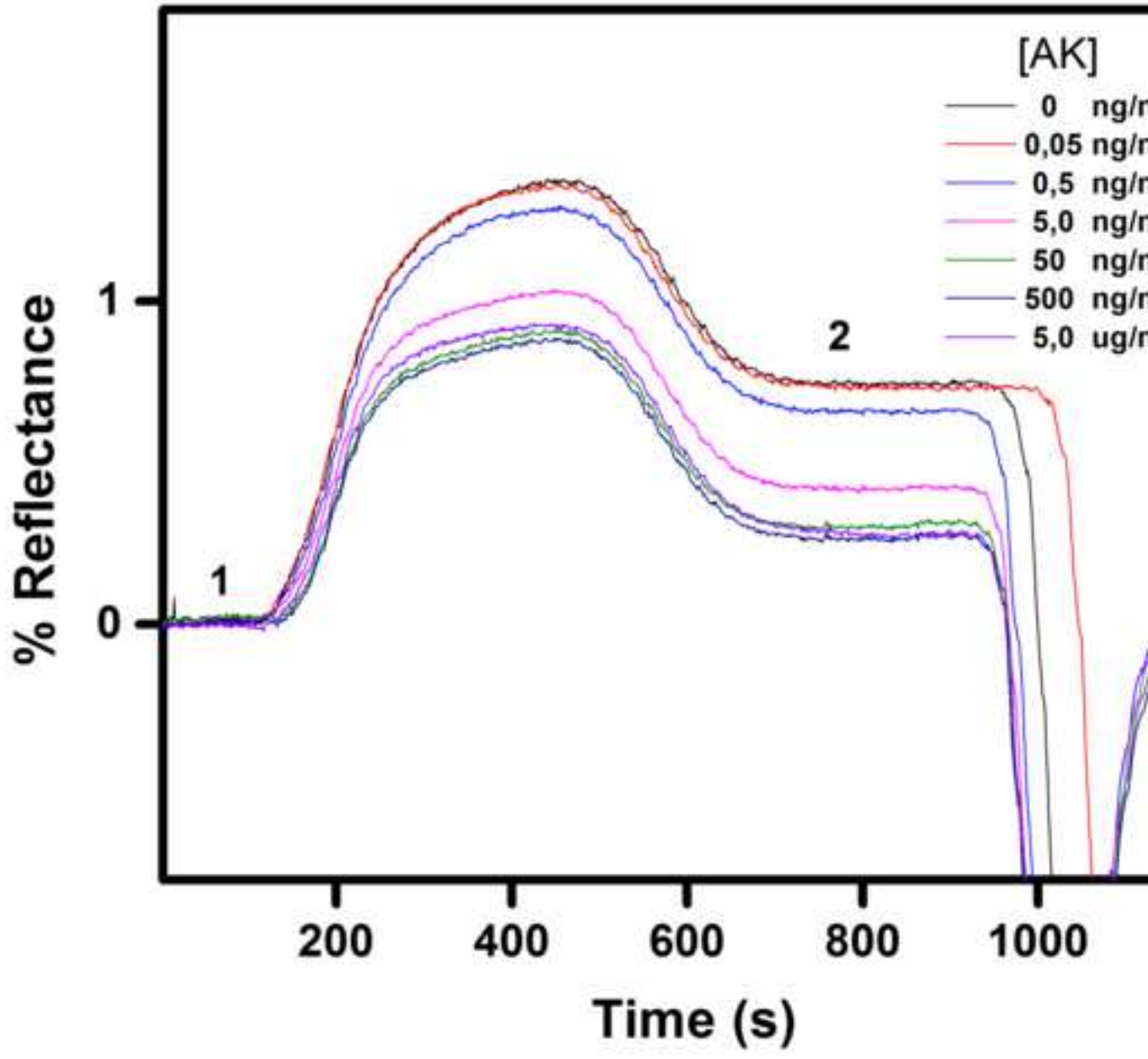




Figure 3

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