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Research Article

Immobilization Techniques and Integrated Signal Enhancement for POC Nanocolor Microfluidic Devices

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Resonance enhanced absorption (REA) nanocolor microfluidic devices are new promising bioassay platforms, which employ nanoparticle- (NP-) protein conjugates for the immunodetection of medically relevant markers in biologic samples such as blood, urine, and saliva. The core component of a REA test device is a PET chip coated with aluminum and SiO_2 thin layers, onto which biorecognitive molecules are immobilized. Upon addition of a sample containing the analyte of interest, a NP-protein-analyte complex is formed in the test device that is captured on the REA chip, for example, via streptavidin-biotin interaction. Thereby, a colored symbol is generated, which allows optical readout. Silver enhancement of the bound nanoparticles may be used to increase the sensitivity of the assay. Herein, we demonstrate that adsorptive immobilization via a cationic polymeric interlayer is a competitive and fast technique for the binding of the capture protein streptavidin onto planar SiO_2 surfaces such as REA biochips. Moreover, we report the development of a silver enhancement technology that operates even in the presence of high chloride concentrations as may be encountered in biologic samples. The silver enhancement reagents may be integrated into the microfluidic assay platform to be released upon sample addition. Hereby, a highly sensitive one-step assay can be realized.

1. Introduction

Modern point-of-care diagnostics strongly relies on low-cost, portable, easy-to-handle platforms especially in regions with limited resources and medical infrastructure.

Microfluidic devices also called μTAS (micro total analysis systems) or lab-on-a-chip systems belong to the most promising technologies studied in this context. A major part of the respective research focuses on the combination of microfluidics with immunoassay technology being the most valuable tool for the detection of medically relevant markers. Compared to conventional immunoassays microfluidic systems offer efficient mass transport and a reduced surface to volume ratio. Thus fast analyses can be performed even with small sample volumes and significantly lower amounts of antibodies and reagents are required [1]. Detection strategies currently studied and employed for microfluidic platforms range from fluorimetric over colorimetric to electrochemical techniques (see, e.g., [2]). The selection of the best suited technology is often a compromise between simplicity and sensitivity. Where quantification is an issue and low detection limits are most critical, electrochemical systems are generally the first choice. For quick tests and screenings, however, a noninstrumental readout may be preferred, which implicates a colorimetric detection strategy.

Resonance enhanced absorption (REA) is an optical phenomenon already described by Leitner et al. [3] in 1993. In 1998, the application of the effect in a bioassay was reported [4]. Its potential as a novel (bio)analytical platform has been studied extensively since then (see [5–7]). In 2009, our group developed a novel microfluidic diagnostic device combining REA technology with an innovative fluidic setup operating by passive capillary force. The manufacture of the device out of PET films by hot embossing or laser micromachining and the physical basics of REA were discussed in detail by Assadollahi et al. [8]. Current research focuses on the optimization of this system as versatile, fast, and sensitive microfluidic POC one-step-immunoassay platform.

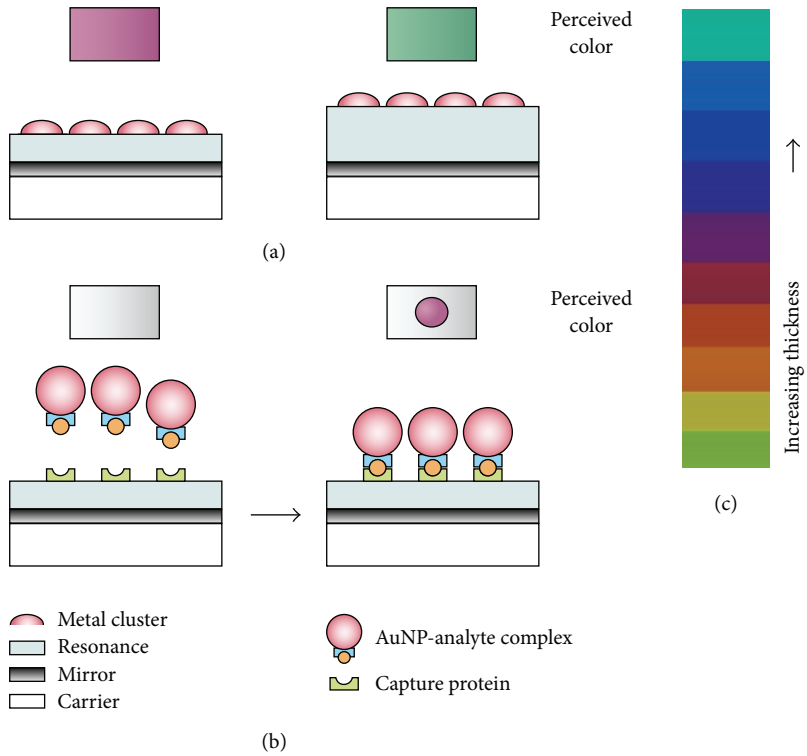

Figure 1: Classical REA setup and bioanalytical REA assay. (a) Schematic drawing of the classical 4-layer REA setup with two different resonance layer thicknesses. The colored squares above the metal cluster layers indicate the film color, which varies with resonance layer thickness. (b) Schematic drawing of a bioanalytical REA assay before (left) and after (right) binding of AuNP-analyte complexes to the capture protein layer. (Without top metal cluster REA materials exhibit the color of the base mirror layer, generally silver to gray.) The NP-analyte complex may comprise further mediator proteins, which are not depicted here. (c) *REA color spectrum*. By stepwise increase of the resonance layer thickness from 0 to 300 nm, a broad range of colors can be obtained.

In brief, resonance enhanced absorption requires a four-layer setup as depicted in Figure 1(a). It comprises a metal mirror layer, a transparent distance layer of nanometric thickness (<300 nm), and a (sub)monolayer of metal nanoclusters.

The sandwich of nanocluster layer and mirror layer at a well-defined distance results in optical resonance. Compared to the absorption spectrum of the nanocluster layer alone this resonance effect causes an 8-fold or higher increase in absorption of electromagnetic radiation of certain wavelengths [5]. As a consequence, a color in the visible range of the electromagnetic spectrum is obtained (which is commonly different from the color of the nanoclusters). The absorption maxima shift depending on the distance layer thickness, which allows the fabrication of REA materials of various colors and multicolored surfaces (see Figure 1(c)).

For analytical purposes REA chips without top metal cluster layer are used as platforms for colorimetric sandwich type immunoassays employing metal nanoparticle-conjugated probes (Figure 1(b)). If the antigen of interest is present in a sample, a complex comprising metal nanoparticle probe, analyte, and—if required—additional mediator proteins is formed and binds to the chip surface. Thereby, the REA structure is completed and a colored symbol appears indicating the positive detection of the analyte. The color intensity is proportional to the concentration of the captured NPs. Like in traditional lateral flow assays (LFAs) all assay reagents are provided in the microfluidic cell in dry form and are released upon sample addition. Such REA immunoassays have already been used for the detection of HSA and IL-6 as reported by Assadollahi et al. [8] and Palkovits et al. [9]. Similar systems based on metal nanoparticle-conjugated probes have been developed by other groups recently (see, e.g., [10–12]). However, those colorimetric tests rely solely on the light absorption of the captured nanoparticle layer. They do not profit from the signal enhancing effect of a REA structure, which may allow reaching even lower detection limits.

In order to provide a flexible platform for a range of analytes, streptavidin should be introduced into the described microfluidic REA technology as capture reagent. Thereby, already established immunoassays employing biotin-tagged antibodies for detection could be combined with the device without the requirement for individual optimization of chip coating procedures.

For low-cost manufacture an efficient and simple technique for streptavidin immobilization must be employed, however. In order to identify a suitable method we performed a screening of published approaches and variations thereof and evaluated their efficiency. The four most successful techniques we identified are described herein. Basically, there are three different approaches that can be taken in protein

immobilization: (1) Adsorption or spotting is a strikingly simple technique but commonly leads to random protein orientation. However, it has proven sufficient for various applications [13]. (2) Covalent immobilization limits the possible orientations of the immobilized protein and thereby can increase or at least homogenize the reactivity of the protein layer. It may, however, also decrease the reactivity if the employed technique entails molecule orientations that hide active sites from potential interaction partners. The choice of the immobilization chemistry depends on the reactive groups that are available or can be created on the protein and the substrate [14]. Recent developments even allow highly selective, oriented conjugation combining the advantages of protein tags with covalent methods [15]. (3) By immobilization via tags (e.g., myc, streptavidin-biotin interaction) highly oriented protein layers can be prepared, even from impure lysates or solutions with low concentration. [14]. However, employing tags for immobilization was not considered in this study as the streptavidin functionalities were required for antibody-antigen capture. Each streptavidin molecule provides four binding sites. The orientation of streptavidin after immobilization should therefore have only minor influence on its binding capacity. Thus, adsorptive as well as covalent immobilization techniques were considered suited for the preparation of streptavidin coatings on the SiO_2 surface of REA chips.

A major drawback of microfluidic systems is the limited number of binding sites that can be created on the commonly flat detection area compared to the generally porous substrates of LFAs. Recent publications suggest that a pronounced 3D structure of the chemical linkers that are used to bind the immobilized molecules to the substrate can be helpful to obtain a high density of binding sites (as reviewed in [16]). Polyethylenimine (PEI) and carboxymethylcellulose (CMC) are two high molecular weight polymers that have been described previously as substrates for protein immobilization [17–19]. Due to its strong positive charge PEI was selected for adsorptive immobilization of streptavidin, which is, with a pI of ~5, negatively charged in the pH-neutral buffers used for chip preparation. CMC was tested as platform for carbodiimide-mediated covalent streptavidin immobilization. As there are several protocols available for the respective conjugation reaction, a screening was performed to identify suitable approaches. To determine the efficiency of the individual immobilization methods and the functionality of the generated streptavidin layers, all chips were tested in a standardized microfluidic immunoassay at defined analyte concentration. As depicted in Figure 1(b) the assay is based on the binding of a nanoparticle-conjugated probe to the analyte in solution and the subsequent capture of this complex on the REA chip surface. However, in contrast to the illustration, a second biotin-tagged probe is required to mediate the binding of the analyte-NP complex to the streptavidin layer on the chip. Two of the most competitive immobilization approaches that could be established are described herein. Chips prepared using CMC or PEI were compared with chips coated via adsorption of streptavidin to 3-APTES. Thereby, it was evaluated whether polymer interlayers actually confer higher biorecognitive capacity to REA bioassay chips.

The limited number of binding sites in microfluidic systems may also be compensated by secondary signal enhancement reactions. Assadollahi et al. described how nanoparticle-catalyzed electroless deposition of metallic silver from solution can be used to boost sensitivity and signal intensity. Even layers of particles so sparsely bound that they are invisible to the eye may provide sufficient catalytic activity for this enhancement method. Optimally the deposited silver, which is generated by in situ reduction of a precursor salt, forms a continuous film and closes the gaps between the captured antigen-antibody-nanoparticle complexes. An increase in signal intensity of up to 13-fold may be achieved thereby. Comparable strategies were employed by, for example, [20, 21]. However, in all three cases, the respective reaction was performed after the actual analyte detection in a secondary step. The silver enhancement solution had to be mixed freshly. Efforts to integrate the enhancement reagents in the assay platform to develop a true one-step device showed that the formulation of Assadollahi et al. was not compatible with the high chloride concentrations often occurring in biologic samples. Silver chloride precipitates formed, which prevented the reduction and precipitation reaction. Accordingly, integrated silver staining demands, first of all, the stabilization of ionic silver in chloride-containing solutions. Furthermore, it must be possible to provide the enhancement reagents, just like the immunoreagents, within the assay platform and store them over a prolonged period of time in this form. Their release, which induces the enhancement reaction, should at best not require additional manipulation by the person performing the assay. It should occur upon or shortly after introduction of the sample into the device. Considering these points a screening of complexing agents and silver staining protocols was performed. Eventually a formulation could be established, which operates stably in the presence of urine. It is entirely based on solid, water-soluble precursors and additives well suited for long-term storage and one-step release. Their performance in the already described REA bioassay system was tested and efficiency and morphology of the respective silver stainings were characterized, as reported herein. It could be demonstrated that the developed formulation is a promising first step towards an integrated enhancement system for nanoparticle-based microfluidic devices.

2. Materials and Methods

2.1. Preparation of REA Chips. A 100 μm thick, aluminum-metalized polyester film (Mitsubishi) was coated with a ~180–220 nm layer of SiO_2 as previously described [8]. (After addition of a metal cluster layer, films of this resonance layer thickness exhibit a pink-violet resonance color, which provides optimal visibility.)

2.2. Streptavidin and Bioassay Reagents. Streptavidin and all reagents required for the bioassay part of the setup (i.e., purified analyte, gold nanoparticle- (AuNP-) conjugated probe, and biotin-tagged analyte binding protein) were kindly provided as a gift. Similar biotin-tagged mediator proteins and AuNP conjugates are available from Sigma-Aldrich (e.g., product number G-0911 or P-6855). For bioassay experiments

AuNP-mediator protein conjugate was used at an OD of ~10.9–11.8 in the final assay mix.

2.3. Streptavidin Coating

2.3.1. Protocol 1 (3-APTES/Streptavidin). A droplet of 3 μL of 2 mg/mL streptavidin in 0.01 M Na-K-phosphate buffer, pH 7.0, was spotted in the middle of a 3-APTES (Sigma-Aldrich) coated chip. The chip was incubated in a wet chamber at RT for 1 h. The chip was washed by shaking in 1x PBS + 0.1% Tween 20 for 3 min. For blocking, the chip was incubated in 2% BSA in 1x PBS + 0.5% Tween 20 for 1 h. The chip was again washed by shaking in 1x PBS + 0.1% Tween 20 for 3 min, rinsed with dH_2O, and used directly for immunodetection.

2.3.2. Protocol 2 (PEI/Streptavidin). A REA chip was coated with PEI (1.6 MDa) by incubation in 10% aqueous PEI solution. A 3 μL droplet of 2 mg/mL streptavidin in 0.01 M Na-K-phosphate buffer, pH 7.0, was spotted in the middle of the PEI-coated chip. The chip was incubated in a wet chamber at RT for 1 h. Washing and blocking were performed as described in protocol 1.

2.3.3. Protocol 3 (3-APTES/Activated CMC Na-Salt/Streptavidin). CMC sodium salt (~700 kDa), carbodiimide, and NHS were all purchased from Sigma-Aldrich. 0.45% (w/v) CMC Na-salt in 0.01 M Na-K-phosphate buffer, pH 7.0, was activated with 200 μM carbodiimide and 50 μM NHS end-to-end rotating at RT for 2 h. ~100–200 μL of the solution was spread on the surface of a 3-APTES-coated chip. The chip was incubated for 30 min in a wet chamber, rinsed with dH_2O, and dried. A 3 μL droplet of 2 mg/mL streptavidin in 0.01 M Na-K-phosphate buffer, pH 7.0, was spotted in the middle of the CMC-coated chip. The chip was incubated in a wet chamber at RT for 17–17.5 h. Washing and blocking were performed as described in protocol 1.

2.3.4. Protocol 4 (3-APTES/CMC Na-Salt-Streptavidin Conjugate). 0.45% (w/v) CMC Na-salt in 0.01 M Na-K-phosphate buffer, pH 7.0, was activated with 200 μM carbodiimide and 50 μM NHS end-to-end rotating at RT for 2 h. Activated CMC was diluted in 0.01 M Na-K-phosphate buffer, pH 7.0, and mixed with streptavidin solution to yield final concentrations of 0.225% (w/v) CMC Na-salt and 2 mg/mL streptavidin. The solution was incubated at RT for 17–17.5 h. 3 μL of the solution was spotted onto a 3-APTES-coated chip. The chip was incubated in a wet chamber for 30 min. Washing and blocking were performed as described in protocol 1.

2.4. Analyte Detection via Microfluidic Assay. Instead of biological samples a standard solution of purified analyte was used. To exclude artifacts that could have resulted from incomplete dissolution of and mixing with the assay reagents (AuNP-conjugated probe, biotin-tagged analyte binding protein, and further additives) the analyte solution was premixed with the respective reagents and incubated briefly before application to the microfluidic cell. Experiments were performed with such assay reagent mixes with an analyte concentration of 342 ng/mL since this was close to the detection limit of the most sensitive chip variants tested. Thus, it allowed distinguishing competitive from less successful immobilization approaches. The chip was placed in a microfluidic cell, the streptavidin-coated side facing a single channel of a width of 6-7 mm and a height of 5–15 μm. The analyte was premixed with the assay reagents, incubated at RT for 1 min, and applied to the microfluidic cell. After 3 min reaction time the assay was stopped by rinsing the chip with dH_2O. The chip was dried and the NP binding density was characterized.

2.5. Silver (Ag) Enhancement. PdNP-conjugated probe and standard human urine were kindly provided as a gift. For grafting the PdNP conjugate was used at an OD of ~30. L-Ascorbic acid (Ph. Eur.) and sodium hydroxide were purchased from Sigma-Aldrich, sodium thiosulfate pentahydrate was purchased from Carl Roth, citric acid was purchased from Merck Chemicals, and silver nitrate was purchased from Ögussa. Ag enhancement tests were performed with the same microfluidic setup as used for the analyte detection experiments described in Section 2.4.

For determination of the maximum enhancement factor of the established formulation the silver enhancement solution was freshly prepared for each test from stock solutions. Standard human urine was used as solvent. The working concentrations in the enhancement solution were 7.5 mM citric acid, 3 mg/mL NaOH, 2.5 mg/mL sodium thiosulfate, 2.25 mg/mL $AgNO_3$, and 6 mg/mL ascorbic acid. Pictures of PdNP-grafted REA chips were taken before Ag enhancement. Then the chips were cut in two in the middle of the grafted area and one half of each chip was subjected to silver enhancement for 2 min. Pictures were taken and the efficiency of the silver enhancement was determined as described in Section 2.7.

2.6. Grafting of REA Chips with PdNP Conjugate. REA chips were coated with PEI using 1%, 10%, or 50% PEI solution in dH_2O to control PdNP-conjugate surface coverage. (Higher PEI concentration in the coating solution results in a higher capacity of the PEI coating to bind the NP conjugate.) PEI-coated REA chips were spotted with 2 μL PdNP-mediator protein conjugate solution and incubated for 1.5 min in a wet chamber. Excess PdNP conjugate was removed by rinsing with dH_2O.

2.7. Image Acquisition, Image Processing, and Quantification of Signal Intensity. To investigate immobilization homogeneity, NP-analyte complex binding capacity, and extent of silver enhancement, it was necessary to measure VIS-light absorption at individual spots of the prepared chips. We found that conventional spectrophotometry was not suited for this purpose. Instead, photos of all chips were taken with a digital camera at constant angle, constant distance, and constant illumination. Image processing software was used to subject all photos to a white correction. For quantification the color profile of the photos was converted to grayscale (8-bit). To evaluate the efficiency of the screened streptavidin immobilization protocols the gray value (in % black, i.e., black =

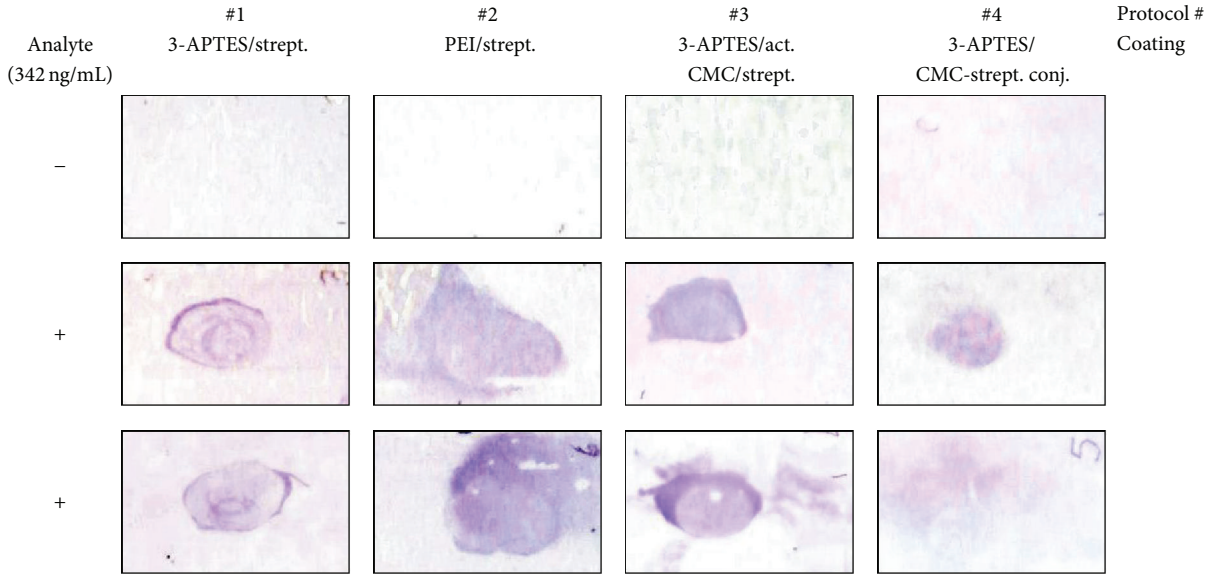

Figure 2: Streptavidin immobilization: protocols 1–4. After coating REA chips according to protocols 1–4, the chips were tested in a microfluidic cell with premixed assay reagent solution of constant analyte concentration (342 ng/mL, indicated by "+"). To analyze whether chips exhibited unspecific binding, instead of analyte solution the corresponding pure buffer was added to the assay reagent mix (−).

100%, white = 0%) of 4 spots in the center and 4 spots in the rim of the spotted area and 4 spots in the noncoated area (= background) was determined for each chip. The term "rim" herein refers to the more or less continuous, ring shaped structure at the outline of the spotted area of some chips, which clearly differed from the central region (= center) due to higher signal intensity (see, e.g., Figure 3(a), top row). Such ring structures were typically encountered on chips coated according to protocol 1. If no such structure was present or if it could not be clearly distinguished from the central area (see, e.g., Figure 3(a), second row), the outmost region of the spotted area within 0.5 mm from the outline was considered as rim instead. (The typical diameter of the spotted area ranged from 5 to 8 mm.) Smear patterns, as described in Section 3.1., were not considered in the quantification if they could be clearly distinguished (due to position, shape, and color intensity) from the spotted area, the rim, and the background. Average values for REA color intensity at rim, center, and background were calculated for each chip individually. The background gray intensity was subtracted from the values calculated for rim and center. Then the average values of all chips of one protocol were used to calculate overall average signal intensities and corresponding standard deviations for the individual protocols. For the determination of the enhancement factor of the developed silver enhancement protocol, the gray value (in % black) at two positions in the PdNP-spotted area of each chip was determined before Ag enhancement. The background signal was determined as described for the streptavidin immobilization tests. After Ag enhancement the gray value at the same two positions and the background signal were determined. After background subtraction the enhancement factor (EF), that is, the ratio of the gray values before and after Ag enhancement, was calculated.

2.8. AFM Imaging. AFM imaging of bare polymer-coated REA chips, chips grafted with PdNP conjugate, and PdNP-conjugate grafted chips after Ag enhancement was performed using a TT-AFM WORKSHOP system with a 15 μm scanner and a Minus-K-vibration-isolation table in contact mode (SICONA-10 cantilevers).

3. Results and Discussion

3.1. Streptavidin Immobilization. Coating protocol 1, that is, adsorption of streptavidin to a 3-APTES-coated surface, resulted in high NP-analyte complex binding capacity at the outer rim of the spotted area (see Figures 2 and 3). In contrast, the spot center showed weak REA signal intensity suggesting a low streptavidin surface density. The phenomenon was described earlier in the context of protein microarrays and is supposed to be caused by the selective diffusion of proteins in droplets of protein solutions to the air-solvent interface [22]. In experiments with other capture proteins we observed that the effect decreased with increasing protein concentrations (>4 mg/mL) and was practically abolished at a protein concentration of 8 mg/mL (unpublished data). Deng et al. [22] reported that the addition of detergent to the spotting solution could suppress ring formation in a covalent immobilization approach. However, there are concerns that such additives may interfere with adsorption processes and may thereby reduce the overall immobilization efficiency of adsorptive methods.

Chips coated by adsorption of streptavidin to a monolayer of high molecular weight PEI (protocol 2) exhibited a significantly higher NP-analyte complex binding capacity in the center of the spotted area (Figures 2 and 3) than protocol 1 chips, but weak ring formation was also observed. Additionally, at one side of the spotted area the chips generally showed

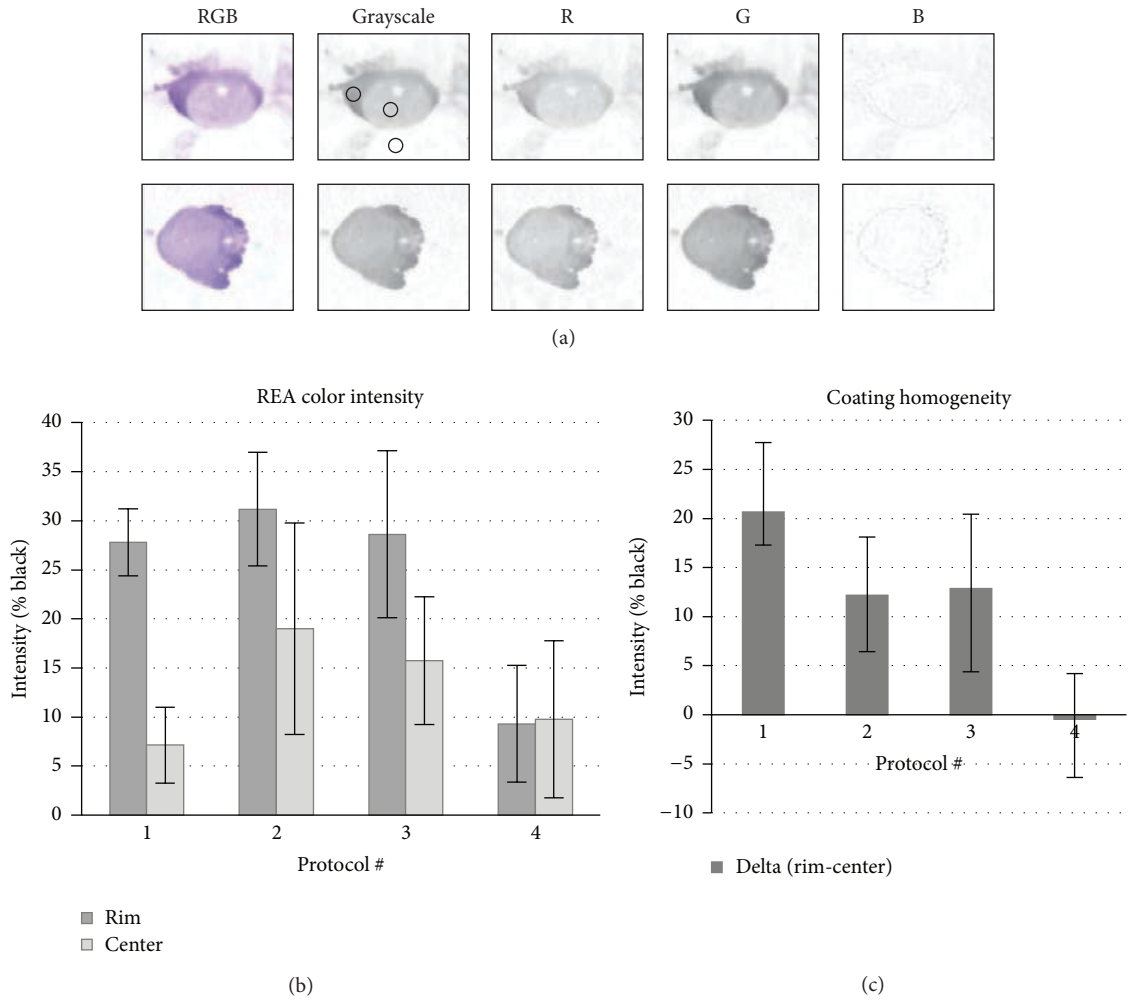

Figure 3: Quantification of REA signal intensity. (a) Photos of REA chips after analyte detection are shown. For quantification the color profile of the images was converted to grayscale (8-bit) and the gray values at rim and center of the spotted area as well as the value of the background were determined (indicated by black circles; for more details, see Section 2). Quantification of the grayscale image was considered to correspond best with the actual REA color intensity and thus was preferred over measuring the contribution of one of the three channels: red (= R), green (= G), and blue (= B). (b) REA color intensities at the rim and center of the spots on the tested chips prepared by protocols 1–4 are shown in % black (black = 100%, white = 0%) (protocols 1 and 2: $n = 4$; protocols 3 and 4: $n = 6$). (c) The differences (delta) of average REA color intensities at rim and center [delta = intensity (rim) − intensity (center)] are shown in % black.

a smear pattern with high signal intensity. Such patterns resulted from the adsorption of streptavidin within the seconds when the spotting solution was washed off the chip. This very brief (unintended) dynamic "application of protein" was apparently more efficient than half an hour of incubation. The effect may be used for other coating techniques but unfortunately complicates spotting methods. Localized spotting of PEI instead of coating the complete chip cannot be employed to yield cleaner spot shapes either, because smearing is also observed here. Thus, when using coating protocol 2 clean circular spots may currently be achieved best by the use of masks. Preliminary experiments on lamination and lacquering techniques for mask application were performed. The suitability of precut commercial self-adhesive films and a variety of polymer lacquers was investigated. Masking could have improved the reproducibility of the spotting experiments as it would have allowed controlling the size of the contact area between chip and spotted protein solution. However, the tests suggested that components of the analyzed masks could interfere with structure or adsorptive properties of the polymer coatings used for immobilization herein. As such effects could not be excluded for any of the examined masking techniques we refrained from using masking in the present study. Structuring of the chip surface to create physical borders for the spotted solution, that is, by laser engraving, was also considered. But any kind of

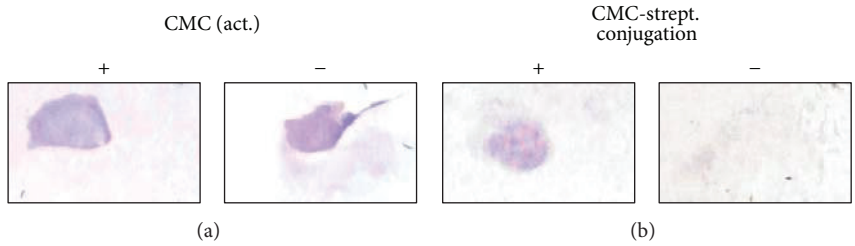

Figure 4: Control experiments: protocols 3 and 4. (a) REA chip coated with streptavidin according to protocol 3 (left). 3-APTES-coated REA chip spotted with 2 mg/mL streptavidin solution and incubated at RT in a wet chamber for 17.5 h (right). Analyte detection was performed on both chips. AuNP-analyte complex binding capacity and the homogeneity of the binding were comparable. (b) REA chip coated with streptavidin according to protocol 4 (left). 3-APTES-coated REA chip spotted with 2 mg/mL streptavidin + 0.225% CMC in 0.01% Na-K-phosphate buffer, pH 7.0, and incubated at RT in a wet chamber for 30 min (right). Analyte detection was performed on both chips. The chip coated according to protocol 4 exhibited significantly higher AuNP-analyte complex binding.

chip surface structuring also affects the flow of the detection solution in the microfluidic test setup. This generally strongly interferes with the performance of the assay leading to artifacts. It must therefore be stated that there is currently no means to control the spot size in the studied immobilization techniques. However, spotting experiments were performed as reproducibly as possible. A defined volume of protein solution was spotted on each chip in the same way and the same area was covered during spotting. In the course of the subsequent incubation, the spotted solution tended to spread over the surface as a result of the hydrophilic properties of the chip surface. However, the extent of spreading was always comparable on chips of the same type (i.e., either pronounced (on PEI) or insignificant (e.g., on 3-APTES)). Thus, the sizes of spots created with the same immobilization technique were not significantly different. The conformation of PEI and PEI derivative molecules in SAMs may be determined by adjusting pH and salt concentration of the employed PEI solutions [23]. However, coating with 10% high molecular weight PEI in dH_2O already allows creating layers of highly globular molecules. Therefore we doubt that the structure or the charge density of the PEI base layer can be significantly optimized further. Solutions of much higher PEI concentrations are not useful for monolayer self-assembly, because their viscosity makes intense washing necessary. It has been observed that this can inflict considerable washing defects to the monolayer.

Layer-by-layer coating of 3-APTES and CMC followed by carbodiimide-mediated covalent protein immobilization (protocol 3) yielded spots with considerably high NP-analyte complex binding capacity. Ring formation was occasionally observed but was in general less pronounced than on chips coated according to protocol 1. However, the requirement of the CMC interlayer is doubtful. By incubation of streptavidin on 3-APTES-coated chips without CMC interlayer for 17.5 h protein coatings with very good NP-analyte capture ability could be created. The results were practically indistinguishable from the chips coated according to protocol 3 (see Figure 4(a)). In both approaches commonly thickening of the spotted coating solution occurred due to evaporation during incubation and in some cases even gel formation was observed. Most probably this led to increased protein concentrations in the coating solutions allowing more homogeneous immobilization, supposedly by unspecific adsorption rather than by covalent binding. However, protein immobilization via drying is generally not recommended. On the one hand, it bears the risk of protein denaturation. On the other hand, experiments with nitrocellulose coated chips showed that fast drying of the spotted solution entails deposition of salts (buffer components), which interferes with adsorptive immobilization and thus prevents homogeneous protein coating.

Protein immobilization protocol 4, conjugation of streptavidin to CMC in solution followed by monolayer self-assembly of the conjugate, is significantly less efficient and less reproducible than the three previously discussed approaches. Streptavidin immobilization is occasionally highly localized but sometimes the streptavidin binding sites appear to be distributed over large areas of the chip in a smear pattern. In a control experiment the CMC-streptavidin conjugate solution was replaced by a mix of nonconjugated CMC and streptavidin at the same concentrations and coating according to protocol 4 was performed. The resulting chips exhibited no visible NP-analyte complex binding capacity (see Figure 4(b)). Adsorption of free streptavidin molecules was obviously prevented by competition with CMC for positively charged groups on the chip surface. It can be concluded that streptavidin immobilization via protocol 4 is not the result of mere adsorption of streptavidin molecules. Efficient CMC-streptavidin cross-linking is critical for this approach and variations in the extent of CMC-streptavidin cross-linking in solution might be the reason for the poor reproducibility of this method.

3.2. Chloride-Resistant In Situ Silver Enhancement. In search of silver staining reagents with good performance in chloride-containing solutions several established protocols and known complexing agents for silver salts were screened including Tris base and histidine. Primarily, reagents were considered, which exhibit good solubility in aqueous solutions. This is most critical for the establishment of a robust in situ enhancement technology as all reagents have to be provided

in the assay platform in a stable form (preferably dry) and it must be possible to release them by addition of the sample without significant further manipulation of the test device. Regarding the stabilization of silver ions against precipitation with chloride, most promising results were achieved by the use of sodium thiosulfate. It was found that silver nitrate could be dissolved in a human urine sample (with a typical chloride concentration of 20 mM) by buffering it with citrate and addition of sodium thiosulfate to a concentration roughly equivalent to the desired molar silver nitrate content. At pH < 7 such a solution was found to be stable for at least 1 h. For Ag enhancement the mixture had to be activated by addition of a reducing agent. Glucose and ascorbic acid were considered as potential candidates here because both easily and quickly dissolve in water. Finally ascorbic acid was selected for it allowed good control of the reduction reaction. It was further observed that the efficiency and speed of the silver enhancement process and the stability of the solution strongly depended on the pH value. We were interested in a fast enhancement process and therefore optimized an alkaline formulation (see Section 2). Eventually, a silver enhancement protocol could be established, which employs exclusively highly water-soluble reagents that can all be provided in an assay device in dry form: thiosulfate, citric acid, and sodium hydroxide for stabilization and buffering and ascorbic acid as reducer. The efficiency of the developed formulation should be characterized on REA chips sparsely grafted with catalytic metal nanoparticles. Already at the start of the screening it had turned out that the AuNP-conjugated probe employed for the screening of streptavidin immobilization techniques did not act as good catalyst for silver reduction (independent of the tested enhancement protocol). The AuNP conjugate was strongly stabilized against precipitation and unspecific binding (by inert proteins and polymers) which might have suppressed its interaction with the silver enhancement reagents. Therefore a less shielded PdNP conjugate was used instead, which exhibited significantly higher catalytic activity but equivalent performance in bioassay applications. REA chips were precoated with PEI and grafted with PdNP conjugate by spotting. Chips with low, medium, and high PdNP density were created. The silver enhancement reagents were dissolved in urine and the PdNP-grafted REA chips were treated with this solution in a microfluidic cell for 2 min. The enhancement efficiency was determined by comparing REA color intensities before and after silver staining. (Only chips exhibiting a visible REA color spot before enhancement were considered in the quantification.) It could be demonstrated that the enhancement factor was especially high for weak signals, that is, if the PdNP density on the chip was low. Even very sparse and therefore invisible PdNP coatings exhibited a clearly visible REA color after treatment of the corresponding chip with the enhancement solution (Figure 5(a)). At low PdNP surface coverage an enhancement factor of up to 13 was observed (Figure 5(b)). With increasing PdNP density the signal enhancing effect decreased logarithmically. However, this is not considered too critical because visibility of the REA signal was already good for those PdNP coatings even before enhancement. AFM imaging revealed that the precipitated silver layer is not smooth but rather crystalline. This also resulted in increased light scattering under very bright illumination, which was not observed in the PdNP-grafted areas before enhancement. Generally the crystalline morphology is not problematic and does not diminish the signal enhancing effect of the silver coating. However, it is an indication that the enhancement reaction still requires fine tuning. Optimally stabilized silver enhancement yields slightly granular to smooth surfaces. Nevertheless, the performance of the developed silver enhancement formulation is competitive and, most importantly, it fulfills all requirements for the integration into microfluidic devices.

4. Conclusions

4.1. Streptavidin Immobilization. Four different protocols for streptavidin immobilization on REA chips could be established and optimized. The techniques employ different chemistry and are based on two different immobilization approaches: adsorption and covalent immobilization, respectively. They were compared regarding coating homogeneity and analyte capture capacity of the prepared protein layers. It was found that by adsorption to a monolayer of high molecular weight PEI the most homogeneous coatings could be generated within a short time. Thus, this method may be best suited for large scale chip manufacture. The only drawback of the approach was smear formation, which prevented the grafting of well-shaped protein spots. By addition of blocking agents to the washing solutions or by use of a mask such random adsorption during the washing step effect may possibly be suppressed. Covalent immobilization to CMC monolayers (protocol 3) also yielded good results but turned out to be much more time consuming. Furthermore, control experiments suggested that immobilization was achieved at least in part by unspecific adsorption rather than by covalent binding and that the CMC base layer was not strictly required. In general, it was observed that linkers with pronounced 3D structure are not absolutely necessary to generate protein layers with occasionally high analyte binding capacity. However, precoating with charged polymers significantly accelerated adsorption of proteins with opposite charge and has the potential to significantly improve capture protein density and coating homogeneity if short immobilization times are required.

4.2. Chloride-Resistant In Situ Silver Enhancement. Silver enhancement reagents could be identified, which operate even in the presence of chloride and can thus be employed for silver staining in samples such as urine, blood, or saliva. All required reagents are water soluble and can be provided in dry form in a microfluidic cell. They can be released upon sample addition (preferably with delay) to react with the captured analyte-nanoparticle complexes on the REA chip and enhance the assay signal. Initial tests in this context have already been performed. For this purpose the enhancement reagents were dried on hydrophilized polymer fleeces and integrated into a laser-structured microfluidic cell as described in Assadollahi et al. [8]. Although mixing of the reagents was not optimal, good signal enhancement could

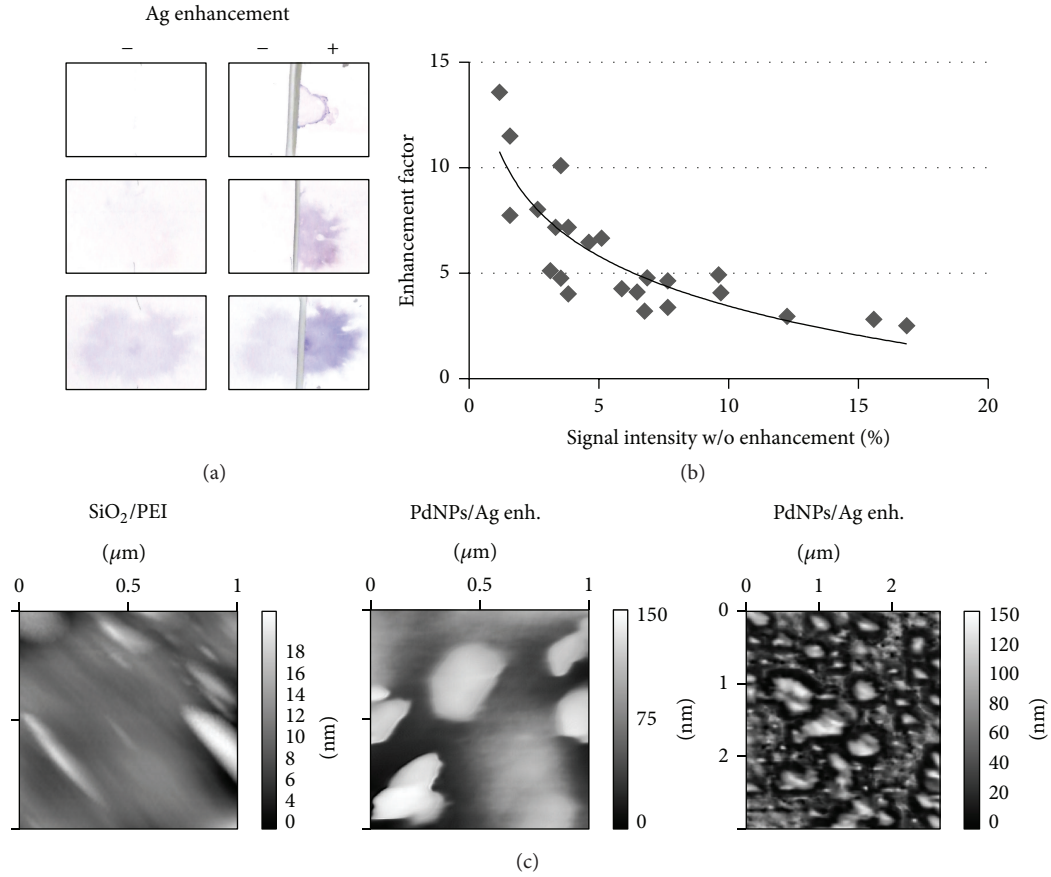

Figure 5: Thiosulfate-stabilized silver enhancement. (a) PEI-coated REA chips grafted with PdNP-conjugated probe (low, medium, and high surface coverage) before Ag enhancement (−) (left). The chips were cut in two and one half of each chip was subjected to Ag enhancement (+) (right). (b) The enhancement factor (EF) strongly depended on the signal intensity before silver enhancement (i.e., the PdNP coverage density) (EF = [signal after Ag enhancement in % black]/[signal before Ag enhancement in % black]). (c) AFM scans of REA chips before PdNP grafting (left) and after PdNP grafting and Ag enhancement (middle and right) (the grafted PdNP conjugate could not be imaged due to the inherent roughness of the chip surface and the polymer layer and the small size of the PdNPs of ≤20 nm.).

be achieved. Thus, optimization of release and mixing of the reagents will be the focus of future efforts towards this kind of integrated enhancement system.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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