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ORIGINAL PAPER

Applying Total Internal Reflection Excitation and Super Critical Angle Fluorescence Detection to a Morphine Assay

Hannu S. Välimäki · Timo Pulli · Kirsi Tappura

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Abstract A surface-sensitive fluorescence measurement platform is utilised in the detection of morphine. The platform is based on a polystyrene parabolic lens that enables the simultaneous application of total internal reflection excitation and supercritical angle fluorescence detection in the measurements. The molecular recognition of morphine is based on two antibodies, one against morphine and the other against the immune complex formed between the anti-morphine antibody and a morphine molecule. The antibodies are applied in a sandwich-like format in a one-step test, where the molecular binding onto a liquid-solid-interface is monitored in real time. Morphine concentrations between 0.6 and 18.2 ng/mL were reliably determined in 60 s, while concentrations down to 2.7 ng/mL were detected already in 20 s. With appropriate recognition molecules the technique is applicable also to other drugs and small analytes.

Keywords Fluoroimmunoassays · Total internal reflection fluorescence (TIRF) · Supercritical angle fluorescence (SAF) · Drug testing · Morphine

Introduction

Pharmaceuticals, drugs of abuse, steroids and toxins form a growing class of small analytes that need to be tested on different occasions by the authorities. For abused drugs, the available commercial diagnostic tests are typically lateral flow based competitive immunoassays. These tests, however, are usually relatively time-consuming to perform, their response-time can be several minutes, and due to their unspecific nature, they generate many false positive results [1]. Immunoassay tests for morphine and heroine, for example, typically cross-react with codeine that is a widely used substance in cough medicaments. Therefore, these tests are generally considered only as opiate-specific, and the positive test results must be confirmed using a more specific laboratory technique such as gas chromatography mass spectrometry.

In order to overcome the problem of the false positive test results and the long testing time, a one-step noncompetitive homogeneous immunoassay has been developed for small analytes using morphine as the model analyte [2]. The assay is based on a pair of engineered antibody Fab fragments, one against morphine (called M1) and the other against the immune complex (IC) formed between the anti-morphine antibody and a morphine molecule (called K11). In this assay, the presence of morphine molecules brings the two antibodies very close to each other. The antibodies are labelled with fluorophores forming a fluorescence resonance energy transfer (FRET) pair so that the close proximity of the antibodies leads to energy transfer from the donor to the acceptor fluorophore. The measured acceptor emission intensity corresponds to the morphine concentration. When tested with a commercial fluorometer, morphine concentrations down to 5 ng/mL were successfully detected within 2 min [2]. Compared to the recommend morphine cut-off concentrations of 40 ng/mL for drug testing [3], the achieved detection limit was very satisfactory.

Despite these achievements, there is still need for improvements, especially related to the speed of the test.

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In laboratory analysis or in the area of point-of-care diagnostics, the test duration of 2 min may be acceptable, but, for example, for a test to be used in the field, a testing time of 2 min is usually too long. An obvious way to further improve the assay speed and sensitivity is to further optimise the antibodies, the labelling ratio, the overall assay conditions as well as the signal to noise ratio in the fluorescence measurement. However, the optimisation of these parameters can improve the assay only to a certain degree. In order to take a more fundamental development step, a new scheme for the assay is proposed in this paper.

We have recently developed a novel fluorescence measurement platform based on a polystyrene parabolic lens [4]. This platform has its starting point in the works of Ruckstuhl et al. [5] and Enderlein at al. [6] and it utilises simultaneously both total internal reflection (TIR) excitation and supercritical angle fluorescence (SAF) detection principles, which makes the platform extremely sensitive to surface-bound fluorescence. The surface-sensitivity in the fluorescence detection provides means to transfer the morphine immunoassay from the pure liquid format into a sandwich-like format exploiting the binding processes occurring on a solid-liquid interface, but in such a way that it still remains essentially as a one-step assay. This new scheme is presented in Fig. 1, where the non-labelled anti-morphine antibody Fab fragments (M1) are immobilised onto a surface and the free sites are blocked against the non-specific binding before the labelled anti-IC antibody Fab fragments (K11) are set free in the sample liquid. The test is initiated by injecting the sample. If it contains morphine, the injection elicits the binding of the labelled K11 antibodies onto the surface. As it is schematically illustrated in the figure, the fluorescent detection can be restricted to the very vicinity of the surface so that no FRET interaction is needed to detect the mutual proximity of the antibodies. The morphine concentration is determined simply by monitoring the surface-bound fluorescence in real time.

The surface-bound assay scheme, where the extremely surface sensitive fluorescence monitoring is substituted for the FRET-interaction, results in some significant advantages. While FRET is a sophisticated method to study molecular interactions, it, as a coupling mechanism, inevitably creates losses, and thus, results in the attenuation of the detected signal. The efficiency of the energy transfer depends strongly ($1/r^6$) on the mutual distance of the fluorophores (r) that should generally not exceed 70 Å [7]. With two antibody Fab fragments we are close to this limit. Also the relative emission and absorption dipole orientations, which are typically out of control, influence the efficiency.

The other advantage of the surface-bound assay is the increased overall fluorescence detection efficiency. First, the molecules of interest are not distributed uniformly throughout the whole liquid volume, but are, due to the binding, concentrating at the interface where the detection is focused. Second, while the unbound fluorescent molecules in free space have an isotropic emission distribution, this is not the case when they are bound to a dielectric interface. The emission of the molecules located close to a dielectric interface is strongly directed towards the half-space of the higher refractive index, so that the usage of the SAF detection principle leads to significantly enhanced emission collection. Third, the evanescent field excitation leads to a decreased generation of background fluorescence in the sample liquid, which typically is one of the most limiting parameters in fluorescent assays. These facts, together with the absence of FRET and its demand for two different labels, are strong motives for transferring to the surface-bound assay scheme. In this paper, we report the first experimental results obtained in morphine detection.

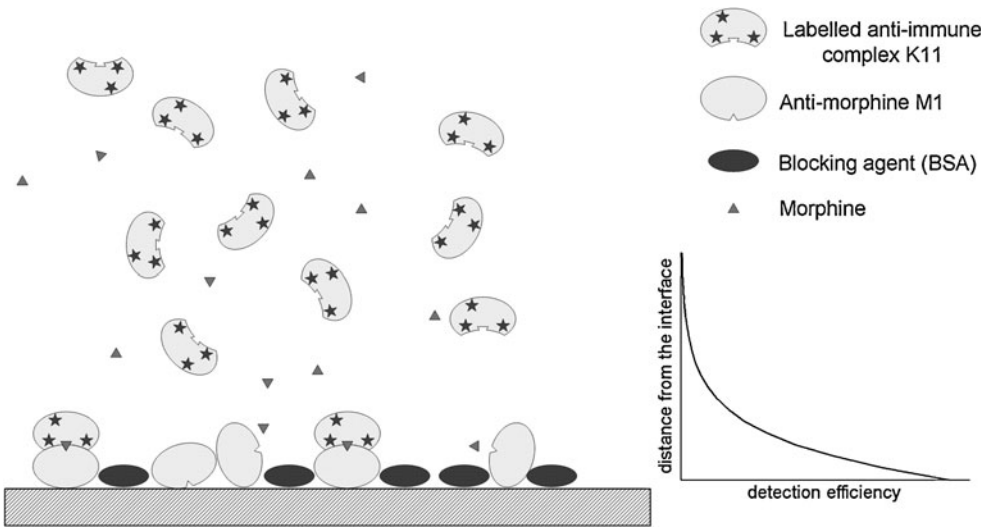

Fig. 1 The scheme of the surface-bound morphine immunoassay

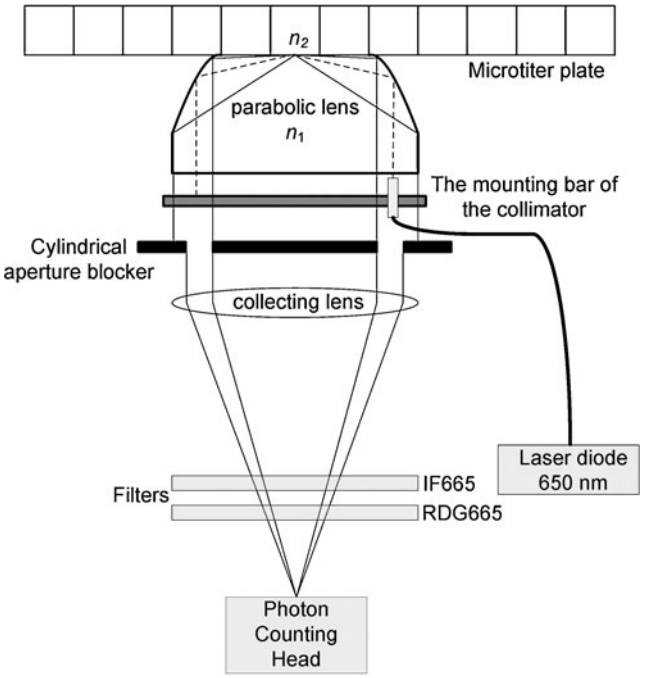

Fig. 2 The fluorescent measurement platform applying simultaneous total internal reflection (TIR) excitation and supercritical angle fluorescence (SAF) detection

Experimental

Antibodies

The antibody Fab fragments M1 and K11 were produced according to the methods described by Pulli et al. [2]. The antibody Fab fragments K11 were labelled with Alexa 647 fluorescence labels at our laboratory according to the instructions of the labelling kit.

Immobilisation

The microtiter wells were first coated with non-labelled anti-morphine antibody Fab fragments M1 that were diluted in phosphate buffer PBS (15 mM, pH 7.4) to a concentration of 10 μg/mL. A volume of 100 μl was pipetted into each well and the plate was left in dark for incubation for 90 min at room temperature. Then the wells were emptied and 100 μl of 0.5% skimmed milk powder dissolved in PBS was pipetted in order to block the remaining free sites against non-specific binding. After the blocking (90 min in dark at room temperature) the wells were washed (5×200 μL) with the blocking solution containing additional 0.05% of Tween20.

Fluorescence measurements

The fluorescence measurements were carried out with the system depicted in Fig. 2. A flat-bottom microtiter plate (F96, 456537, type MaxiSorp from NUNC) is placed on a polystyrene parabolic lens that is utilised in simultaneous TIR excitation and SAF detection. The set-up restricts the fluorescence read-out to the very vicinity (< 100 nm) of the surface so that the binding of the labelled antibodies onto the surface can be monitored in real time. The technical details and the system characteristics are described elsewhere [4]. In the present study, each individual measurement point corresponds to the number of photons counted during the single illumination time of 200 ms. The time interval T between individual measurements was set to T=10 s.

The injection procedure for morphine detection was as follows: First, the microtiter wells, coated with non-labelled M1 antibody fragments and the blocking agents as described above, were filled with 50 μL of PBS buffer. Then the fluorescence recording was started, and Alexa-647

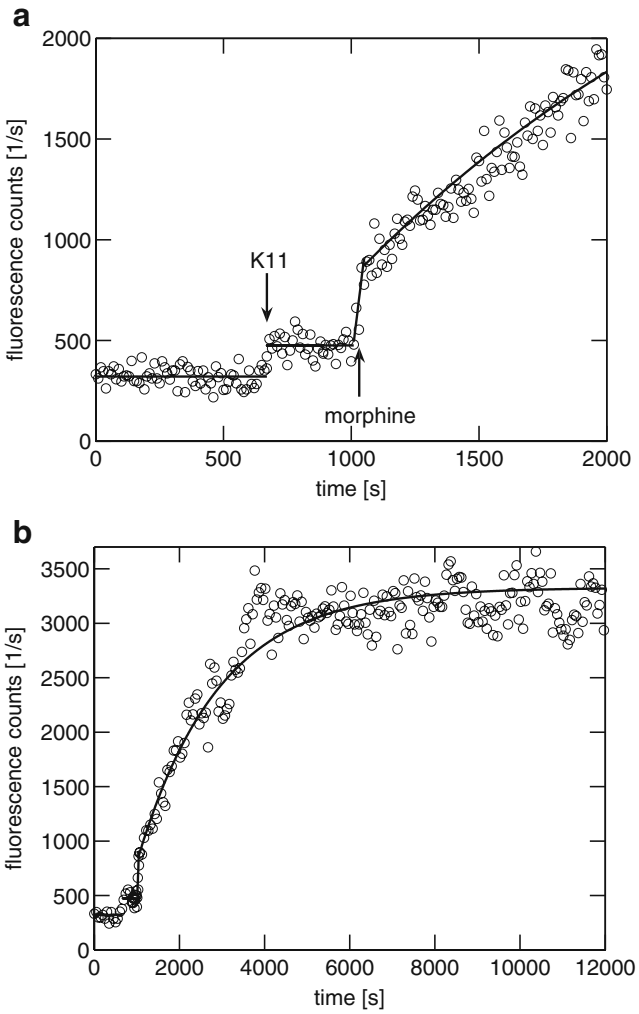

Fig. 3 (**a**) The first stages in morphine induced binding process: the injection of the Alexa-647 labelled K11 anti-IC antibodies into the final concentration of 5.0 μg/mL in 100 μL PBS, and the injection of morphine sample into the final concentration of 5.8 ng/mL in PBS. (**b**) The binding curve in its entire length. For the sake of clarity, only every fifth measurement point is shown. The *open circles* (o) represent the actual measurement points, the *solid lines* (-) the fits

labelled K11 antibody fragments, diluted in 50 μL of PBS, were injected into the well to the final concentration of 5 μg/mL. Finally, the morphine sample (10 μL in PBS) was injected into the well to obtain the desired final morphine concentration.

Results and discussion

A typical morphine measurement is shown in Fig. 3a and b, where the former figure presents the early phase of the measurement and the latter the entire recording. When only PBS buffer is present in the well (coated with M1 antibody fragments, as described above), the measured fluorescence intensity is shown to fluctuate around the value of 320 cst/s (Fig. 3a). Then at the timepoint t=670 s, Alexa-647 labelled K11 antibody fragments, diluted in 50 μL of PBS, are injected into the well to the final concentration of 5 μg/mL. This immediately shifts the measured intensity close to the level of 475 cts/s. It is noteworthy that the signal remains constant after the instant shift. This means that the blocking against non-specific binding is efficient, and that the shift corresponds only to the increased amount of the background signal stemming from the injected fluorescent labels in the sample liquid. Then at t=1,010 s, the morphine sample (volume 10 μL, concentration 64 ng/mL in PBS), is injected into the well leading to the final morphine concentration of 5.8 ng/mL. After the injection the signal starts to rise as the morphine molecules now bind to the M1 antibodies on the surface forming immune complexes that are capable to bind with the labelled K11 antibodies present in the sample liquid.

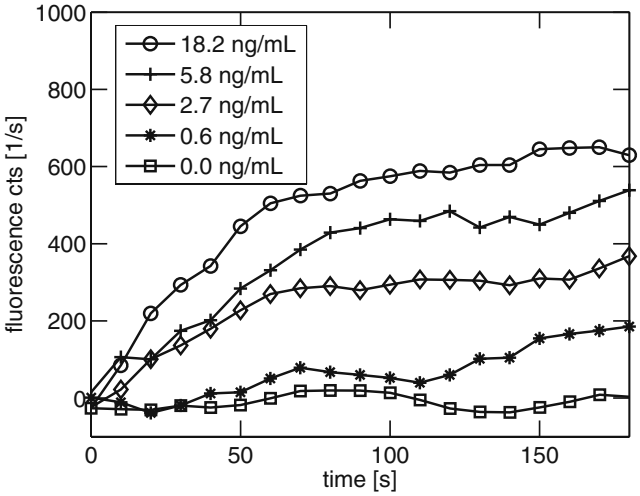

Fig. 5 A close-up of the responses of the Fig. 4 during the first 3 min of the measurements. The data is smoothed with a five-point moving average (backward)

The binding process of the K11 antibodies shows some peculiarities. First there is a brief phase (lasting 40–60 s) of rapid binding, and then a long phase (2–3 h) exhibiting a slower binding process. As a small molecule, morphine can quickly diffuse through the sample liquid, bind to M1 antibodies available at a high concentration in the early phase of the measurement, and thus very rapidly increase the amount of the available binding sites for the K11 antibodies, which, at the very beginning, are also available at a high concentration. These facts presumably induce the sharp initial rise in the response curve (marked as a straight,

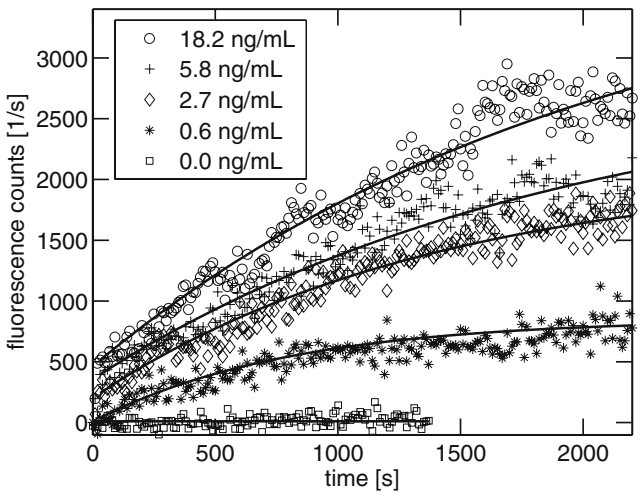

Fig. 4 The response of the system to different concentrations of morphine. The *markers* (o, +, ⋄, *, ·) present the actual measurement points, and the *solid curves* (-) the fits. The background levels are subtracted from the data

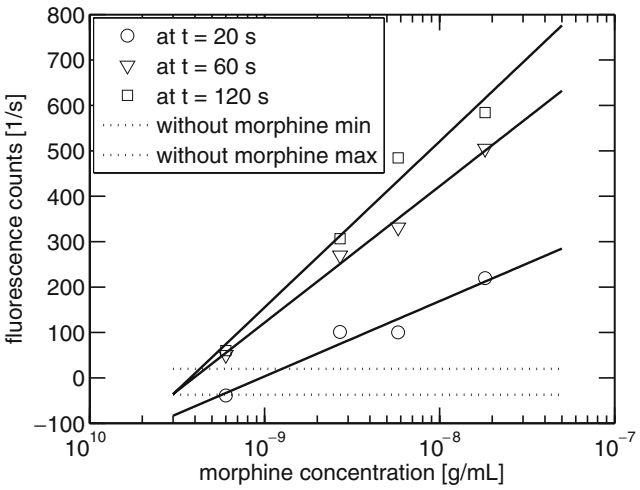

Fig. 6 The system response to different morphine concentrations at three timepoints after the morphine injection. The open markers denote the actual measurements, the *solid lines* (-) the fitted straight lines and the dashed lines the range of variation of the responses without morphine

almost vertical, solid line in the Fig. 3a). When the concentration of free morphine, as well as the concentration of the free morphine binding sites has decreased (and the number of the binding sites for K11 antibodies has started to saturate), and when the K11 antibodies closest to the surface are consumed, the binding rate of antibody K11 begins to slow down and show more a typical one-to-one binding kinetics. This assumption is supported by the good fit (shown as the solid line in Fig. 3b) of the one-to-one association model [8]

$$y = y_\infty\left(1 - e^{-kt}\right) \quad (1)$$

to the data starting at 40 s after the morphine injection. The best-fit parameter values are y_∞=2,456 cts/s and k=5.235×10^{-4} /s.

The response of the system to different concentrations of morphine is shown in Fig. 4. The background fluorescence level, measured after the injection of the K11 antibodies but before the injection of morphine, is subtracted from these curves. Together with the actual data points, also the best-fit curves of Eq. 1 are shown. The figure shows that both the initial rising velocity and the final equilibrium level depend logically on the morphine concentration, that is, the higher the morphine concentration, the higher are the rising velocity and the final equilibrium level.

Fig. 5 presents a close-up of the first 3 min of the response curves of Fig. 4, smoothed with a five-point moving average to the backward direction. The figure shows that the response curves differ logically from each other already in the very beginning of the measurements. To examine the early responses in more detail, the response values at three different time points, t=20 s; t=60 s; and t=120 s, extracted from Fig. 5, are plotted against the morphine concentration in Fig. 6. The actual data is presented with open markers and the fitted straight lines are plotted as solid lines. At all the time points, the dependence between the morphine concentration and the fluorescence intensity value is shown to be linear in the semilogarithmic scale, and the slope of the fit increases along with the time point value. The range of variation of the responses without morphine (describing the fluorescent background level) is shown for comparison with the dotted lines. The lowest measured concentration of 0.6 ng/mL gives a negative response at t=20 s that can not be distinguished from the background, but at the later time points all responses are strictly positive and differ clearly from the background. From these results it can be deduced that morphine concentrations at least down to 2.7 ng/mL can be detected already in 20 s, and that all the morphine concentrations studied (from 0.6 to 18.2 ng/mL) can reliably be predicted within 60 s.

Compared to the FRET-based assay reported earlier [2], we have now shown that the testing time can be reduced from 120 s to 20 s by transferring the molecular recognition from the sample liquid onto a liquid-solid interface and by applying both TIR excitation and SAF emission collection in the detection. In addition, more than eight times smaller morphine concentrations were reliably determined in 60 s with the new set-up. Furthermore, it should be noted that the concentrations of the K11 anti-IC antibodies were relatively small in the experiments presented above. With higher K11 concentrations also higher sensitivities are expected. Another way to obtain further enhancement in the response curves is to use site-directed immobilisation of M1 antibodies onto the surface with hydrophilic polymers with very low non-specific binding in between [9].

Conclusions

A very rapid and sensitive detection of morphine was realised with the surface-bound fluorescence immunoassay utilising simultaneous TIR excitation and SAF detection. The morphine concentrations between 0.6 and 18.2 ng/mL were reliably determined in 60 s, while the concentrations down to 2.7 ng/mL were shown to be detected already in 20 s. With appropriate recognition molecules the technique is applicable also to other drugs and small analytes.

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