A comparative analysis of polyurethane hydrogel for immobilization of IgG on chips

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Abstract

Hydrogels are considered an optimum material for protein chip surfaces, since they provide a quasi-liquid environment which allows protein activity to be maintained and shows good spot morphology as well as excellent immobilization capacity. In the following, we present a polyurethane (PU) chip that electrostatically binds IgG. The PU surface is optimized with regard to layer thickness (∼200 nm), hydrogel (2%) and immobilized antibody concentration (0.5 mg mL\(^{-1}\); 0.3 ng spot\(^{-1}\)), pH and ionic strength of the print buffer as well as to blocking solution. Evaluation is done in a direct IgG immunoassay using the Nexterion slide H as a reference. It is shown that higher IgG loading is achieved on the PU chip than on slide H, no matter whether 1× PBS (pH 7.2), Sørensen (pH 5.8) or Nexterion buffer was used as a spotting solution. Moreover, the crossreactivity with goat IgG, human IgG and monoclonal anti-CRP spotted in Nexterion buffer was as low as ≤0.74% (slide H: ≤3.34%).

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1. Introduction

Numerous strategies have been developed for immobilizing proteins on solid substrates for microarray application [1–3]. These range from adsorption on hydrophobic materials, such as polystyrene, and covalent binding on functional polymers [4] to DNA-mediated, oriented immobilization [5] on activated glass or plastic supports. For the development process, high immobilization capacity as well as prevention of protein denaturation are important issues. Furthermore, the surface needs to be robust, stable and reproducible. The most widely used substrate for protein microarrays are membranes, as they provide a high binding capacity. With this approach, 1 pg mL\(^{-1}\) of sample [6] was easily detected. For this reason, filter membranes, such as nitrocellulose or nylon, are now attached to glass and used in the microarray format. Other 3D surfaces are mostly based on hydrogels [7–9] and dendrimers [10] (branched polymers). In addition to polyacrylamide [7,8] and respective copolymers, agarose [9] has been reported as being easy to prepare as a 3D matrix for proteins using activated aldehyde groups for protein binding.

Hydrogels are defined as colloidal gel polymers in which water is the dispersion medium. This insoluble network of polymer chains has the ability to swell in aqueous solutions, thus providing a semi-liquid environment for attached proteins. As hydrogels are able to react to external conditions, such as changes in pH, ionic strength and temperature, they provide great potential for a variety of fields of application; among these, drug delivery and enzyme sensors are the most prominent.

Yet hydrogels are rather difficult to prepare manually, hence commercially available slides tend to be expensive. Until now only a limited number of 3D hydrogel surfaces have been available on the market. The most well known of these are the hydrogel slides based on polyacrylamide from Perkin-Elmer, dating back to the works of Mirzabekov [11], and the Nexterion slide H from Schott.

Slides coated with hydrogels are usually quite thick, up to 30 μm in fact. The protein spotted on such a surface evaporates slowly, forming extremely homogeneous spots, and subsequent denaturation is prevented. Moreover, a hydrogel surface allows a better separation of protein spots, since these soak into the polymer and are quite limited in movement. In the follow-

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ing, we report on a 3D polyurethane (PU) surface for simple, one-step immobilization of protein for application in an on-chip immunoassay. The PU chip is optimized with regard to layer thickness, hydrogel and immobilized antibody concentration, pH and ionic strength of the print buffer as well as to blocking solution. The immobilization capacity and assay performance of the PU chip is compared with commercial Nexterion H slide for evaluation.

2. Materials and methods

2.1. Materials

Silane Prep™ slides (25 mm × 75 mm) (Sigma, St. Louis, MO, USA, S-6451), adhesive slides (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany, no. 08 100 00) and –NCS modified SAL1-Slides (Asper Biotech, Tartu, Estonia) were used as the substrate. Nexterion® Slide H from Schott (Schott Jena Glass GmbH, Jena, Germany) was employed as a reference. The print buffer recommended for slide H consists of 0.3 M phosphate buffer (pH 8), 0.005% CHAPS (3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate hydrate) (Fluka, Sigma–Aldrich Chemie GmbH, Buchs SG, Switzerland) and 10 µg mL⁻¹ BSA and will be referred to as Nexterion buffer in the manuscript. Anionic hydrophilic polyether polyurethane hydrogel PU was acquired from Cardiotech International (Wilmington, MA 01887, US). Cysteaminehydrochloride (CAHCl), mercaptoethanesulfonic acid (MESA) and polystyrenesulfonic acid were obtained from Fluka. Chitosan (food grade) was from Dalwoo (Dalwoo-ChitoSan, Seoul, Korea) and monochlorotrisazinyl-ß-cyclodextrin (MCT) was acquired from Wacker (Wacker Chemie AG, Munich, Germany). Dodecyl sulfate sodium salt (SDS) was provided by Merck (Merck KGaA, Darmstadt, Germany) and phosphate buffered saline (PBS) by Gibco. 0.2 M Sörensen buffers of pHs 5.8, 7.2 and 8.6, obtained from Electron Microscopy Sciences (Hatfield, PA 19440, USA), were diluted 1:1 with MiliQ water. Buffer additives aminosulfobetain (ASB-14) and sodium deoxycholate were from Sigma whereas 3-(decyldimethylammonio)propanesulfonate inner salt (SB3-10) and hexadecyltrimethylammonium bromide (CTAB) were from Fluka. Tween-20 was purchased from Fluka and Tween-80 from Sigma. All other reagents were analytical grade.

2.2. Chip fabrication

Two to 10% PU was dissolved in EtOH/H₂O (95/5) and dip-coated onto glass slides using the KSVD dip coater by KSV Instruments (velocity: 100 mm min⁻¹; retention time in solution: 60 s; retention time between layers: 2 min). Two percent PU layers were modified with a solution of either 3.8 mg mL⁻¹ MESA or 2.6 mg mL⁻¹ CAHCl in 1 × PBS (pH 7.2) for 30 min to create an anionic and cationic surface, respectively.

2.3. Surface characterization

AFM studies were performed with a NSOM/AFM 100 from Nanonics Ltd., Israel. Samples were scanned in contact mode with Mikromasch probes having spring coefficients of 0.03 N m⁻¹. Images were analyzed using SPIP—a commercial software package from Image Metrology A/S. Layer thicknesses were measured over 2.5 mm × 2.5 mm surface areas using the Wyko NT1100 optical profiling system (Veeco, Veeco Instruments GmbH, 68165 Mannheim, Germany) and Vision32 Veeco software. The values in Table 1 are mean values for two measurements.

2.4. Microarray printing

Three replicates of 0.03–1 mg mL⁻¹ rabbit IgG (technical grade, Sigma) in various print buffers were arrayed onto the respective hydrogel surfaces using the OmniGrid contact spotter by GeneMachines (pin SMP3). Unless stated otherwise, 1 × PBS (pH 7.2) was used as print buffer. The spot-to-spot distance was 400 µm, spot volume was 0.6 nL. For crossreactivity studies, 0.125 mg mL⁻¹ goat IgG (Sigma), human IgG (Sigma) and monoclonal anti-CRP (C reactive protein) from Exbio (EXBIO Praha, a.s., 252 42 Vestec, Prag) were used.

2.5. Postarraying and blocking

After arraying, the slides were incubated in a humid chamber at 4 °C overnight to complete probe immobilization. Surface blocking was performed for 30 min using blocking solutions I–IV consisting of: (I) 1 × PBS (pH 7.2)/0.1% Tween-20; (II) 1 × PBS (pH 7.2)/0.1% Tween-80; (III) 1 × PBS (pH 7.2)/3.8 mg mL⁻¹ MESA; and (IV) 1 × PBS (pH 7.2)/2.6 mg mL⁻¹ CAHCl. This was done in order to wash off

<table>
<thead>
<tr>
<th>Support</th>
<th>PU conc in %</th>
<th>No. of layers</th>
<th>Layer thickness in nm Before</th>
<th>Layer thickness in nm After</th>
<th>Spot diameter in nm</th>
<th>Fluorescence at 0.05 mg mL⁻¹ IgG</th>
<th>% CV at 0.05 mg mL⁻¹ IgG</th>
<th>Fluorescence at 0.5 mg mL⁻¹ IgG</th>
<th>% CV at 0.5 mg mL⁻¹ IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminosilane</td>
<td>2</td>
<td>1</td>
<td>235</td>
<td>202</td>
<td>159 ± 31</td>
<td>16230</td>
<td>11</td>
<td>46244</td>
<td>30</td>
</tr>
<tr>
<td>Aminosilane</td>
<td>4</td>
<td>1</td>
<td>721</td>
<td>686</td>
<td>165 ± 34</td>
<td>7124</td>
<td>11</td>
<td>11782</td>
<td>36</td>
</tr>
<tr>
<td>Aminosilane</td>
<td>10</td>
<td>1</td>
<td>1761</td>
<td>2300</td>
<td>165 ± 22</td>
<td>649</td>
<td>44</td>
<td>532</td>
<td>85</td>
</tr>
<tr>
<td>Adhesive</td>
<td>4</td>
<td>1</td>
<td>734</td>
<td>742</td>
<td>152 ± 31</td>
<td>6915</td>
<td>12</td>
<td>13904</td>
<td>32</td>
</tr>
<tr>
<td>–NCS</td>
<td>4</td>
<td>1</td>
<td>851</td>
<td>768</td>
<td>158 ± 32</td>
<td>4749</td>
<td>21</td>
<td>7854</td>
<td>51</td>
</tr>
<tr>
<td>Aminosilane</td>
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<td>5</td>
<td>291</td>
<td>254</td>
<td>143 ± 33</td>
<td>13724</td>
<td>24</td>
<td>25839</td>
<td>41</td>
</tr>
</tbody>
</table>

Percent CV (coefficient of variation), spot diameter and fluorescence (a.u.) data were calculated from 18 spots (spots in triplicate; two slides each; three subarrays per slide).
unbound protein and deactivate reactive surface groups. Finally, the slides were washed twice in 1 × PBS (pH 7.2) and then blown dry using compressed air or spun dry in the centrifuge (900 rpm for 3 min).

2.6. Immobilization capacity

Immobilization capacity in fmoles mm$^{-2}$ was calculated by taking the median fluorescence minus the local background of 18 replicate spots of Dy633-labelled anti-IgG before and after blocking (30 min) multiplied by spotted protein concentration and divided by molecular mass of labelled protein and square radius of the spot. The calculation was done according to the formula. The factor $1.9 \times 10^6$ is calculated from the volume of the protein solution per spot (0.6 nL spot$^{-1}$) and the PI.

$$I = \frac{1.9 \times 10^6}{F_A M_{LP} R^2} C_{LP}$$

where $I$ is immobilization capacity; $F_B$ is fluorescence before blocking; $F_A$ is fluorescence after blocking; $M_{LP}$ is molecular mass of labelled protein; $R$ is spot radius; $C_{LP}$ is concentration of spotted protein.

2.7. Direct immunoassay

Protein slides were processed with 4 ng $\mu$L$^{-1}$ Dy633-labelled anti-Rabbit IgG ($\lambda_{exc} = 635$ nm, $\lambda_{em} = 670$ nm) (DyAB) (Dyomics) in 1 × PBS (pH 7.2)/0.1% Tween-20 at 4°C for 3 h, then washed twice in 1 × PBS (pH 7.2) and spun dry in the centrifuge (900 rpm for 3 min).

2.8. Fluorescence detection

Slides were stored in the dark and scanned on the same day the immunoassay was performed. Fluorescence measurements were taken using a GenePix™ 4000B non-confocal scanner by Axon Instruments. For data comparison, the PMT (photomultiplier tube) was kept constant within single experiments. All fluorescence (a.u.) data is background-corrected. Additionally, data flagged as bad, according to parameters set in the Genepix software (e.g. spot diameter 40–220 µm; signals >200 a.u. fluorescence), was filtered.

3. Results and discussion

3.1. Effect of substrate, hydrogel concentration and number of layers on immobilization capacity and assay performance

Polyurethane layers of 2%, 4% and 10% PU and one or five layers of 2% PU, on either an amino, isothiocyanate or adhesive substrate, were evaluated in a direct IgG immunoassay. The fluorescence (a.u.) thereby achieved for 0.05–1 mg mL$^{-1}$ rabbit IgG processed with 4 ng $\mu$L$^{-1}$ Dy633-labelled anti-rabbit IgG represents a measure for the immobilized active IgG available for the immunoassay and consequently for the suitability of the hydrogel surface for protein chips. Figures of merit for the PU surfaces are compiled in Table 1. The data in Table 1 reveals that increasing hydrogel concentration results in increased layer thickness: layers of 4% PU are three times thicker and 10% PU 7.5 times thicker than those of 2% PU, regardless of which type of substrate was used. Multiple layers of 2% PU resulted in about the same layer thickness as one layer of PU; thickness increased by only 50 µm. This might be due to the fact that a deposited, additional layer dissolves the previous one during retention (2 min) in the dip-coating solution. The ability of the surface to immobilize processable IgG, as expressed by the slope of the loading curve (change in fluorescence signal per concentration unit), decreases with increasing hydrogel concentration: fluorescence values (a.u.) are about eight times higher for 2% PU than for 4% PU, whereas no significant signal was measured for 10% PU. Multiple layers of PU resulted in a deterioration of assay performance, yet still led to stronger signals than surfaces of 4% or 10% PU. The poor assay performance on 10% PU might be due to an increased density of functional groups on the surface and thus to more closely packed immobilized antibodies, which are thereby sterically hindered from reacting with the target. In fact, when spotting 0.5 mg mL$^{-1}$ Dy633-labelled anti-IgG (0.3 ng spot$^{-1}$) onto surfaces made of 2%, 4% and 10% PU 88%, 98% and 95% of the initially deposited material was immobilized which corresponds to 388 fmoles mm$^{-2}$, 677 fmoles mm$^{-2}$, and 651 fmoles mm$^{-2}$, respectively. This problem is usually solved by mixing functionalized and non-functionalized polymers in order to control the density of reactive groups and, accordingly, immobilized antibodies [12]. Another reason might be the decreased transmission of light in layers of high polymer concentration, leading to more light being absorbed by the polymer than by the fluorescent label to be activated. The same is true for the light emitted by the fluorophores (not just the excitation light is being absorbed and/or scattered, but also the emitted light).

The optimum PU concentration is therefore 2%, and the optimum probe concentration 0.5 mg mL$^{-1}$ (0.3 ng spot$^{-1}$). With respect to data reproducibility (see coefficients of variation (CV) in Table 1), there was no difference between 2% and 4% PU, yet 10% PU showed a tremendously high CV, while there was almost no effect for amino, adhesive and NCS slides when used as activated substrates to promote better adhesion and binding of the hydrogel, allowing the layer to expand in only one dimension. Clearly, the CVs of PU-covered amino (36%), adhesive (32%) and NCS slides (51%) indicate that the fluorescence signals obtained are comparable (one has to keep in mind that variation was calculated from raw data, in order to show actual differences in slide performance, while data filtering and normalization techniques applied for routine applications, would result in much lower CVs).

3.2. Surface characterization

Fig. 1 presents the surface topography of the 2% PU surface. As can be seen from the AFM image the surface is isotropically flat without preferred direction of the texture providing a peak-to-peak roughness of ~8 nm. The average roughness $S_A$ is 0.619 nm, whereas $S_q$ (RMS roughness) is 0.787 nm. The surface can be considered as non-porous, as there are only 0.183
The mechanical stability of the PU layers was estimated by measuring the layer thickness before and after incubation with Dy633-labelled anti-IgG using a profilometer. The incubation time was set to 3 h, as this was the typical incubation time for the direct on-chip immunoassay. As is obvious from Table 1, increasing the PU concentration leads to improved mechanical stability: the loss of hydrogel on aminosilane glass covered with 2% and 4% PU was 14% and 5%, respectively, whereas respective 10% PU layers showed no loss in material. On the contrary, the thickness increased by 30% indicating swelling of the polyurethane. When comparing the mechanical stability of PU layers (4% PU) on aminosilane (−5%), adhesive (+1%) and −NCS glass support (−10%), the mechanical stability was similar.

3.3. Modification of PU

Functionality was introduced by the addition of functional or charged reagents (polystyrenesulfonic acid, cysteaminhydrochloride, dextran sulfate and chitosan) or by employing bifunctional crosslinkers (monochlortriazinyl-ß-cyclodextrin). However, no improvement in assay performance was achieved (unpublished results) indicating that simple one-step adsorption of antibody on anionic polyurethane is the immobilization method of choice. In order to investigate the effect of surface charge on IgG binding, PU layers were modified with mercaptoethanesulfonic acid, to create an even more anionic surface, and cysteaminhydrochloride, to produce a cationic surface. The results show that signals on PU and cysteamin-modified PU were about the same, whereas the signals obtained on surfaces modified using mercaptoethanesulfonic acid were enhanced by 30%, suggesting that electrostatic binding of IgG is strongly promoted on very anionic surfaces.

3.4. Effect of print buffer on IgG adsorption

The choice of print buffer for a certain chip surface determines the protein binding capacity and stability as well as the spot morphology and in consequence the signal strength and data reproducibility [13–16]. Therefore, the printing solution is optimized by use of additives and with regard to optimum reaction pH and ionic strength.

3.4.1. Buffer additives

In addition to widely used printing solutions, such as 3× SSC, 3× SSC/1.5 M betaine and 1× PBS (pH 7.2), commercial buffers as well as 1× PBS (pH 7.2) containing various anionic, zwitterionic and cationic detergents usually used in electrophoresis to promote solubility of proteins encountering the hydrophobic sites of the proteins and prevent protein aggregation were employed. Furthermore, kosmotropes, such as sodium sulfate, which enhance hydrophobic interactions and promote protein adsorption, were also used.

Interestingly enough, additives often recommended, such as BSA [14], trehalose [15,16] or glycerol [4], did not significantly enhance fluorescence. On the other hand, the addition of betaine to 3× SSC led to reduced signals and the addition of sulfobetain (ASB14) resulted in about a 50% increase in signals. It is interesting to note that, when comparing the most appropriate additives, which were ASB14 (zwitterionic), SB3-10 (zwitterionic), sodium deoxycholate (anionic), CTAB (cationic) and Tween-20 (non-ionic), there is no correlation between the charge of the additive and the signal-to-noise ratio measured.

3.4.2. Ionic strength

Protein adsorption on hydrophilically neutral surfaces tends to be relatively weak, whereas adsorption of proteins on hydrophobic surfaces is usually very strong and often partially irreversible. Adsorption of proteins on charged surfaces tends to be a strong function of the charge character of the protein, the pH of the medium, and the ionic strength. The influence of ionic strength on microarray signals was investigated using 0.5×, 1× and 2× PBS (pH 7.2) as the printing solution. The ionic strength of the buffers is also reflected in the conductivity of the solution, which is 9.18, 17.42, and 32.30 mS mm⁻², respectively. As shown in Fig. 2, the fluorescence increased with increasing buffer salt concentration (except for the lowest probe concentration: 0.03 mg mL⁻¹ IgG). Up to 40% stronger signals were achieved when using 2× PBS instead of 1× PBS. This might be...
due to the fact that higher ionic strength in the printing solution leads to slower evaporation of spotted IgG (spotting volume is only 0.6 nL) and thus to longer interaction between the spotted IgG and the chip surface resulting in an increased immobilization yield. However, the effect of ionic strength at 0.5 and 1 mg mL\(^{-1}\) IgG remains ambiguous. From the standard deviations in Fig. 2 it is obvious that both signals can be distinguished significantly, yet this is not always the case with 0.5 \(\times\) and 1 \(\times\) PBS. However, the effect on the assay performance is rather small when compared with the huge difference in ionic strength and conductivity of the tested buffers (2 \(\times\) PBS provides 4 \(\times\) and \(\sim 3.5 \times\) greater ionic strength and conductivity, respectively, than 0.5 \(\times\) PBS, while there is no effect at 0.03 mg mL\(^{-1}\) IgG, about 0.5 times at 0.13 and 0.25 mg mL\(^{-1}\) and 0.2 times at 0.5 and 1 mg mL\(^{-1}\) IgG). The effect is obviously more strongly expressed at low antibody concentrations.

3.4.3. pH of print buffer

The effect of pH on IgG adsorption was investigated when printing 0.03–0.5 mg mL\(^{-1}\) IgG (0.018–0.3 ng spot\(^{-1}\)) in commercial Sörensen sodium phosphate buffers of pH 5.8, 7.2 and 8.0. The strongest signals were measured in Sörensen buffer of pH 5.8, and the signals detected for IgG spotted in buffers of pH 7.2 and pH 8.0 were of about the same strength, but 25–60\% lower than in buffer of pH 5.8. In conclusion, except for pH 5.8, the effect of the buffer pH on the fluorescence signal is negligible, an observation that has been previously reported [13] and investigated over four pH units (pH 4.5–pH 8.5) by Kusnezow et al. [16]. The stronger signals at pH 5.8 can be attributed to greater immobilization capacity at pHs below the isoelectric point of the antibody, since more antibody is bound to the anionic polyurethane due to stronger electrostatic adhesion of the more positively charged IgG (the isoelectric point of IgG is 6). Clearly, the loading ability of the PU surface is increased at pH 5.8, which produces stronger signals at all tested immobilized IgG concentrations. However, the increase in signal with immobilized antibody concentration follows the same mechanism as at lower pHs, since the linear coefficients are very similar and the calibration curve is merely shifted upward, as may be seen in Fig. 3.

3.5. Choice of blocking solution

Four different blocking solutions were tested in order to create PU surfaces of low fluorescence background noise: (I) 1 \(\times\) PBS (pH 7.2)/0.1% Tween-20, (II) 1 \(\times\) PBS (pH 7.2)/0.1% Tween-80, (III) 1 \(\times\) PBS (pH 7.2)/mercaptoethanesulfonic acid, and (IV) 1 \(\times\) PBS (pH 7.2)/cysteaminhydrochloride. In using different blocking solutions, we aimed at creating surfaces of different hydrophilicity and surface charge. Surfaces that are more hydrophilic are known to decrease non-specific adsorption, whereas charged surfaces were expected to promote either specific or non-specific binding. In order to compare the hydrophilicity of the blocked surfaces, 0.01, 0.05 and 0.1 mg mL\(^{-1}\) labelled antibody in 1 \(\times\) PBS (pH 7.2) was spotted onto the PU chip. Spot diameter was then taken as a measure for the surface hydrophilicity and calculated as a mean value for 54 spots (spots in triplicate, six arrays per slide, three slides). The greatest influence on the spot diameter/hydrophilicity was observed when anionic mercaptoethanesulfonic acid and non-ionic Tween-80 were used: the spots increased by 35 \(\mu\)m in comparison to the non-blocked surface. The mean spot diameters obtained for non-blocked PU surfaces and PU surfaces blocked with solutions I–IV were 125 \(\mu\)m, 134 \(\mu\)m, 159 \(\mu\)m, 158 \(\mu\)m and 120 \(\mu\)m. Fig. 4 shows the mean fluorescence (a.u.) for 27 spots (spots in triplicate, three arrays per slide, three slides) obtained with 0.05–1 mg mL\(^{-1}\) IgG after blocking in solutions I–IV and processing with 4 ng \(\mu\)L\(^{-1}\) Dy633-labelled anti-rabbit IgG. The strongest signals by far were achieved when the surface was blocked with Tween-20 and Tween-80. The latter produced signals that were enhanced by 25\% compared with Tween-20, whereas solutions III and IV resulted in signals reduced by a factor of 2–5. In summary, neither the surface charge – blocking with anionic MESA and cationic CAHCL – nor the hydrophilicity or wettability of the PU surface affected blocking efficiency (compare the diameter of surfaces blocked with MESA and Tween-80).
3.6. Comparison with slide H and crossreactivity

PU slides were compared with Nexterion® Slide H in a direct IgG immunoassay spotting 0.063–0.5 mg mL\(^{-1}\) rabbit IgG in 1× PBS (pH 7.2), Sörensen (pH 5.8) and Nexterion buffer. Immobilization of IgG on polyurethane was due to electrostatic adsorption, whereas immobilization on slide H was covalent through amino groups of amino acids side chains on the protein surface. The immobilization on the PU chip occurs in a random manner, whereas binding on slide H is “statistically oriented” as some orientation is given due to binding between reactive groups of the surface and certain units of the antibody (e.g. NH\(_2\) groups in the arginine units). Fig. 5 shows the respective loading curves calculated from 18 spots (three subarrays on two slides, probes spotted in triplicate). Highest loading was achieved for the PU chip using PBS and Nexterion buffer. Comparable results were obtained for slide H at 0.5 mg mL\(^{-1}\) IgG. However, in contrast to slide H, which shows an almost linear loading curve, the PU chip displays a logarithmic dependence on the immobilized probe concentration. While the PU chip shows saturation at 0.25 mg mL\(^{-1}\) IgG in Nexterion buffer, slide H can be still loaded with antibody, as also stated in the slide H data sheet (recommened probe concentration: 0.1–1 mg mL\(^{-1}\)). This can be advantageous in especially sandwich immunoassays, where high immobilization yield leads to increased assay sensitivity, however, then again may limit the assay sensitivity because probe material available for spotting is limited (e.g. medical diagnostics, biopsies). In any case, the choice of printing solution strongly effects the IgG loading: when using Nexterion buffer for the PU chip saturation occurs at 0.25 mg mL\(^{-1}\) IgG, whereas when using Sörensen buffer the concentration of immobilized IgG is linearly increased up to 0.5 mg mL\(^{-1}\) IgG producing fluorescence signals that are comparable to those achieved with slide H using the Nexterion buffer. As a consequence, the loading capacity on the PU chip can be tuned with respect to the individual application by using printing solutions of different composition.

The reproducibility of measurement reflected by the coefficient of variation (%CV) calculated from 18 spots is 35%, 38% and 29% for the PU chip and 28%, 14% and 10% for slide H when using Nexterion buffer, Sörensen buffer (pH 5.8) and 1× PBS (pH 7.2), respectively. From the fluorescence signals in Fig. 5 it is obvious that there is no effect of immobilized biomolecule orientation on the assay performance. One reason for this might be found in the fact that IgG is a very stable molecule, thus requiring no special conditions for surface immobilization; another factor promoting IgG binding might be the surface charge combined with the gel character of the 3D surface and the additional fact that orientation plays a less important role than often reported in literature, especially in the case of antibodies. In fact, when Wacker et al. [5] compared direct spotting, DNA-directed immobilization and streptavidin–biotin attachment, they observed that all three tested formats led to comparable detection limits, signal intensity as well as assay sensitivity and reproducibility. Kusnezow et al. [16] reported that there was no difference in signal-to-noise ratios when the carbohydrate groups of antibodies were activated and the antibodies were immobilized in an oriented manner on the slide. On the contrary, activation and subsequent purification were both time consuming and led to a loss of up to 40% of antibody. In [17] poly-l-lysine, slides for electrostatic adsorption as well as aldehyde surfaces for covalent immobilization were judged easy to prepare and robust. The authors reported both very good signal-to-noise ratios and interfer coefficients of variation. Even though according to our profilometry measurements the sensitive layer of the PU chip is more than 100 times thicker than slide H, no binding problems due to slowed diffusion or decreased fluorescence signals as a result of increased background were observed. On the contrary, the PU surface might have the potential of further enhancing assay performance when producing stable hydrogel layers that are thinner than \(\sim 200\) nm.

Furthermore, both chip surfaces were tested for crossreactivity with 0.125 mg mL\(^{-1}\) goat IgG, human IgG and monoclonal anti-CRP. Crossreactivity was calculated from 18 spots (three subarrays on two slides, probes spotted in triplicates) as percentage of the specific fluorescence signal achieved at 0.125 mg mL\(^{-1}\) rabbit IgG. The respective fluorescence images for 0.125 mg mL\(^{-1}\) rabbit IgG, goat IgG, human IgG and monoclonal anti-CRP spotted in Nexterion buffer are shown in Fig. 6.

![Fig. 5. Fluorescence signals obtained for: ◆ 3% PU and ▐ Nexterion® Slide H in 1× PBS (full lines), Sörensen (pH 5.8) (dotted lines) and Nexterion buffer (dashed lines).](image-url1)

![Fig. 6. Fluorescence images showing the crossreactivity in % with 0.125 mg mL\(^{-1}\) goat IgG, 0.125 mg mL\(^{-1}\) human IgG and 0.125 mg mL\(^{-1}\) anti-CRP using the PU-chip (left) and slide H (right).](image-url2)
Percent crossreactivity given in the images indicate that the PU chip reveals up to five times lower crossreactivity (≤0.74%) than the Nexterion slide H (≤3.34%). Specificity of the PU chip was superior over slide H in all tested print buffers, however, crossreactivity was lowest in Nexterion and highest in Sörensen buffer (PU chip ≤8.5%; slide H ≤13.6%).

3.7. Storage stability

The PU surfaces were stored in the refrigerator or at RT for 2 months without loss in stability or deterioration of assay performance. In contrast to slide H, which is recommended to be stored at −20 °C to prevent hydrolysis of amine-reactive groups, storage of the PU chip at −20 °C led to decreased signal strength and reduced assay reproducibility.

4. Conclusions

Herein, we have presented a polyurethane surface capable of binding proteins via electrostatic adsorption. The PU layer was characterized and optimized in terms of layer thickness (∼200 nm) and glass substrate (adhesive glass), hydrogel concentration (2%), surface topography (Sa = 0.619 nm, Sq = 0.787 nm) and IgG loading (388 fmoles IgG mm⁻² on 2% PU). Compared with Nexterion slide H the PU chip reveals higher loading capacity in 1× PBS (pH 7.2), Sörensen (pH 5.8) and Nexterion buffer and up to five times lower crossreactivity with goat IgG, human IgG and monoclonal anti-CRP indicating that simple IgG adsorption on the PU chip is a powerful alternative to covalent IgG binding using Nexterion slide H.

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