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Influence of Pluronic F127 on the distribution and functionality of inkjet-printed biomolecules in porous nitrocellulose substrates

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ABSTRACT

The distribution of inkjet-printed biomolecules in porous nitrocellulose substrates often results in a non-homogeneous spot morphology commonly referred to as 'doughnut-shaped' spots. We have studied the influence of Pluronic F127 (an amphiphilic surfactant) on the functionality of inkjet-printed primary antibody molecules and on the final assay result by performing a one-step antibody binding assay in the nitrocellulose substrate. The primary antibody was printed with and without Pluronic, followed by the addition of double-labelled amplicons as antigen molecules and a fluorophore-labelled streptavidin as detection conjugate. The distribution of the fluorescence intensity down into the nitrocellulose substrate was investigated by confocal laser scanning microscopy in 'Z' stacking mode. Each horizontal slice was further analysed by applying a concentric ring format and the fluorescence intensity in each slice was represented in a colour-coded way. The mean and total fluorescence intensity of the antibody binding assay (fluorescent streptavidin) showed a peak at 0.2% (w/v) Pluronic F127. In addition, an improved spot morphology was observed also peaking at the same Pluronic concentration. Subsequently, we investigated the direct influence of Pluronic F127 on the location of the primary antibody molecules by labelling these molecules with the fluorophore Alexa-488. Our results show that upon increasing the concentration of Pluronic F127 in the printing buffer, the spot diameter increased and the number of primary antibody molecules bound in the spot area gradually decreased. This was confirmed by analysing the distribution of fluorescently labelled primary antibody molecules down into the membrane layers.

We conclude that a particular ratio between primary antibody and Pluronic F127 molecules in combination with available substrate binding capacity results in an optimal orientation, that is F*ab*-UP, of the primary antibody molecules. Consequently, an increased number of antigen molecules (in our case the labelled amplicons) and of the fluorescent detection conjugate (streptavidin) will give an optimal signal. Moreover, distribution of the primary antibody molecules was more homogeneous at the optimal Pluronic F127 concentration, contributing to the better spot morphology observed.

1. Introduction

Non-contact arraying is a modern tool for printing biomolecules [1] on a range of porous and non-porous substrates. Researchers have shown that high-quality microarrays are produced on porous substrates like nitrocellulose [2,3], porous silicon [4], alumina [5–7] and hydrogels [8] as compared the non-porous substrates like glass [9] and polystyrene [10]. On porous substrates, a lower limit of detection can be obtained as compared to non-porous substrates[11]. The 3-D matrix of the porous substrates [12] enables more molecules to bind as projected per unit surface area and this results in a better signal-to-noise (S/N) ratio [13]. Of all the available porous substrates, the nitrocellulose membrane has been used extensively in diagnostic applications [2,14–16] and for producing biochips[3,17].

It has been commonly observed that, upon printing, biomolecules often distribute non-homogeneously which results in 'doughnut-shaped' spots, also referred to as the 'coffee-stain' effect[18–20]. Researchers have successfully demonstrated that

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for DNA microarrays, incorporation of various additives like DMSO [21], polyvinyl alcohol[22], betaine[23] and Triton-X 100[24] can result in a more homogeneous spot morphology. However, the chemical aspects of DNA microarrays cannot be easily translated to the production of protein microarrays due to the fundamental biophysical and biochemical differences between these two classes of biological substances.

Recently, Norde and Lyklema have shown that Pluronic molecules enhance the functionality of deposited antibodies by forcing them to adsorb at the substrate surface with their antigen binding sites oriented towards the solution[25]. They have also reported that in the presence of Pluronic (P75), the adsorbed IgG molecules showed both improved stability and biological activity. Pluronics are tri-block copolymers with repeating polyoxyethylene-polyoxypropylene-polyoxyethylene (PEO-PPO-PEO) units where the central part (PPO) is hydrophobic and the ends (PEO) are hydrophilic in nature. For our study, we have used Pluronic F-127 which belongs to a class of polymers called as poloxamers [26].

However, Pluronic P75 is very different from Pluronic F127 used by us. Earlier studies performed by Alexandridis *et al.* [27] have shown significant difference in the properties of the two classes of tri-block polymers. More specifically, the molar mass of F127 is 12600 Da whereas that of P75 is 4150 Da and the percentage of PEO in F127 is 70% whereas in P75 it is only 50%. This corresponds to $(PEO)_{100}$-$(PPO)_{65}$-$(PEO)_{100}$ for F127 and $(PEO)_{24}$-$(PPO)_{36}$-$(PEO)_{24}$ for P75, the subscripts indicating the average number of monomers in the polymer blocks. Thus the two Pluronics differ strongly in length of the respective PPO and PEO blocks. The larger PPO block in F127 allows this Pluronic to attach more strongly to the substratum surface and, more importantly, the longer PEO moieties in F127 allows for a much stronger influence on the orientation of IgG molecules on the substratum surface (see Sections 3.1 and 3.3). On the other hand, Pluronic F127 has been used for inkjet printing applications [28,29] of protein microarrays. Wolter *et al.* have successfully demonstrated that the application of Pluronic F127 improves the signal as well as the limit of detection on a 2-D substrate surface [30]. Therefore, we have selected Pluronic F127 for studying its influence on the functionality of inkjet-printed primary antibody molecules, the final assay result, spot morphology as well as the distribution of biomolecules in nitrocellulose membrane pads.

We have assessed the influence of various concentrations of Pluronic F127 (0 to 1% (w/v)) on the spot morphology and functionality of the primary antibody (anti-FITC). Using a non-contact inkjet printer, primary anti-FITC antibodies were printed on nitrocellulose membrane slides, and the functionality of the primary antibodies was investigated by performing a one-step diagnostic antibody assay based on the binding of a double-labelled amplicon (FITC- and biotin-labelled), as was reported for the nucleic acid microarray immunoassay (NAMIA) [2,9,10]. The distribution as well as the functionality of the primary antibody was judged from the distribution of the fluorescence signal of the final assay (i.e. fluorescent streptavidin). Using confocal laser scanning microscopy, the NAMIA spots were sliced horizontally ('Z' stack method) and the signal distribution profile in each slice was calculated using a concentric ring format. The results are presented in a colour-coded format. Additionally, we also investigated the direct influence of the amphiphilic surfactant (Pluronic F127) on the distribution of the primary antibody molecules that had been labelled with Alexa-488.

2. Material and methods

2.1. Reagents

Pluronic F127 was purchased from Sigma Aldrich (St. Louis, MO, USA), and a 1% (w/v) stock solution was prepared in 150 mM phosphate buffered saline (PBS, pH 7.4). Running buffer (100 mM borate buffer containing 1% BSA and 0.05% Tween-20) was used as a diluent for the conjugates/labels and also during the intermediate washing steps.

2.2. Biomolecules

Anti-fluoroisothiocynate (FITC) was purchased from Bioconnect (Huissen, The Netherlands). The DNA templates for *Corynebacterium bovis* were provided bythe Animal Health Service, Deventer, The Netherlands. Streptavidin-Alexa-633 for labelling biotinylated amplicons was from Invitrogen (Bleiswijk, The Netherlands).

2.3. Substrates for printing biomolecules

Nexterion nitrocellulose membrane coated slides in 16-pad format were purchased from Schott AG (Mainz, Germany). The dimension of each pad was 6×6 mm, whereas the thickness was ~ 11 μm.

2.4. Labelling of the primary anti-FITC antibody

The primary anti-FITC antibody molecules were labelled with Alexa-488 fluorophore using an Alexa-Fluor 488 antibody labelling kit (Invitrogen, Oregon, USA). The labelling procedure was carried out as described by Invitrogen, and the concentration of the fluorophore-conjugated antibody (anti-FITC-Alexa-488) was measured using a Nanodrop-1000-v3.6 spectrophotometer (Wilmington, DE, USA).

2.5. Printing of antibodies on NC membrane slides

Anti-FITC and Alexa-488 conjugated anti-FITC antibodies were diluted (200 $\mu g mL^{-1}$) in various Pluronic F127 concentrations (0%, 0.01%, 0.05%, 0.1%, 0.2%, 0.4, 0.8% 1.0%w/v) and loaded into the wells of a Genetix microplate (384 wells). The diluted antibodies were printed on the nitrocellulose membrane pads using a non-contact inkjet printer, sciFLEXARRAYER S3 (Scienion AG, Berlin, Germany). The printer was placed in a hood to maintain constant temperature and humidity. Spotting of the biomolecules was performed at room temperature and $70 \pm 1\%$ humidity. The voltage and pulse of the piezo-dispensing capillary (PDC) were optimized to print a droplet of ~ 250 pL throughout the experiment. The slides were stored overnight in a sealed Al pouch.

2.6. PCR reaction

DNA template from *Corynebacterium bovis* was amplified using primers Cb-F2 and Cb-R3 according to a published protocol[10]. The PCR protocol was optimized to 30 minutes using Phire Hot Start polymerase (Finnzymes) and the Piko thermal cycler (Finnzymes). The forward primer was labelled with a FAM tag and the reverse primer with biotin. The amplification resulted in a double stranded amplicon with at each end a tag, FAM or biotin. Gel electrophoresis confirmed that the amplicon obtained after PCR had the correct band length[31].

2.7. Nucleic acid microarray immunoassay (NAMIA)

To investigate the influence of Pluronic F127 in the printing buffer of the primary anti-FITC antibody a one-step diagnostic assay was performed on the NC membrane slides based on the principle of the Nucleic Acid Microarray Immuno Assay, NAMIA [2,9,10]. Anti-FITC antibodies (specific for the FAM tag) were diluted in various concentrations of Pluronic F127 and printed onto the NC membrane slides. Prior to performing NAMIA, the NC