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RESEARCH PAPER

A paper-based lateral flow assay for morphine

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Abstract Morphine was used as a model analyte to examine the possibility of using cellulose, physically modified by papermaking and converting techniques, as a capillary matrix in a lateral flow type of diagnostic assay. This research was directed toward low-cost, disposable, and portable paper-based diagnostics, with the aim of addressing the analytical performance of paper as a substrate in the analysis for drugs of abuse. Antibody Fab fragments were used as sensing molecules, and gold nanoparticle detection was employed. Inkjet printing was used to pattern sensing biomolecules as detection zones on paper. To validate the usefulness of paper as a diagnostic platform, the principle of a direct sandwich assay, based on immunocomplex formation between morphine and the anti-morphine Fab fragment and detection of the formed immunocomplex by another Fab fragment, was implemented. Results were compared with that achieved by using nitrocellulose as a reference material. Possible interfering from the sample matrix on assay quality was investigated with spiked oral fluid samples. Under optimized conditions, a visually assessed limit of detection for the sandwich assay was 1 ng/mL, indicating that the paper-based test devices developed in this work can perform screening for drugs of abuse and can fulfill the requirement for a sensitive assay in diagnostically relevant ranges.

Keywords Paper-based · Drugs of abuse · Recombinant antibody · Immunocomplex assay · Point-of-care diagnostics · Low resource · Capillary action

Introduction

Lateral flow tests have gained wide acceptance as simple, inexpensive, and robust platforms for detecting a variety of analytes, even in a complex physiological environment and belong to a valuable analytical discipline of point-of-care (POC) diagnostics [1–3]. Among the most important application areas of lateral flow tests intended for point-of-care diagnostics are tests to detect human chorionic gonadotropin (hCG), cholesterol, hemoglobin A1c, markers of infectious diseases, and drugs of abuse. In clinical laboratories, lateral flow tests are in most instances used to detect drugs of abuse, hypercholesterolemia and other lipid metabolism disorders, markers of infectious diseases; cancer antigens; and cardiac markers including creatine kinase MB, troponins, and natriuretic peptides [4]. In the lateral flow assay format, a sample liquid applied at one end of a porous lateral flow membrane is allowed to traverse through the porous structure by capillary action. The analyte in the sample is captured by the reagent on a sensing area in order to create a detectable signal. At present, the majority of lateral flow assays use nitrocellulose as an analytical membrane, although this carries potential disadvantages, such as complexity of production, flammability, and imperfect analytical reproducibility.

Diagnostic devices using paper have become a focus of active research, and this eventually has led to the invention of microfluidic paper-based devices (μPADs) reviewed among others by [5–11]. Paper possesses a fundamental property in

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transporting liquids by capillary action without any external equipment. Standard chromatography papers like Whatman 1 have so far almost exclusively been used as substrates to fabricate μPADs. In paper microfluidics, hydrophilic and porous channels of micrometer-scale are created on paper by patterning with hydrophobic agents. Another method has been to shape paper by cutting in two dimensions with a programmable knife. Here, the hydrophilic paper is bordered by air instead of hydrophobic material [12]. Yet, another example of recently developed paper-based diagnostic test devices is an approach which closely resembles conventional ELISA. These test devices were designed to fit in the same dimensions as 96-well plate but use much smaller reagent volumes, since the detection reactions occur in the paper matrix [13–15]. Moreover, assay platforms, which enable detection of multiple analytes, have been developed [16]. Paper substrate has been combined to such sophisticated detection methods as surface-enhanced Raman spectroscopy (SERS) in determining chemical residues [17] and for cancer biomolecular screening [18] or implementing new method in preparation of silver nanoparticle surfaces for SERS on paper chips [19]. Usually, the detection of signal developed in a specific reaction in paper-based diagnostics is based on visual detection, though portable devices, which use light reflectance and LED light source, have been successfully applied in quantitation of various analytes [20]. Patterning methods to create hydrophobic boundaries first used with paper have been widened to include nitrocellulose in fabrication of 3D-stacked microarrays for multiple analytes [21]. Moreover, the concept of lateral flow assay is further in the focus of active research and, for instance, a new method to pretreat samples and detect metal ions based on complexation reaction and competitive assay format has emerged [22].

Precise control of lateral flow in a fibrous network presents one of the essential requirements in assays, which utilize capillary flow to transfer and separate molecules in the sample liquid through the porous network. Capillary flow in straight capillaries can be predicted by the Washburn equation:

$$L = (\gamma r t \cos\theta / 2\eta)^{1/2} \quad (1)$$

where L is the distance the liquid advances under capillary pressure into a capillary in time t, γ is the surface tension of the liquid, r is the average radius of the capillary, θ is the contact angle, and η is the viscosity of the liquid [23]. Evidently, the flow of liquid in lamellar cellulose fiber structures can deviate much from the flow occurring in straight capillaries. Recently, in conjunction with the development of paper-based assays, an understanding of capillary flow and dynamics of flow in porous materials has become vital. The behavior of liquid imbibition was measured on constructs where nitrocellulose was cut into different shapes to provide the possibility of simplifying the conventional lateral flow test arrangement [24] or to develop more sophisticated assay devices on paper, where more than one reagent is taken to test the system in the capillary flow in the designed two-dimensional paper strips with sample inlets [25, 26].

The physical properties of paper are predominantly determined by the source of native fiber and how the fibers and fibrous network are modified and treated during the papermaking [27]. Inter-fiber hydrogen bonds, which form during the drying of manufactured paper, control the mechanical properties of paper. Hydrogen bonds break when fibers are exposed to water solutions and fibers detach from each other. Fibers also swell in water, and swelling is dependent on the content of cellulose, hemicellulose, and lignin in fiber. Cellulose I, the most prevalent form of cellulose in plants, exists in crystalline and less ordered amorphous structures. Crystalline regions are less sensitive to penetration by water and to chemical modifications than are amorphous regions. The stability of sensing molecules on paper upon storage and in ambient conditions during the completion of a bioassay is dependent on the properties of paper. Evidence of a fairly good stability of antibodies was obtained in a study, which mimicked environmental conditions of mass manufacturing, even though a combination of high temperature and high relative humidity accelerated conformational changes of the antibodies studied [28]. On the contrary, slightly decreased stability of antibodies on paper was proposed in a study, where activity of an antibody in preparations supplemented with various stabilizers was investigated by flow cytometry [29]. Besides studies with antibodies, enzymes are shown to retain their activities, when deposited by thermal inkjet printing method on paper surface [30].

Determination of drugs and their metabolites comprises a significant proportion of all diagnostic tests carried out in clinical laboratories or in sites of POC diagnostics including roadside drug testing, workplace drug testing, and screening hospitalized patients or athletes for either intentional or non-intentional use of drugs [31, 32]. Various sample matrices like blood, saliva, urine, sweat, and hair have been used in screening of drugs depending on the invasiveness of the sampling procedure, possibility to adulteration during sampling, the need of discrimination between actual drugs of abuse or their metabolites, and the time between exposure and analysis of drug. Immunoassay procedures have quite largely been adopted in the diagnostics of drugs regardless of lack in specificity due to cross-reactivity. Nevertheless, they suit extremely well for prescreening purposes [33, 34]. The diagnostic sensitivity, specificity, and efficiency of the available immunological methods for analysis of drugs of abuse in oral fluid have been evaluated by Bosker et al. [32]. For confirmatory analyses, liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) with modifications are used. Morphine belongs to the group of opioids and, in fact, in the majority of commercially available immunoassays

for opiates, antibodies are raised against morphine and simultaneously structurally similar opiates as codeine, heroin, 6-monoacetylmorphine, and hydromorphone are detected [35].

In this research, the applicability of paper as an analytical membrane in a lateral flow assay was demonstrated using morphine as a model analyte. Small-analyte immunocomplex assay made use of antibody Fab fragments derived from immunized Fab libraries or from non-immunized IgM libraries. Reproducibility, degree of nonspecific binding, detection of the developed signal, and antibody binding on cellulose were investigated. Spiked concentrations of morphine in a real human oral fluid sample were detected. By the use of recombinant antibody fragments and cheap mass manufactured paper, the directions toward diagnostic applications, which are easily accessible and available in settings with limited resources, were further justified.

Materials and methods

Reagents and materials

Anti-morphine Fab fragment and Fab fragment directed against the immunocomplex between morphine and anti-morphine Fab fragment, designated as M1 and K11, respectively, were generated as previously described [36]. Purified Fab fragments were dissolved in phosphate-buffered saline (PBS), pH 7.4. Anti-human $F(ab')_2$ polyclonal IgG was purchased from Rockland (Gilbersville, USA). Paper substrate, 0.15 mm in thickness and with a weight of 80 g m^2, was manufactured on a laboratory scale from eucalyptus fiber, as described in detail previously [37]. Poly(aminoamide)-epichlorohydrin) (PAE) was used as a wet strengthening chemical in the manufactured paper sheets. Bovine serum albumin (BSA), polyvinylpyrrolidone (PVP40), and a nonionic surfactant Tween-20 were obtained from Sigma. Fab fragments M1 and K11 were labeled to colloidal gold (40 nm, OD 10) by British Biocell (Cardiff, UK). Protein G-coated magnetic beads (Magne™ Protein G Beads) were from Promega (Madison, WI). All other reagents were of analytical grade and used as received. Purified water from Millipore, Direct-Q system was used for preparation of buffer and reagent solutions. Nitrocellulose membrane HiFlow180 was purchased from Millipore (Bedford, MA, USA). Whatman Fu-5 and Whatman CF6 materials were obtained from Whatman (Kent, UK). Plastic backing with an adhesive GL-187 was manufactured by G&L Precision Die Cutting (San Jose, California).

Inkjet printing of test zones on paper and on nitrocellulose

A working solution for antibody printing was PBS, pH 7.4 containing 0.1 % Tween-20. Anti-morphine Fab M1 and anti-human $F(ab')_2$ were diluted in this buffer at concentrations of 1.0 and 0.5 mg/mL, respectively. The diluted antibody inks were filtered through 0.22-μm-diameter syringe-driven filters (Millex GV, Millipore) prior to insertion into disposable inkjet print cartridges (piezo-based inkjet print cartridges, each with 16 nozzles, 254 μm spaced and 21.5 μm in diameter, DMC-11610, Fujifilm, Santa Clara, USA). The drop size was 10 pl. Anti-morphine Fab M1 and anti-human $F(ab')_2$ were printed to form test and control lines, respectively, 5 mm apart from each, using a piezoelectric inkjet printer (Dimatix DMP-2831 materials printer from Fujifilm Dimatix Inc., Santa Clara, USA). Drop spacing was adjusted to 10 μm in order to deposit 1 μg of antibodies into the $1 \times 5\text{-mm}^2$ signal detection area on paper or on nitrocellulose. The temperature of the cartridge was controlled at 30 °C during printing. A standard waveform with a single pulse developed by the manufacturer as well as firing voltages and frequencies were adjusted to achieve regular print patterns and consistent drop formation. The printed materials were stored at 4 °C and protected from moisture until use.

Fabrication of paper-based lateral flow devices

Materials to compose the paper-based lateral flow test device consisted of sample application pad (20 mm × 10 cm), conjugate pad (10 mm × 10 cm), immunochromatographic membrane, either paper (32 mm × 10 cm) or nitrocellulose (25 mm × 10 cm), absorption pad (25 mm × 10 cm), and a backing card (Fig. 1a). Sample application and conjugate pads were made of glass fiber (Whatman Fu-5), and absorbent pad was a mixture of glass fiber and cellulose (Whatman CF6). The conjugate pad was prepared by dispensing 80 μl of K11 gold conjugate diluted in 800 μl of 25 mM sodium borate buffer (pH 8.5) containing 2 % BSA, 3 % sucrose, 0.6 M NaCl, and 0.2 % Tween-20. After being dried, the conjugate pad was stored at +4 °C. Paper or nitrocellulose membrane was blocked by soaking in a selected blocking buffer and gently shaken at room temperature for 30 min. After drying, the blocked membrane and other parts of the test device were affixed onto the plastic backing. The sample and conjugate pads at the sample end as well as the absorbent pad at the distal end overlapped 2 mm with paper or nitrocellulose membrane to generate imbibition of liquid through the test device after sample application. After assembly, the test device was cut into individual 5-mm-wide strips with a roller cutter.

Analysis of morphine using paper-based lateral flow devices

Morphine solutions in concentrations ranging from 0 to 100 ng/mL were prepared in PBS with 1 % BSA and 0.25 % Tween-20, pH 7.4, in order to carry out an immunocomplex assay. Morphine standard solutions were applied in the volume of 80 μl. The sample solution was allowed to migrate

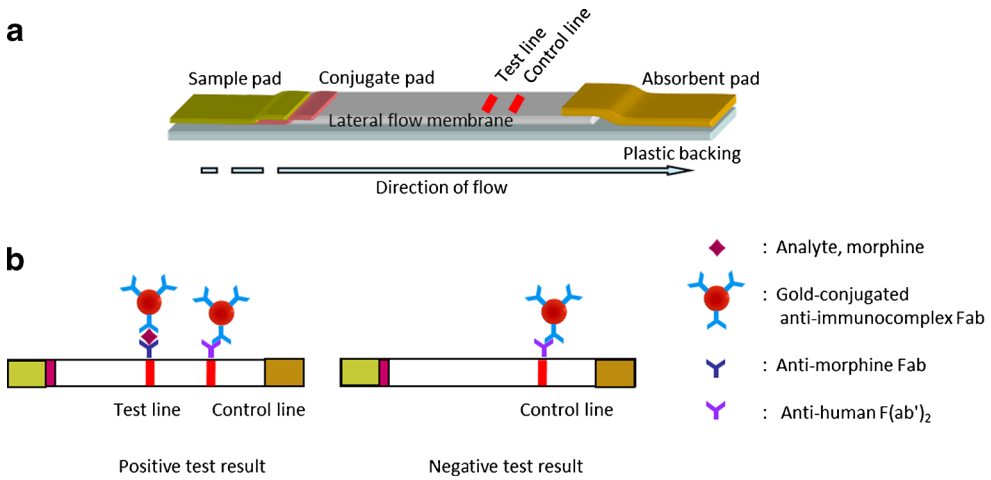

Fig. 1 Schematic diagram of the assembly of the paper-based lateral flow test (**a**) and the illustration for interpreting test results in an immunocomplex assay (**b**). Aqueous sample containing morphine is applied to sample pad, dissolving the gold conjugate dried on conjugate pad. During capillary flow, morphine is captured by the immobilized anti-morphine Fab on test line, and the immunocomplex formed is recognized by the gold-conjugated anti-immunocomplex Fab. On control line, gold-conjugated anti-immunocomplex Fab is recognized by anti-human F(ab′)$_2$ both with and without morphine in the sample thus serving as a control of a successful assay

along the paper or nitrocellulose membrane toward the absorption pad by capillary action. The signal was qualitatively estimated visually after 5–20 min, and intensities of the test and control lines were quantitatively determined by inserting the test strip into a POCTER reader [38] followed by the measurement of the intensity of reflected light. The scanned images of the test strips were acquired by a flatbed scanner (Epson Expression 1680; Epson, Tokyo, Japan). In order to study the matrix effect of real samples on the developed paper-based assay, saliva from a non-addict volunteer was collected by using a saliva collection device OnTrack OraTube (Varian, Lake Forest, CA). The saliva sample was diluted 20 times in PBS with 1 % BSA and 0.25 % Tween-20 and centrifuged at 14,000 rpm for 10 min. The immunoglobulin fraction from the diluted saliva sample was removed to eliminate cross-reactivity against gold-conjugated Fab fragment K11 through selective binding to protein G-coated magnetic beads. A volume of 5 μl of protein G beads was added into 1 mL of diluted saliva and incubated with agitation for 30 min at RT. After collection of the protein G beads by a magnet, the supernatant was spiked with morphine to prepare a concentration series of 0, 0.01, 0.1, 1.0, 10, and 100 ng/mL morphine in diluted saliva.

Results and discussion

Immunocomplex lateral flow assay for morphine on paper

Cellulose paper manufactured of eucalyptus fibers was chosen as a matrix for lateral flow assays according to the results of research, in which papermaking and converting methods were employed to tailor the paper structure to have properties suitable for a lateral flow membrane [37]. The functionality of the test platform developed was evaluated through morphine, a routinely screened candidate of drugs of abuse, using as recognizing elements anti-morphine Fab M1 and anti-immunocomplex Fab K11 antibody pair. Being a small antigen, morphine (MW 285 g/mol) can be difficult to detect as a single molecule by antibodies in a sandwich immunoassay, where two antibodies bind simultaneously the antigen. Therefore, a test system was developed, where morphine is recognized, when bound to an antibody (anti-morphine Fab M1) by another antibody (anti-immunocomplex Fab K11). It is believed that in the epitope of the immunocomplex, there are structural determinants of morphine and anti-morphine Fab fragment. In the immunocomplex assay, morphine as an analyte was transferred in the sample buffer along the test strip by capillary action and was captured by Fab M1, which was immobilized on the test line. The formation of an immunocomplex was verified by the formation of red color on a test line as a result of the binding and accumulation of gold-conjugated Fab K11. The higher the concentration of analyte in the sample, the higher is the amount of gold conjugate bound with the immunocomplex and intensity of red color. Thus, the intensity of red color on the test line would be proportional to the concentration of the analyte in the samples and would be unchanged after saturation point at higher analyte concentrations. Sample without morphine was used to evaluate the level of nonspecific binding of the antibodies or compounds from the sample, which would trigger nonspecific binding of the gold conjugate in the absence of morphine. The formation of red color on control line resulting from binding of the anti-immunocomplex Fab fragment K11 with anti-human F(ab′)$_2$ was used as verification of the performance of the assay including sufficient transfer of

liquid and gold conjugate through different parts of the test device into the absorbent pad. In order to carry out quantitative determination of morphine concentrations in the samples, the intensities of test and control lines were acquired by a portable reader. Thus, the use of portable reader enabled numerical justification of the test result in addition to a visual interpretation.

Effect of nonspecific interaction on assay quality

In order to develop a paper-based assay device, which would be sensitive enough to measure reliably morphine in concentrations commonly found in screening and quantitation of drugs of abuse, among the parameters to be optimized is the degree of nonspecific binding. The reactive groups in paper network, which would interfere with binding of the antibodies with their analytes or which can directly bind the detection antibody, have to be eliminated. To address this event, five different blocking buffer compositions were compared, containing compounds which are very widely used to reduce nonspecific binding in surface-capture bioassays. BSA was selected as a commonly used blocking agent to prevent protein binding on cellulose, and nonionic surfactant Tween-20 was selected to reduce any hydrophobic interactions and to decrease surface tension of the sample buffer. In addition, blocking was carried out at two different pH values: pH 7.4 and pH 8.8. Figure 2 shows that highest intensity both on test line and on control line was achieved for 1 ng/mL morphine, when the paper surface was blocked with PBS buffer, pH 7.4, with 3 % BSA and 0.1 % Tween-20. The reason why in higher BSA concentration highest signal intensity was observed could be saturation of the high surface area of cellulose fiber

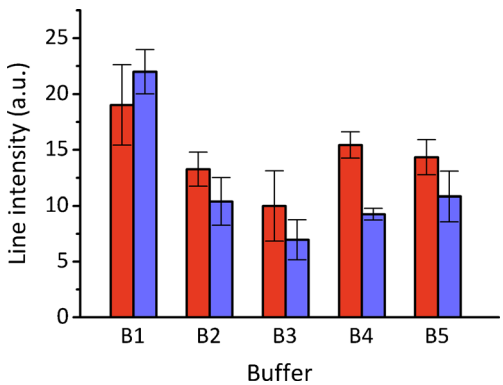

Fig. 2 Lateral flow assays were carried out to determine the effect of blocking buffers on the intensity of test line (*red*) and control line (*blue*). The compositions of buffers were (*B1*) 3 % BSA, 0.1 % Tween-20; (*B2*) 1 % BSA, 0.1 % Tween-20; (*B3*) 1 % BSA, 1 % sucrose, 1 % PVP, 0.1 % Tween-20; (*B4*) 1 % BSA, all dissolved in PBS, pH 7.4; and (*B5*) 1 % BSA, 0.1 % Tween-20 in 100 mM sodium borate, pH 8.8. The concentration of morphine in the test sample was 1 ng/ml in a sample buffer, 1 % BSA and 0.25 % Tween-20 in PBS, pH 7.4, and the volume of the sample was 80 μl in lateral flow assay. *Each column* is the mean of three assays and the *error bars* represent the standard deviations of the assays

network, which hinders further nonspecific attachment of components of the immunoassay on cellulose surface. The chosen two pH values did not seem to significantly affect the measured line intensities. To further characterize the role of blocking on the quality of the developed paper-based lateral flow assay, assays were run in a series of 0–100 ng/mL of morphine in the sample on unblocked and blocked paper (Fig. 3a, b). It was observed that without blocking procedure, the signal intensities are somewhat higher in lower morphine concentrations, i.e., at 0.01 and 0.1 ng/mL, while the standard deviations of the assays are little higher especially in higher morphine concentrations. As a conclusion, a blocking step using PBS buffer with 3 % BSA and 0.1 % Tween-20 (Fig. 2, B1) was employed in the following experiments.

Basic mechanisms by which molecules interact with the surface are primarily dependent on whether the surface is hydrophilic or hydrophobic [39]. Evidently, there are two main factors responsible for nonspecific binding of solutes on a pure cellulose surface or on cellulose modified by papermaking chemicals. Firstly, the analytes may bind on the regions outside detection zones, or other compounds in the sample may replace or bind to the immobilized antibodies on the detection zone. Secondly, the antibody gold conjugate may interfere with the cellulose or may be physically trapped inside the fiber structure. Cellulose is slightly negative in charge and is largely hydrated in aqueous solutions. Similarly, proteins are largely hydrated through hydrogen bonds between water and hydrophilic clusters on protein surfaces. From the viewpoint of free energy, the change is small whether proteins bind to cellulose from solution or reside in solution, thereby being quite different situation compared to the immobilization of proteins on more common assay surfaces such as nitrocellulose and hydrophobic plastic surfaces. Regarding these more common assay surfaces, a conformational change of a protein is necessary for becoming immobilized from solution on hydrophobic surface at least in a certain degree to allow interaction between a hydrophobic surface and the more hydrophobic core of a protein. More specifically, in a study in which the adsorption behavior of BSA on hydrophilic and hydrophobic surfaces was investigated, it has been proved that BSA in a PBS buffer, pH 7.4, had almost entire coverage on a hydrophilic surface studied. This was partly explained by the observed formation of BSA–phosphate surface complexes [40]. It was also shown that BSA with a quite flexible structure may undergo conformational changes in order to align with cellulose chains, and at pH 7.4, interactions occur between positively charged clusters of BSA surface and negatively charged cellulose or between carboxyl groups of hemicellulose present as residual polysaccharides in the paper.

Yet, another possibility for interaction between proteins and cellulose is the additives used in papermaking. Cellulose used as a lateral flow membrane in this research was treated

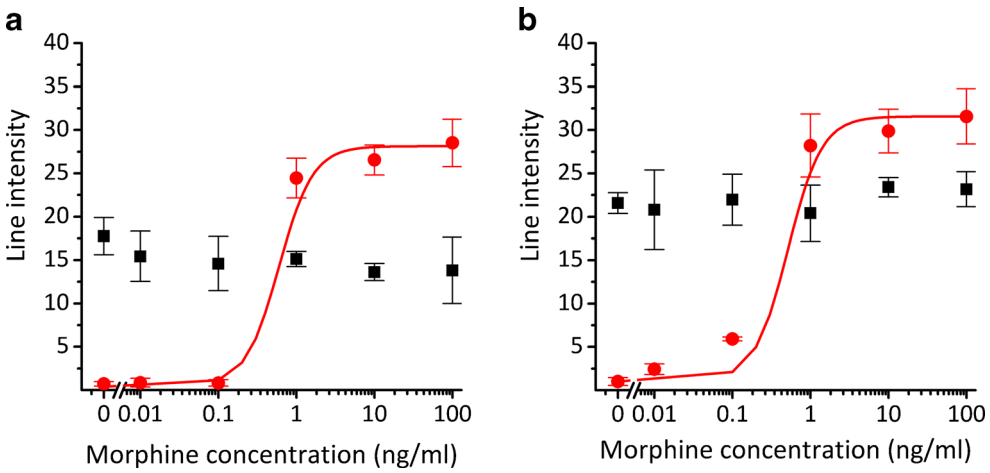

Fig. 3 Comparison between blocked (**a**) and unblocked (**b**) paper sheets for the detection of morphine at concentrations from 0 to 100 ng/ml in 1 % BSA and 0.25 % Tween-20 in PBS, pH 7.4. Intensity on the test line is shown as *red circles* and *red line* and intensity on the control line as *black squares*. Each data point is the mean of three assays, and the *error bars* represent the standard deviations of the assays

with PAE in order to enhance the stability of the fiber structure in aqueous conditions. It has been presented that PAE reacts with cellulose by forming a cross-linked cationic polymer network on the fiber surfaces. Thus, the attachment of cellulose fibers with each other in water occurs not only via hydrogen bonds of cellulose but also through cationic PAE and the hydroxyl groups of cellulose [41]. Thus, PAE may also interact with proteins via positively charged groups.

Gold nanoparticles were employed as signal transducers in this paper-based lateral flow assay due to their intensive red color and well-known conjugation chemistry for proteins. In general, gold nanoparticles interact by hydrophobic interactions, through ionic interaction of the negatively charged gold nanoparticle surface and through binding via sulfur or nitrogen atoms to other molecules such as proteins [42]. It is likely that gold, which is negatively charged and hydrophobic, would not have any selective adsorption to cellulose. However, the observed quite high intensity of red color outside test and control lines on paper and thereby lower visual quality of the assay may be a result of diffusion and entrapment of Fab fragment gold conjugate between layers of fibers or inside the fiber lumen. Detailed characterization of the paper structure has shown that fibers contain pits, which can be up to 4–6 μm in diameter and are only partially compressed during papermaking [43]. This size is large enough to enable entrapment and residence of gold conjugate nanoparticles inside the fiber lumen.

Effect of liquid flow rate on assay quality

The sensitivity and specificity of a lateral flow assay are much affected by the flow rate of fluid, dissolved analytes, and detecting molecules in sample liquid. A high flow rate is needed to transport a large enough amount of analytes over the test zone and to diminish the background by washing out unbound detecting molecules in order to avoid generation of false-positive signals. Moreover, available linear range is narrower at lower flow rates as a consequence of evaporation of the traveling sample liquid during lateral flow. Conversely, lower sensitivities can also be the result of too high a flow rate, as there is not enough reaction time for immunocomplexes to form. In the context of nitrocellulose, the desired flow rate is adjusted by selecting the type of material according to pore size and by the design of the assay procedures, of which selection of a blocking buffer is of great importance. Higher concentrations of proteins decrease the flow rate, whereas the presence of surfactants aids in rewetting the matrix by lowering its surface tension. Undoubtedly, the same also holds for paper, although the interactions between blocking agents and paper are different.

The sample volumes for paper and nitrocellulose were 80 and 50 μl, respectively. The higher water-absorbing capacity of paper compared to nitrocellulose required a higher sample volume in order for the sample to enter the absorption pad at the end of the test strip. Earlier research in our lab demonstrated that it was possible to adjust the capillary flow time of paper to fall into the same range as nitrocellulose by modifying the physical properties of paper [37]. Essentially, this was achieved by selecting the native source of the fiber species and through modifying the pore size and density of the paper. As the fibers absorb water, the space between fibers, which acts as a pore structure to transfer liquids, becomes smaller. Swelling of the fiber wall during water exposure also results in a smaller volume between fibers and inside fiber lumen. Moreover, wetting of paper is a cause of partial detachment of individual fibers from each other in parallelly organized fiber layers. When wetted paper is let to dry freely without compression, the opened fiber structure remains and fibers collapse together. Consequently, the mean pore size is higher than in a never-wetted paper. Water evaporation causes lowering of pressure and fibers coalesce with each other and hydrogen bonds can form between fibers. Prior to measurement of capillary flow time, the main difference between blocked and unblocked paper was that the former was let to dry without compression

after blocking step and the latter was compressed during papermaking. Thus, in blocked paper, the pores were larger and flow rate higher than in unblocked paper. Notably also, blocked paper was treated with a blocking agent containing surfactant Tween-20, which gives rise to flow rate by lowering surface tension. These events most probably lie behind the observation that the capillary flow time was shorter in blocked paper than in unblocked paper (Electronic Supplementary Material Fig. S1). Yet, another issue is that treating of blocked paper with surfactant may contribute to faster liquid flow due to the decrease in surface tension. Blocked paper was once wetted and dried, and unblocked paper had not been in contact with water after papermaking process and prior to measuring the capillary time. According to the Washburn equation, which describes the capillary flow of liquids in narrower straight capillaries, rate of the movement of the liquid front is a direct correlation of a square root of the diameter of the capillary or size of the pore in case of paper. The pore structure of nitrocellulose is much more rigid, and the difference in measured capillary times was not so remarkable between unblocked and blocked nitrocellulose membrane. There might be other issues which in fact can decrease the flow rate after blocking such as the space-filling effect of proteins, which attach to the pore walls from the sample liquid. This could have led to the measured higher capillary time in blocked nitrocellulose.

Analytical performance of paper-based LFA for morphine

Figure 4 reports the lateral flow assay in the immunocomplex format in a concentration series ranging from 0 to 100 ng/mL of morphine on both paper and nitrocellulose. Nitrocellulose served as a reference material, for which the construction of the test device as well as the method of blocking were the same as for paper. The test result became detectable by the naked eye 6–10 min after the sample application, but the final interpretation of the test was made when the test device was fully air-dried. A detectable positive test signal was obtained at a concentration of 1.0 ng/mL of morphine for both paper and nitrocellulose and can as such regarded as a qualitative limit of detection. A detection limit of 5 ng/mL was suggested in a previous study using the same Fab fragments in a FRET fluorescence immunoassay for morphine [36].

Figure 4 and the standard curve of morphine assay on blocked paper in Fig. 3a show that there was a steep increase in the signal intensities after concentration of 1.0 ng/mL, and after this inflection point, only moderate change is observed in the intensity over a concentration range from 1 to 100 ng/mL. This similar behavior in signal intensities was detectable when nitrocellulose served as an immunogromatographic membrane. No obvious test line intensity could be observed, when morphine was absent in the sample buffer. In order to assess the possibility of the developed assay on paper to become a

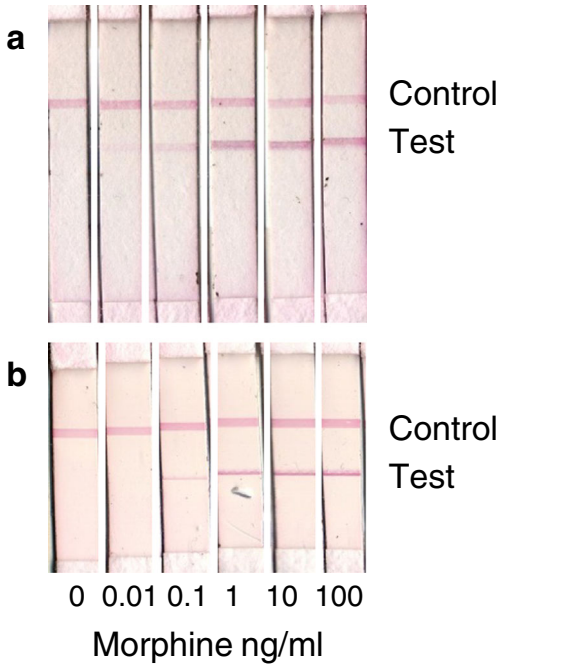

Fig. 4 Images of the immunocomplex assay results from 0 to 100 ng/ml of morphine per test strip (**a**) on paper and (**b**) on nitrocellulose

quantitative test and to find out linear relationship between morphine concentrations and signal intensities, assays in a narrower range, i.e., less than 5 ng/mL of morphine, were carried out. According to Fig. 5a, b, it can be seen that the developed assay, in fact, allowed quantification of morphine in an unknown sample only at concentrations less than 1–2 ng/mL, where the intensity of the red color developed on the test line was proportional to the concentration of morphine in the standard solutions. The data presented in Fig. 5b was acquired by measuring the signal intensities along the test strip by the reader. This indicates that peaks with regular shapes at the detection and test lines were possible to obtain. Moreover, as illustrated in Fig. 5b, the variation in the background outside the peak area is relatively high, but the measured level of background light reflection intensity was always lower than the level of the intensity of the lowest measured morphine concentration (0.05 ng/mL). The observed narrow range of linearity may be explained by the fact that capturing antibodies detach from the test line in aqueous sample solutions because these are immobilized only by physical absorption and therefore the binding capacity on the test line is saturated at a 2-ng/mL concentration of morphine. The amount of the Fab fragment immobilized on the paper becomes a limiting factor for binding morphine and the gold conjugate. However, on nitrocellulose, the signal generation for morphine appeared to become visibly detectable at the same morphine concentrations as for on paper. Under these circumstances, the steep rise in signal intensity above concentration of 2 ng/mL of morphine could be a consequence of the binding kinetics of the Fab fragments in such a way that if the affinities

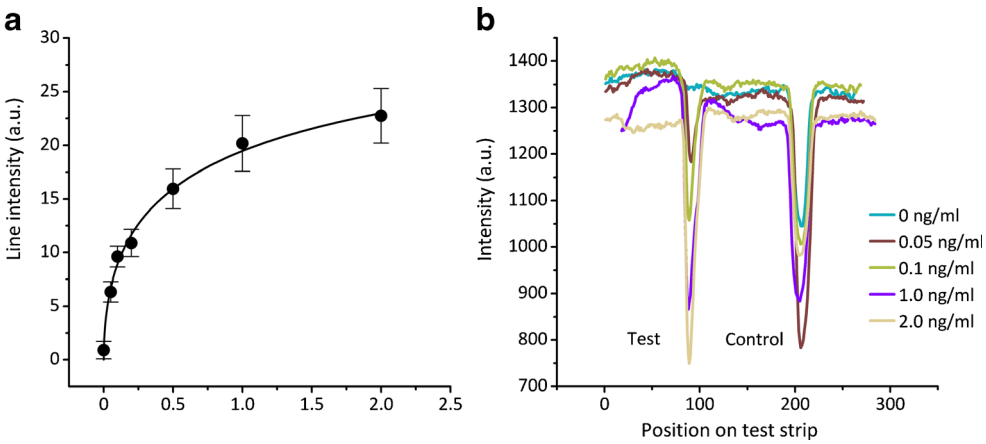

Fig. 5 Calibration plot for morphine in a concentration range from 0 to 2.0 ng/ml (**a**). Each data point is the mean of three assays, and the *error bars* represent the standard deviations of the assays. Reflectance values measured by a strip reader along the test strip for each assay with concentrations of 0, 0.05, 0.1, 1.0, and 2.0 ng/ml morphine standards in sample buffer (**b**)

of the Fab fragments are low at higher concentrations, morphine is not bound by the immunocomplex but is in free form.

Figure 3a also shows that there is a correlation between the intensities of test and control lines, as the intensities of controls tended to decrease with increasing concentrations of morphine and with higher intensity test lines. In an ideal lateral flow assay, the response on the control line is constant throughout the assay series. This observed variability in the responses on control lines may be caused by uneven flow of the antibody-conjugated gold nanoparticles, and it may be possible that not all of the conjugate reaches the control line. This becomes more pronounced at higher morphine concentrations, when the amount of the conjugate decreases locally after the test line.

Attachment of Antibodies on the Paper Surface

The technique by which test and control lines are patterned on paper most obviously have a considerable impact on the qualitative performance of the developed paper-based lateral flow assay. In this study, piezoelectric inkjet printing was applied. It was considered to be very suitable for printing antibody containing inks on paper substrate being, in general, contactless, fast, and easy, with no need for predesigned templates. Moreover, when the volume of the printed drop was only 10 pl, the spreading of the deposited spot due to capillary forces before drying could be minimized. By means of inkjet printing, it was possible to form well-defined printed patterns with sharp boundaries on paper. The drawback in piezoelectric printing is the very narrow window of viscosity and surface tension of water containing biological inks. For optimal jettability from a piezoelectric print head, viscosity should be between 8 and 14 mPas [44]. The viscosity of water solutions is very low, less than 1 mPas, and surface tension is very high, over 58–60 mPas dynes/cm. Antibodies to be printed were dissolved in PBS buffer pH 7.4 supplemented with 0.1 % Tween-20 without any printing additives. The composition of the ink also affects the quality of the printed features on paper and the penetration of liquid and dissolved components into the paper matrix, as well as the amount of antibodies retained on the paper network after being subjected to the flow of liquid during the assay procedure. If the printed antibodies penetrate too far into the paper network, the signal originating from the binding event of a deeply located capture antibody would not be detectable on the surface. A number of printing additives have been presented in order to enhance the printing quality as well as stability and adhesion of printed proteins on more commonly employed surfaces such as glass, silicon oxide, gold, and nitrocellulose [45]. These include trehalose, sucrose, glycerol poly(ethylene glycol) (PEG), and polyvinyl alcohol (PVA). The length of the polymers is also a determining factor. It has been found that polymers such as PVA performed better in their higher molecular weight forms in immobilizing antibodies on nitrocellulose during flexographic printing by intertwining the antibodies on the surface [46]. The surface chemistry of cellulose significantly differs from that of surface materials more commonly used in diagnostics, and hence, the adhesion of antibodies on cellulose is different compared to, for example, more hydrophobic nitrocellulose. Cellulose surface can be beneficial to antibody stability owing to the hydrophilic nature and rigid structure, but the level of binding can be lower than on other diagnostic surfaces, since hydrophobic interactions are stronger than ionic interactions. Presumably, the physical attachment of antibodies on cellulose is enabled in a neutral buffer with pH higher than the pI values of most antibodies, if the ionic concentration is high and the electric repulsions are low. This condition always holds after printing of antibodies, when water evaporates from the printed layer. To overcome possible drawback in the analytical performance of paper-based assays due to the limited level of immobilization of antibodies on paper surface, several methods to increase the surface density of antibodies have been developed including chemical modifications of cellulose [47] and utilization of specific building blocks, which consist of a cellulose binding domain and an antibody binding domain [48].

Detection of Morphine in Real Samples

The validity of the paper-based morphine assay in actual diagnostic settings and the background effects of a biological sample were investigated by an oral fluid spiked with morphine. Oral fluid samples are preferred in screening drug abuse in routine controls because this method is noninvasive and samples can be taken under controlled conditions, thus avoiding the adulteration of samples. There are certain differences in levels and occurrences of the metabolites of opiates as well as background effects depending on whether oral fluid, serum, or urine is used for testing for drugs of abuse [31]. The saliva sample was taken with the aid of an oral fluid collector to avoid the disturbance arising from the high viscosity of saliva. The oral fluid was passed through a swab into a sample tube. Oral fluid sample was diluted 1:20 with 1 % BSA and 0.25 % Tween-20 in PBS, pH 7.4, and centrifuged to remove cellular or other debris from the sample. For a specific need, immunoglobulins present in oral fluid were depleted by magnetic beads coupled to protein G. This additional step was necessary to perform due to the incompatibility of the anti-immunocomplex antibody Fab K11 with human samples, since anti-morphine Fab K11 fragment was originally isolated from a naïve human antibody fragment library and carried antigenic determinants, which were of human. Human antibodies also present in saliva would occupy the binding sites of the anti-human $F(ab')_2$ antibody, which served as a control line, and hence, the appearance of a visible control line would have been blocked. Additions of morphine were made in diluted, centrifuged, and immunoglobulin-depleted saliva sample. Untreated oral fluid did not travel either on paper or on nitrocellulose. It is known that morphine may remain bound with the components of saliva. Figure 6 shows the images and calibration curve of the immunocomplex morphine assay on paper and nitrocellulose for a range of concentrations of standards between 0 and 100 ng/mL in an assay buffer. By visual interpretation, the qualitative limit of detection is both on paper and nitrocellulose 1 ng/mL, although a faint band is seen on both already at 0.1 ng/mL. Considering that the original oral fluid sample was diluted 1:20, the limit of detection in oral fluid would be 20 ng/mL. Quantitative image analysis (Fig. 6c, d) reveals a significant matrix effect on paper as the variation in assay results is higher than in nitrocellulose, even though also the overall intensities are higher on paper, which might preferably reflect inter-assay variation rather than a true situation. The calculated CV% (percentage of standard deviation divided by mean) varied from 10 to 28 %, in samples where a signal was detectable. The red color on the test line started to develop 6 min after application of the sample, and after 20 min, no further development of the color was observed. Irregularities in the flow of both sample liquid and gold conjugate are the most obvious reasons for the lower reproducibility of the assay. Evaporation of the sample liquid becomes more pronounced as the flow of the liquid proceeds, and changes in concentrations

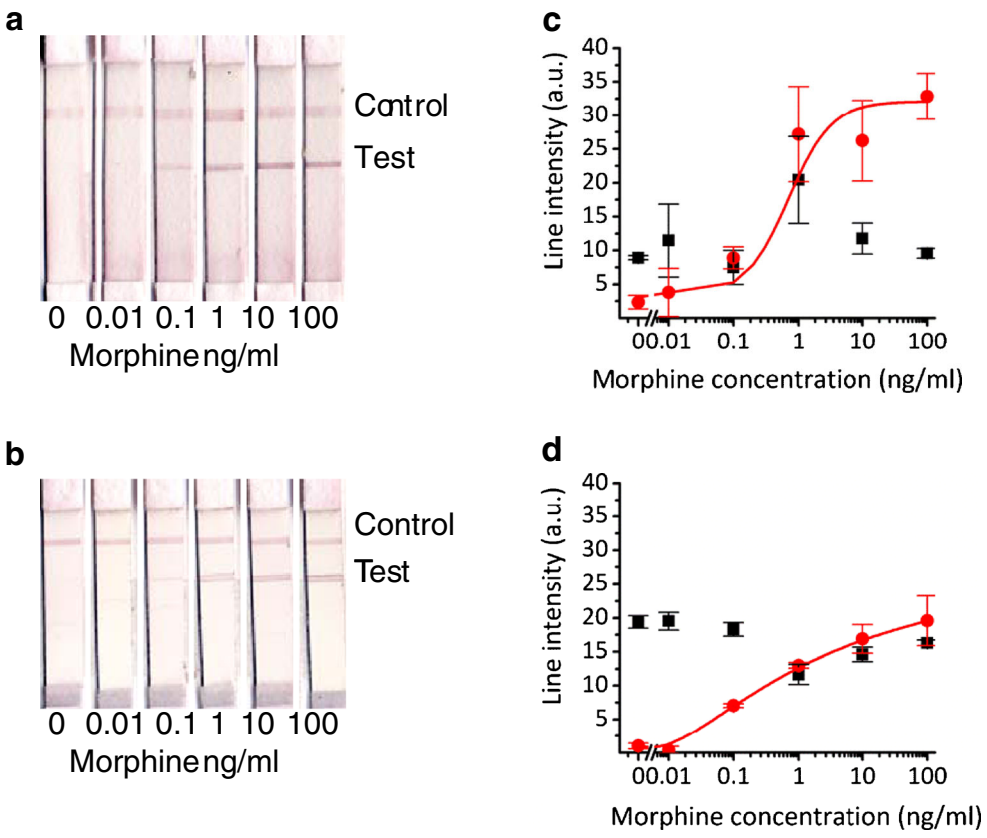

Fig. 6 Performance of the paper-based assay in actual diagnostic specimen. Immunocomplex assay of morphine in diluted oral fluid sample on paper (**a** and **c**) and on nitrocellulose (**b** and **d**). Diluted saliva was spiked with morphine in a concentration range of 0 to 100 ng/ml. Intensity on the test line is shown as *red circles* and *red line* and intensity on the control line as *black squares*. Each data point is the mean of three assays, and the *error bars* represent the standard deviations of the assays

can markedly affect the equilibrium amounts of bound antibody conjugates on test and control lines. General guidelines for workplace testing present cutoff value of 40 ng/mL for morphine in blood [49, 50]. Yet, an issue to be considered is that concentrations of morphine as well as other drugs of abuse in blood cannot be equivocally deduced from concentrations in oral fluid. To become a fully portable and suitable for point-of-care diagnostics and for testing in field, additional functionalities which allow filtering and diluting of the saliva sample as well as including a sample application port which can accommodate exact sample volumes for quantification need to be incorporated in the paper-based lateral flow assay devices. Obviously, the additional compartments increase the overall cost structure, albeit paper as a raw material is ultra-cheap even compared to nitrocellulose. The cost of mass-manufactured paper with size of 100 cm^2 was estimated as $0.00086 and $1.6, respectively, for a type of nitrocellulose often being incurred in research. As a summary, the developed paper-based assay for morphine may be considered to fulfill the diagnostic requirements for testing drugs of abuse already at this stage of development.

Conclusion

A lateral flow assay device, where nitrocellulose as an analytical membrane was substituted by paper, has been realized. Paper served as a matrix for wicking of liquid and dissolved sensing molecules and particles and also as a reaction zone for signal generation. Low cost of the developed platform was attained through the use of recombinant antibody fragments, physical absorption of antibodies on test zones without chemical modification, use of printing in antibody deposition, and applying simple fabrication method. It has been confirmed that the detection of morphine was successful in diluted oral fluid in concentrations sufficiently low indicating that the fabricated paper-based platform can become adapted to point-of-care applications and rapid detection of other diagnostically important analytes.

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