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Adenosine reagent-free detection by co-immobilization of adenosine deaminase and phenol red on an optical biostrip

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Adenosine detection in human serum is important because this ribonucleoside has established clinical applications, modulating many physiological processes. Furthermore, a simple and cheap detection method is useful in adenosine production processes. Adenosine can be determined enzymatically using either *S*-adenosyl-homocysteine hydrolase and ^{3}[H]-adenosine, or adenosine kinase combined with GTP and luciferase, or an amperometric biosensor carrying adenosine deaminase (ADA), purine nucleoside phosphorylase, and xanthine oxidase. We developed a simple and cheap method relying on a transparent biostrip bearing ADA and the indicator phenol red (PR), co-immobilized to polyacrylamide, itself chemically adhered to a derivatized glass strip. The ADA-catalyzed conversion of adenosine to inosine and ammonia leads to a local pH alteration, changing the absorbance maximum of PR (from 425 to 567 nm), which is measured optically. The biostrip shows an analytical range 0.05–1.5 mM adenosine and is reusable when stored at 4°C. When the biostrip was tested with serum, spiked with adenosine (70 and 100 μM), and filtered for protein and adenosine phosphates depletion, it showed good adenosine recovery. In summary, we show the proof-of-concept that adenosine can be determined reagent-free, at moderate sensitivity on an easy to construct, cheap, and reusable biostrip, based on commercially available molecular entities.

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1 Introduction

The ribonucleoside adenosine, with established clinical applications [1], is derived in vivo from rapidly metabolized ATP, via adenosine mono- and diphosphates, interacting with specific purinergic receptors of the cell surface [2] and modulating many physiological processes [1]. Adenosine is of interest to cardiology, as an anti-arrhythmic agent [3], in the evaluation of coronary stenosis [4], and in myocardial contrast echo cardiography [5]. Furthermore, adenosine receptors are potential targets for analgesic and anti-inflammatory agents [6,7].

Several methods have been proposed for adenosine determination, relying either on HPLC analysis [8,9] or enzyme catalysis, employing either reduced *S*-adenosyl-homocysteine hydrolase and ^{3}[H]-adenosine [10], or adenosine kinase combined with GTP and luciferase [11], in non reagent-free techniques. An earlier work focused on an amperometric biosensor featuring three co-entrapped enzymes (adenosine deaminase (ADA), purine nucleoside phosphorylase, and xanthine oxidase) in a composite lactobionamide and amphiphillic polypyrrole matrix around a Pt microelectrode [12]. Another biosensor was based on modulating the activity of a DNAzyme by an appending inhibitor consisted of an oligonucleotide strand, containing an adenosine-specific aptamer [13]. An electrochemiluminescent aptamer biosensor was proposed [14], based on the interaction between an ssDNA (capture DNA) immobilized on gold electrode and another DNA containing an adenosine-specific aptamer, modified on the elec-

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Abbreviations: ADA, adenosine deaminase; **BSA,** bovine serum albumin; **PAc,** polyacrylamide; **PAc-PR,** polyacrylamide with entrapped phenol red; **PR,** phenol red.

trode surface through hybridization with the capture DNA.

The present method relies on a simple, cheap, and easy to make transparent sensing bilayered strip ("biostrip") bearing two commercially available molecular entities, the enzyme ADA (EC 3.5.4.4) from bovine spleen and the pH-indicator dye phenol red (PR), both chemically co-immobilized on polyacrylamide (PAc), which itself was chemically adhered to a transparent supporting glass-strip. The signal transduction results from the ADA-catalyzed conversion of adenosine to inosine with concomitant release of ammonia, which brings about a local pH change affecting the visible absorbance (λ_{max}) of the polyacrylamide-entrapped indicator dye PR. The method requires no added reagents but only the adenosine-containing sample. The biostrip was put to the test with adenosine samples in buffer and in filtered human serum spiked with known amounts of adenosine. This proof-of-concept assay could be the basis for the construction of optic biosensors for adenosine determination.

2 Materials and methods

2.1 Materials

Acrylamide (99%), *N,N′*-methylene-bis-acrylamide, *N,N,N′,N′*-tetramethylethylene-diamine (99%), ammonium persulfate, glutaraldehyde (grade II, 25% aqueous solution), pH indicator dyes (analytical grade, ACS), potassium phosphate monobasic, adenosine (99%), and ADA (A5043, type X from calf spleen, 151 units/mg, solution in 50% glycerol/5 mM potassium phosphate) were purchased from Sigma (Germany). ADA working solutions were prepared immediately before use in 100 mM phosphate buffer, pH 7.5. Glutaraldehyde and bovine serum albumin (BSA) were diluted with or dissolved in buffer as above, respectively. Human serum was obtained from clinical laboratories and kept in deep freeze.

2.2 Construction of the biostrip

2.2.1 *Step 1. Entrapment of the indicator dye phenol red to polyacrylamide (PAc-PR)*

In a vacuum conical flask (nominal volume 250 mL) were added 2.5 mL Tris–HCl buffer (0.5 M, pH 6.8), 1.7 mL solution containing acrylamide (CH_2=CHCONH$_2$) and *N,N′*-methylenebis(acrylamide) ($(CH_2(CH_2$=CHCONH$)_2$) (3 g : 0.08 g in 10 mL water), and 5.8 mL water. The mixture was degassed on a water-pump for 15 min, prior to the addition of 3 mg PR (0.03% w/v final concentration) and sonication to effect solubilization. To the clear solution were added 50 μL ammonium persulfate solution (1.5% w/v) and 10 μL TEMED. The mixture was slowly poured into the space formed between two glass plates (working volume 8.5 mm × 7.5 cm), standing 0.75 mm apart and ensuring that no bubbles had been trapped. To the free surface of the casted liquid approx. 1 mL solution of 1-butanol/water (1:1 v/v) was carefully applied in order to deplete the polymerization reaction of oxygen and was left for 2 h at room temperature. After completion of the reaction, the butanol solution was removed by adsorption on a paper filter and the polyacrylamide carrying entrapped dye (PAc-PR) was gently washed several times with water to remove unreacted monomers, chemicals, and unbound dye, prior to equilibrating in 100 mM phosphate buffer, pH 6.9, and storing at 4°C.

2.2.2 *Step 2. Adherence of PAc-PR gel to glass (bilayered strip)*

Prior to adhering PAc-PR to the glass-strip, the latter was first surface-functionalized with aminopropyltriethoxysilane in toluene or acetate buffer. In a round bottom flask (nominal volume 100 mL) were added 30 mL of 5% (v/v) solution of aminopropyltriethoxysilane in either toluene or 0.1 M acetate buffer, pH 5.5. In the flask four glass strips (20 mm × 5 mm × 1 mm each) were carefully added, previously treated with boiling 0.5 N HCl for 1 h, ensuring that they do not get in contact with each other. The flask was placed in an oil-bath and the reaction mixture was brought to reflux where it was maintained for either 12 h (toluene) or 4 h (acetate buffer). Upon completion of the reaction, the glass strips were taken out and washed thoroughly with either ethanol (when the reaction was run in toluene) or water and ethanol (when the reaction was run in acetate buffer). The adherence was effected by applying evenly 0.5 mL fresh glutaraldehyde solution (25% v/v, made in 0.1 M phosphate buffer, pH 7.5) on the animated glass surface and immediately laying on top a strip of PAc-PR gel (0.75 mm thick) of the same dimensions as the glass strip. The bilayered strip was left to react at 37°C for 19 h in humid environment.

2.2.3 *Step 3. Activation of the PAc-PR part of the bilayered strip with glutaraldehyde*

Four bilayered strips from *Step 2* were transferred in a beaker (nominal volume 250 mL) containing a fresh solution of glutaraldehyde (24 mL, 25% v/v) and 0.1 M phosphate buffer, pH 7.5 (18 mL). The beaker was hermetically sealed and left to react at 37°C for 19 h, under gentle shaking. After completion of the activation reaction, the bilayered strips were freed from uncoupled glutaraldehyde by washing with 100 mM phosphate buffer, pH 6.9 (20 mL), followed with water (20 mL), prior to incubation in 100 mM phosphate buffer, pH 7.5, for 4 h at 25°C. The success of the activation reaction was confirmed by visual inspection of glutaraldehyde-treated and untreated (control) strips, on reaction with a solution of 6-aminohexyl-Procion® MX-8B dye (0.4% w/v, 10 mL, 100 mM phosphate buffer, pH 7.5) for 30 min at 40°C. After completion of the dyeing reaction and exhaustive washing of

the PAc-PR part of the strip with water, only the one activated successfully was colored red.

2.2.4 *Step 4: Immobilization of adenosine deaminase and albumin to the glutaraldehyde-activated bilayered strip ("biostrip")*

Enzyme immobilization to the activated bilayered strip was done immediately after the strip from *Step 3* was obtained. On the surface of a freshly activated bilayered strip (PAc-PR part: 20 mm × 5 mm × 0.75 mm), previously placed in a plastic cuvette (nominal area 5 mm × 20 mm) and slightly dried at 4°C for 30 min, 20 μL of an ADA–BSA mixture (made by adding 10 μL 100 mM phosphate buffer, pH 7.5, and 10 μL commercial ADA (0.3 units, 2 μg) in a tube containing approx. 10 μg BSA), were carefully and evenly applied. The cuvette with the strip was fixed on the surface of a polystyrene piece floating in a beaker with water, which was hermetically sealed and left at 4°C for 2 days. Upon completion of the immobilization reaction, the bilayered strip (termed as *biostrip*) was incubated with chilled immobilization buffer (1 mL) for 30 min, under gentle shaking at 4°C to remove unbound ADA and BSA. The liquid was discarded and the process was repeated twice with the last one extended to 1 h. The biostrip was finally stored in 100 mM phosphate buffer, pH 7.5, at 4°C.

2.3 Effect of pH on the optical absorption of the free and immobilized systems

2.3.1 *Free system*

PR solution (20 μL from 0.03% w/v) was added in a cuvette containing 600 μL 5 mM phosphate buffer in a pH range 6.5–10.0 (in 0.5 pH unit increments) and the optical absorption spectrum was recorded in the range 650–400 nm.

2.3.2 *Immobilized system*

A strip of glutaraldehyde-activated PAc-PR gel (5% v/v, 20 mm × 0.75 mm × 5 mm) was equilibrated in 5 mM phosphate buffer in a pH range 6.5–10.0 (in 0.5 pH unit increments) for 10 min, prior to recording the optical absorption spectrum in the range 650–400 nm. For every different buffer used, prior to spectrum recording, the gel was washed exhaustively with water until it had reached its original color and absorption intensity (yellow).

2.4 Standard assay for adenosine deaminase

One unit of enzyme (ADA) is defined as the amount of enzyme that converts 1.0 μmole of adenosine to inosine and ammonia per minute at pH 7.5 at 25°C. The Sigma's spectrophotometric assay was implemented either in quartz or synthetic cuvettes suitable for UV measurements (1.5 mL nominal volume). The stock solutions required and the quantities added in the cuvette are described in Supporting information, Table S1. The reaction was followed by recording the $-\Delta A_{265}$, whereas, the enzyme units were calculated from the slope of the linear section of the assay curve.

2.5 Determination of adenosine using the new biostrip

For creating the adenosine calibration curve, the biocatalytic bilayered strip (20 mm × 5 mm × 0.75 mm) was placed in a plastic cuvette (nominal volume 0.5 cm × 1 cm × 2.5 cm) along with 1 mL of phosphate buffer (1 mM, pH 7.0) and left to equilibrate for 15 min at 25°C. The liquid was discarded, adenosine solution of known concentration (1 mL, 50–1500 μM) was introduced and the reaction was allowed to proceed until no further increase of absorbance, due to ammonia release, was observed and a stable red color (567 nm) obtained. As a control (background) reading was taken the absorption in the absence of adenosine in the same buffer. Before assaying different adenosine concentrations, the biostrip was first equilibrated in 1 mM phosphate buffer, pH 7.0, until steady background absorption had been reached (yellow color).

The procedure for adenosine determination in human serum was essentially the same as above, except for using adenosine samples prepared in a solution of 10% human serum in phosphate buffer, 1 mM, pH 7.0. The adenosine-in-serum samples (1 mL), prior to assaying, were filtered through a column containing 0.5 mL each of Cibacron Blue 3GA-agarose (top) and diethylaminoethyl (DEAE)-agarose (bottom). Filtered human serum (10% v/v) was used to set zero readings.

2.6 Stability of the biostrip

The daily routine followed to monitor the enzyme stability and calculate the biostrip's useful lifetime, was as follows: the cuvette with the biostrip was transferred from the refrigerator (4°C), where it was kept sealed in phosphate buffer (100 mM, pH 7.5), to equilibrate at 25°C for 1 h, prior to assaying the immobilized ADA using the standard assay (Section 2.4). The biostrip was then rinsed with fresh buffer and kept at 4°C until next day's assay. The enzymatic activity at 25°C, on the day of enzyme immobilization was taken as 100% of enzyme activity. The enzyme activity was monitored also for the biostrip, which is used on a routine daily basis for experimental purposes.

3 Results and discussion

The present method relies on a signal transduction principle similar to that applied for the determination of albumin [15], penicillin, urea and glucose [16], lecithin [17], carbaryl and dichlorvos [18], and atrazine [19]. However, only some of the techniques described earlier are reagent-

free [15–17], yet employing laborious synthetic protocols for their sensing elements.

3.1 Choice of the indicator dye

To identify the most suitable indicator dye, two dyes, showing working pHs around that of the enzyme (7.5), PR (pH 6.4–8.0) and cresol red (pH 7.2–8.8), were tested for their ability to shift their λ_{max} during the conversion of adenosine to inosine by free ADA. PR was the one chosen for it combines the ability of a bathochromic shift upon ADA reaction (from yellow to red, Fig. 1A, insert) with the ability of reliable entrapment to PAc and maintaining these properties after gel-activation by glutaraldehyde and ADA immobilization. Figure 1A and Supporting information, Figure S1 present the absorption spectra of PR in immobilized and free form, respectively, in various pH values. Each spectrum shows two maxima, the second of which (567 nm in PAc and 557 nm in solution; Fig. 1A and Supporting information, Fig. S1, respectively) offers substantially higher change in optical absorbance. Furthermore, after PR immobilization to PAc (to obtain PAc-PR), a bathochromic shift of the λ_{max} (557 nm in solution and 567 nm in PAc) is observed with progressive increase of pH. Subsequent treatment of the PAc-PR with glutaraldehyde (for ADA co-immobilization) had no further effect on the absorption spectra.

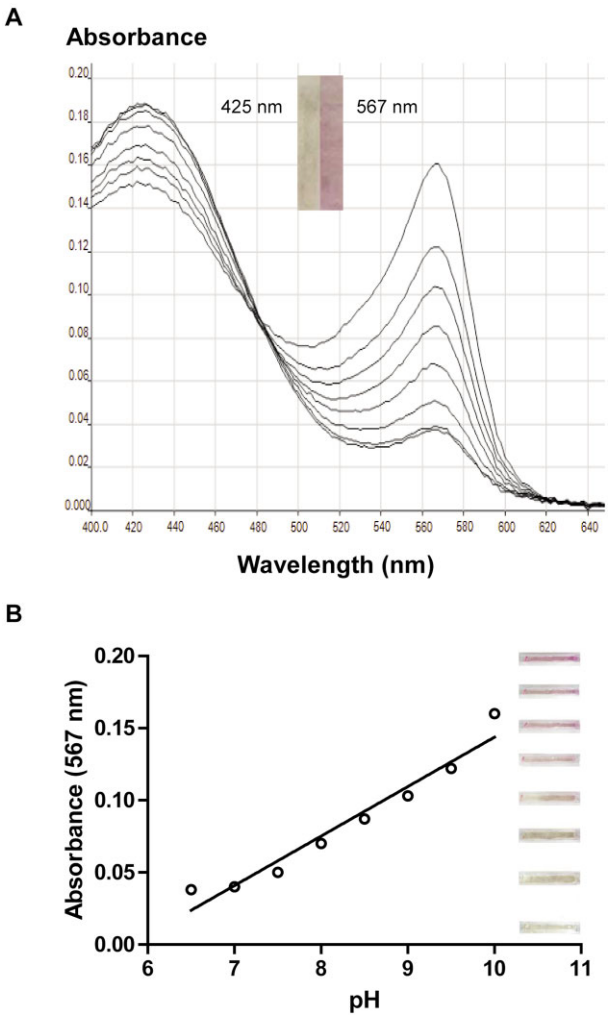

Figure 1. The effect of pH on the absorbance of immobilized phenol red. (**A**) Superimposed absorbance spectra of indicator phenol red entrapped to glutaraldehyde-activated polyacrylamide strip for various pH values (from bottom up: 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0). Insert: optical analytical signal change of biostrip as observed during adenosine determinations; before addition of adenosine analyte equilibrated with 1 mM phosphate buffer, pH 7.0 (left) and after addition of adenosine and reaction accomplishment by immobilized adenosine deaminase (right). (**B**) Indicator titration curve for various pH values at peak wavelength 567 nm. Insert: color effect of sensing strip upon pH changes as in (**B**), respectively.

From Fig. 1, one is able to identify the pH value suitable for the present method. If one chooses a near neutral pH to initiate the enzymatic reaction, where the 425 nm peak prevails (yellow color), as the reaction proceeds, ammonia is continuously released and the pH increases, thus leading the 567 nm peak to be the dominant one (red color). This phenomenon is analogous to the bathochromic shift of PR absorption spectrum upon pH increase (Fig. 1A). Therefore, the study of the effect of pH on the PAc-PR optical absorption reveals the effective pH range (Fig. 1B) from which the pH value 7.0 was chosen for the present method. Notably, this value is close to pH 7.5, proposed in Sigma's standard assay protocol described in Section 2.4.

3.2 Construction of the biostrip

Figure 2 presents the route followed to construct the biostrip, whereas the respective experimental procedures were described in Section 2.2. Preliminary experiments were implemented with PAc-PR gels composed of different monomer concentrations (5–15% v/v). After recording their absorption spectra, the 5% PAc-PR was finally chosen for subsequent work due to its superior linearity over the tested pH values. The λ_{max} of the immobilized dye chosen for subsequent work (567 nm), offers a higher dynamic absorption change (≈0.12), compared to the λ_{max} at 425 nm (≈0.035) (Fig. 1A). Robustness is endowed to the sensing PAc-PR by adhering it to derivatized glass of the same dimensions. In order to couple the amine groups of PAc-PR to glass with glutaraldehyde, first, amino-groups were introduced to the glass surface after reaction with aminopropyltriethoxysilane in aqueous [20] or organic solvent [21]. Prior to silanization, the number of surface hydroxyl (silanol) groups was maximized by boiling the glass strips in acid (HCl) and drying at 150°C, in order to cleave most siloxane bridges. Following glutaraldehyde-promoted adherence, the resulted bilayered strip shows excellent mechanical stability and can be used for many assays (Section 3.4). Furthermore, the unreacted (free)

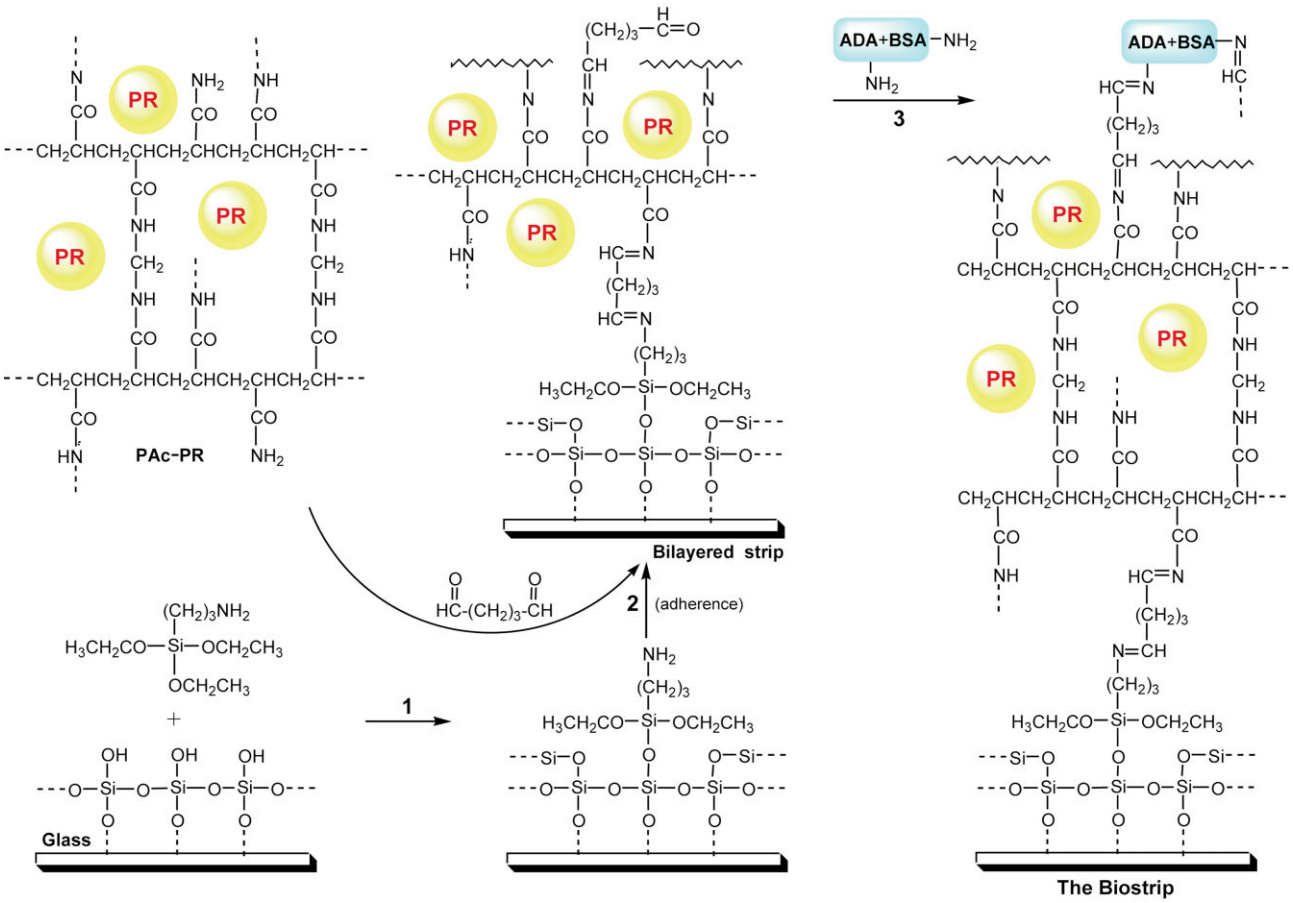

Figure 2. The construction of the biostrip. (**1**) Introduction of amino-groups on the surface of a glass strip using aminopropyl-triethoxysilane. (**2**) Glutaraldehyde-effected adherence of aminated glass to polyacrylamide carrying the indicator dye phenol red (PAc-PR), to form the bilayered strip (glass–PAc-PR). (**3**). Immobilization of adenosine deaminase (ADA) and albumin (BSA) on the glutaraldehyde-activated PAc-PR part of the bilayered strip via its free aldehyde-groups.

aldehyde functions of the activated bilayered strip will readily react with proteins, thus, forming a three-dimensional network together with the PAc part of the bilayered strip. To stabilize this network mechanically and protect the enzyme activity, BSA was used together with ADA to a 5:1 ratio. Immobilization experiments of ADA on glutaraldehyde-activated PAc-PR in the presence and absence of BSA showed (Sigma's assay) that BSA co-immobilization with ADA led to improvement of the catalytic activity by approximately 3.4-fold.

3.3 Determination of adenosine using the biostrip

Figure 3 shows the influence of adenosine concentration (buffered samples with starting pH 7.0) on the optical absorption of the biostrip at 567 nm (three repeats with SD 1.3–5.1%, Supporting information, Table S2). The change of absorption remains linear for up to a 30-fold dynamic range of adenosine concentration (R^2 = 0.9887), with a lower detection limit of 50 μM. Although this sensitivity limit is relatively high, compared to earlier reported opto-electronic [15–17] and fiber-optic enzyme biosensors [18, 19], nevertheless, the dynamic range of analysis offered by the biostrip compares favorably to similar immobilized enzyme systems, which varied in a 6- to 10-fold range [15–18,22].

The method proposed here was tested with biological samples originating from fresh human serum spiked with known amounts of adenosine. Human serum contains substances, other than adenosine (e.g. proteins and adenosine phosphates), able to bring about a slight bathochromic shift of the λ_{max} of bilayered strips carrying or lacking ADA activity, thus, leading to background readings. Therefore, the adenosine-in-serum samples (1 mL), prior to assaying, were filtered through a double mini column, containing 0.5 mL each of Cibacron Blue 3GA-agarose and DEAE-agarose, to remove proteins and adenosine phosphates, respectively. Filtered adenosine-in-serum samples showed approx. 97% reduction of protein content and were freed of adenosine phosphates

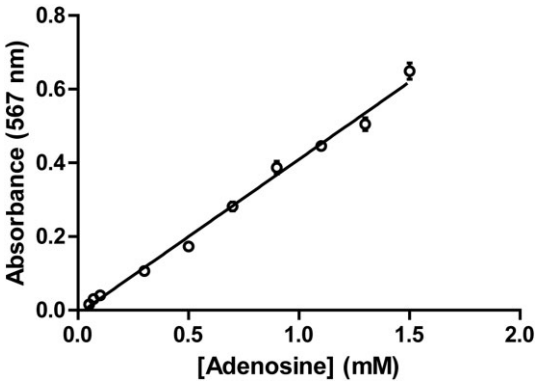

Figure 3. Adenosine calibration curve. The optical change at 567 nm is used as analytical signal for drawing the calibration curve. Each point represents the mean of three determinations (complete experimental data are presented in Supporting information, Table S2).

(Supporting information, Table S3). Filtered human serum (10 v/v) lacking adenosine, does not shift the λ_{max} of the biostrip, and it was used to set the zero readings.

The biostrip was shown to work successfully when assaying adenosine in filtered human serum. The adenosine recovery in diluted serum spiked with known amounts of adenosine (70 and 100 μM) near to the method's sensitivity limit, was ≈103% (found 103.1 ± 0.5 and 103.2 ± 1.9 μM, respectively; Supporting information, Table S4). The sensitivity of the biostrip for adenosine is limited to ≈50 μM, whereas its application range extends to 30-fold (0.05–1.5 mM, Fig. 3). A three-enzyme microelectrode biosensor for adenosine determination offers less than 1 μM sensitivity and 10-fold application range [12]. However, this biosensor is based on non-commercial enzymes (except ADA) and its construction is laborious and expensive, thus, limiting its applicability. An aptamer-based biosensor [14], though very sensitive for adenosine (3.0×10^{-11} mol/L) and able perform up to 30 assays, it is not a reagent-free one, and its construction is rather complex, requiring non-commercially available sensing materials.

3.4 Stability of the biostrip

The biostrip was assayed daily for its functionality using adenosine as substrate (Supporting information, Fig. S2). Although the decay of ADA activity, in nominal terms, was faster compared to other enzyme systems of similar technology [17–19], the biostrip maintained its actual useful functionality (approx. 10% of the initial "first day" activity) for more than a month and was used for ≈50 assays, being kept at room temperature for several hours. The amount of active ADA (≈0.03 units) left on the 100 mm^2 biostrip after approximately 1 wk since the original loading with ≈2 μg ADA (0.3 units) and ≈10 μg BSA, is still sufficient for many adenosine assays. For daily stability determinations, the biostrip was equilibrated for 1 h in phosphate buffer (1 mM, pH 7.0) at 25°C prior to assaying. The biostrip was then washed in phosphate buffer (100 mM, pH 7.5) and stored in the same buffer at 4°C.

4 Concluding remarks

An optical method for adenosine determination is presented. It relies on a transparent biostrip carrying ADA and the pH indicator PR, co-immobilized to PAc, itself chemically adhered to a glass strip. The signal generation is based on the ADA-catalyzed conversion of adenosine to inosine and ammonia that brings about a local pH change affecting the λ_{max} of PR (from 425 to 567 nm). The biostrip is reusable for several times and functional for weeks, if stored at 4°C, with working range 0.05–1.5 mM adenosine. The biostrip is applicable to diluted human serum containing adenosine (filtered through a mini-column for protein and adenosine phosphates depletion). This biostrip is moderately sensitive, but novel for it is regent-free, cheap, easy to construct and it is based on commercially available molecular entities.

The authors declare no financial or commercial conflict of interest.

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