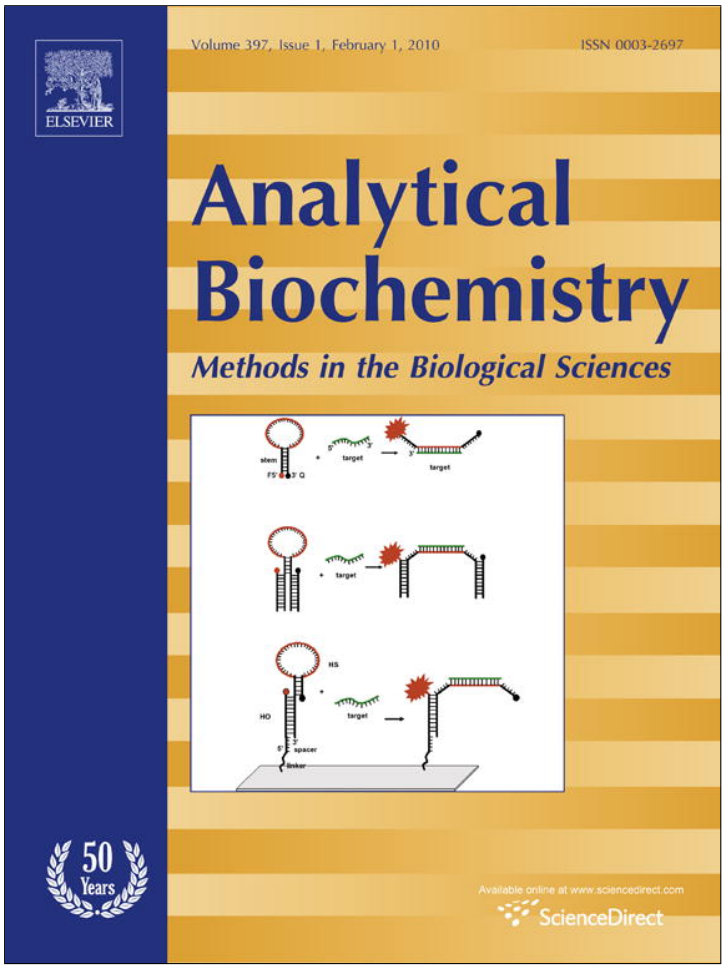

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Coating of nitrocellulose for colorimetric DNA microarrays

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ABSTRACT

We report on the modification of a nitrocellulose film with copoly(DMA–NAS–MAPS), a tercopolymer based on *N*,*N*-dimethylacrylamide (DMA), *N*-acryloyloxysuccinimide (NAS), and 3-(trimethoxysilyl)propyl-methacrylate (MAPS). The chains of this polymer, interacting with nitrocellulose fibers, introduce active ester functionalities that promote the covalent binding of short oligonucleotide fragments to the nitrocellulose thin film. Using colorimetric detection, naked eye visible DNA microarrays are developed for easy identification of foodborne pathogens. The fast and robust procedure of nitrocellulose functionalization opens the opportunity to implement this material in disposable analytical microdevices that do not require sophisticated readout systems.

Nitrocellulose is a polymeric material that has been widely used for 30 years in traditional molecular biology assays such as Southern and Northern blots. Nitrocellulose membranes are used as solid-phase immobilization supports for DNA, RNA, and proteins. DNA, RNA, and proteins bind to the membranes in a noncovalent but irreversible manner by a mechanism that is not fully understood. Hydrophobic interactions are believed to dominate under normal conditions even if electrostatic interactions may be involved as well [1]. The microporous polymeric surface of nitrocellulose is ideally suited for microarrays and offers an alternative to functionalized glass slides that is easy to use and offers high reproducibility [2]. The two most important advantages of nitrocellulose-based microarray slides are binding capacity and the fact that most researchers have knowledge of this material. Nitrocellulose-coated slides contain a 20- to 30-μm-thick membrane and, thus, provide a three-dimensional matrix into which molecules can attach. The three-dimensional nature of the coating provides a much greater binding capacity than do two-dimensional surfaces such as glass slides functionalized with amino or aldehyde groups. A disadvantage of nitrocellulose surface compared with functionalized glass is that the white nitrocellulose membrane reflects excitation light, which typically elevates background noise in microarray experiments. Nitrocellulose is not intrinsically fluorescent, but it reflects light because white surfaces reflect all of the colors in the visible spectrum, including laser light from microarray scanners and white light from microarray imagers [3]. The resulting high background noise, which reduces assay sensitivity, is only partially compensated by the high binding capacity of nitrocellulose. On the other hand, the white color of nitrocellulose is advantageous when using colorimetric detection. Even though fluorescent dye labels remain the dominant label used in conjunction with microarrays [4], the increasing demand for low cost and simplicity in microarray technology makes colorimetric assays for naked eye visible arrays an important field of research. Paper-based diagnostic devices, for example, are promising technology for applications in which low cost and simplicity of fabrication must be combined with microfluidic functions [5]. Examples of these types of devices for monitoring the quality of water and environment have been shown recently [6]. To the best of our knowledge, only few examples of DNA microarrays on nitrocellulose have been reported [2,7], probably due to the difficulty in binding oligonucleotide probes to this support. For example, Mao et al. [8] reported on a colorimetric DNA microarray for the study of hepatitis B virus gene. However, to achieve the sensitivity of fluorescence methods, the assay is carried out on silanized glass slides and the targets are transferred to nitrocellulose for colorimetric detection.

In this work, we modify nitrocellulose slides to covalently bind DNA molecules and demonstrate the suitability of this material as microarray support for colorimetric detection of pathogens. The modification procedure is robust and simple, being based on the interaction of nitrocellulose fibers with copoly(DMA–NAS–MAPS), a tercopolymer based on *N*,*N*-dimethylacrylamide (DMA),[1] *N*-acryloyloxysuccinimide (NAS), and 3-(trimethoxysilyl)propyl-methacrylate (MAPS) [9,10]. The polymer self-adsorbs onto the nitrocellulose from a diluted aqueous solution in 30 min without

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[1] *Abbreviations used:* DMA, *N*,*N*-dimethylacrylamide; NAS, *N*-acryloyloxysuccinimide; MAPS, 3-(trimethoxysilyl)propyl-methacrylate; NBT/BCIP, nitro blue tetrazolium/**5**-bromo-4-chloro-3-indolyl phosphate; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; rDNA, ribosomal DNA.

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the need for any surface pretreatment. Using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) colorimetry, naked eye visible DNA microarrays are developed for easy identification of foodborne pathogens.

Materials and methods

Reagents and equipment

Tris, ethanolamine, standard saline citrate (SSC), ammonium sulfate, sodium dodecyl sulfate (SDS), DMA, MAPS, and phosphate-buffered saline (PBS) stock solution were purchased from Sigma (St. Louis, MO, USA). NAS was obtained from Polysciences (Warrington, PA, USA). FAST Slides (nitrocellulose-coated glass slides) were purchased from Whatman (Sanford, ME, USA). Oligonucleotides were synthesized by MWG (Martinsried, Germany). *Staphylococcus aureus* subsp. *aureus* (ATCC 700699D) and *Listeria monocytogenes* (ATCC 19115D) genomic DNA were obtained from LGC Promochem (Teddington, UK). Polymerase chain reaction (PCR) kits were purchased from Promega (Madison, WI, USA). Streptavidin–alkaline phosphatase from *Streptomyces avidinii* was purchased from Sigma. NBT/BCIP stock solution was purchased from Roche (Mannheim, Germany).

Coating of FAST slides

Copoly(DMA–NAS–MAPS) was synthesized and characterized as described in Ref. [9].

FAST slides were immersed for 30 min in a copoly(DMA–NAS–MAPS) solution (1% [w/v] in water solution of ammonium sulfate at 20% saturation level). The slides were then rinsed with water and dried under vacuum at 80 °C.

Oligonucleotide microarrays

A SciFlexArray spotter (Scienion, Berlin, Germany) was used to pattern six subarrays of amino-modified oligonucleotides (0.1–15 μM) labeled with biotin or Cy3 (P4 and P5) and dissolved in 150 mM sodium phosphate (pH 8.5) as well as to pattern one or four subarrays of 5′-amino-modified oligonucleotides (P1, 10 μM). In our experimental conditions, the volume of spotted drops was 400 pl. Printed slides were placed in a humid chamber and incubated at room temperature overnight.

Unreacted sites on the slides were blocked in 0.1 M Tris–HCl/50 mM ethanolamine for 15–20 min at 50 °C, rinsed with water, and then washed in 4× SSC/0.1% SDS for 15–20 min at 50 °C.

The subarrays spotted with P1 were hybridized with P2 or P3 (2 h, 65 °C), and then they were washed in 2× SSC/0.1% SDS buffer (10 min, 65 °C) and rinsed with 0.2× SSC (1 min), followed by a rinse with 0.1× SSC (1 min). Scanning for fluorescence evaluation was performed with a Scan Array Express scanner from Packard Bioscience (Boston, MA, USA). For colorimetric development, the arrays were hybridized with 2 U/ml streptavidin–alkaline phosphatase in 1× PBS for 20 min at room temperature, washed with 1× PBS for 10 min, and rinsed with water. They were then hybridized with a solution made of 200 μl NBT/BCIP stock solution in 10 ml of 0.1 M Tris–HCl (pH 9.5, 20 °C) and 0.1 M NaCl for 10 min in the dark and washed with water. The results were visible with the naked eye.

Microbial genotyping

The sequences and details of the oligonucleotides used for microbial genotyping are reported in Table 1. Oligonucleotide probes AUR16S, LIS16S, and UNIBACT at the concentration of 20 μM were patterned in two subarrays according to the spotting scheme reported in Fig. 4A. Printed slides were placed in a humid chamber and incubated at room temperature overnight. After blocking as described above, the slides were subjected to hybridization with PCR products of bacterial genomes from *S. aureus* subsp. *aureus* and *L. monocytogenes* amplified by the use of primers F-16SR and R-16SR (see Table 1 for details) as described in Ref. [11]. The forward primer F-16SR is labeled with biotin, and the amplicons generated are approximately 370 bp in length (checked by agarose gel electrophoresis). The PCR was performed for 40 cycles (94 °C, 25 s; 55 °C, 30 s; and 72 °C, 25 s) starting from 1.6 μg of bacterial DNA. Hybridizations were carried out with a total volume of 40 μl (20 μl PCR product in 5× SSC, 0.1% SDS, and 0.02% bovine serum albumin [BSA]) denatured at 95 °C for 10 min, immediately applied to microarrays, and kept in a humidified chamber for 2 h at 50 °C. After hybridization, the slides were washed (2× SSC and 0.1% SDS) at hybridization temperature for 5 min. This operation was repeated twice and followed by two washing steps (0.2× SSC and 0.1× SSC for 1 min at room temperature).

Colorimetric development was performed adding streptavidin-conjugated alkaline phosphatase (final concentration 2 U/ml) to an NBT/BCIP solution in 0.1 M Tris–HCl (pH 9.5) and 0.1 M NaCl.

Table 1
Sequence of the oligonucleotides used in this work.

Code	Sequence 5′–3′
P1	NH_2-GCCCACCTATAAGGTAAAAGTGA
P2	Cy3-TCACTTTTACCTTATAGGTGGGC
P3	biotin-TCACTTTTACCTTATAGGTGGGC
P4	Cy3-TCACTTTTACCTTATAGGTGGGC-NH_2
P5	NH_2-TCACTTTTACCTTATAGGTGGGC-biotin
AUR16S	GCTCCTAAAAGGTTACTCCACCGGCT-NH_2
LIS16S	ACTGAGAATAGTTTTATGGGATTAGG-NH_2
UNIBACT	GTACAAGGCCCGGGAACGTATTCACC-NH_2
F-16SR	biotin-AACTGGAGGAAGGTGGGGAT
R-16SR	AGGAGGTGATCCAACCGCA

Results and discussion

DNA binding experiments

Nitrocellulose slides coated by copoly(DMA–NAS–MAPS) were tested for their ability to covalently bind biomolecules using an amino-modified oligonucleotide (P4) fluorescently labeled with Cy3 (see Table 1). The oligonucleotide was spotted on the slides as described in Materials and methods. After an overnight binding, its attachment efficiency was quantified by scanning the slides for fluorescence evaluation before and after several washing steps. Unmodified and copoly(DMA–NAS–MAPS) modified nitrocellulose were compared side by side. Coated glass slides were added to the experiment as a control because the copoly(DMA–NAS–MAPS) also generates a stable coating on glass [9].

The results, summarized in Fig. 1A, represent the average fluorescent values of oligonucleotide spots (16 replicates for each concentration) ranging from 0.1 to 15 μM. The images of fluorescence scanning of the three substrates are shown in Fig. 1B.

The spot fluorescence intensity after washing off the excess of unbound oligonucleotide on the coated and uncoated nitrocellulose is comparable for every oligonucleotide concentration. However, the experiment confirms the superior binding capacity of nitrocellulose over glass slides coated by copoly(DMA–NAS–MAPS) resulting from its three-dimensional structure and demonstrates that the polymeric coating does not alter the porous structure of nitrocellulose. As shown in Fig. 1B, the background noise provided by the copoly(DMA–NAS–MAPS)-coated nitrocellulose and glass

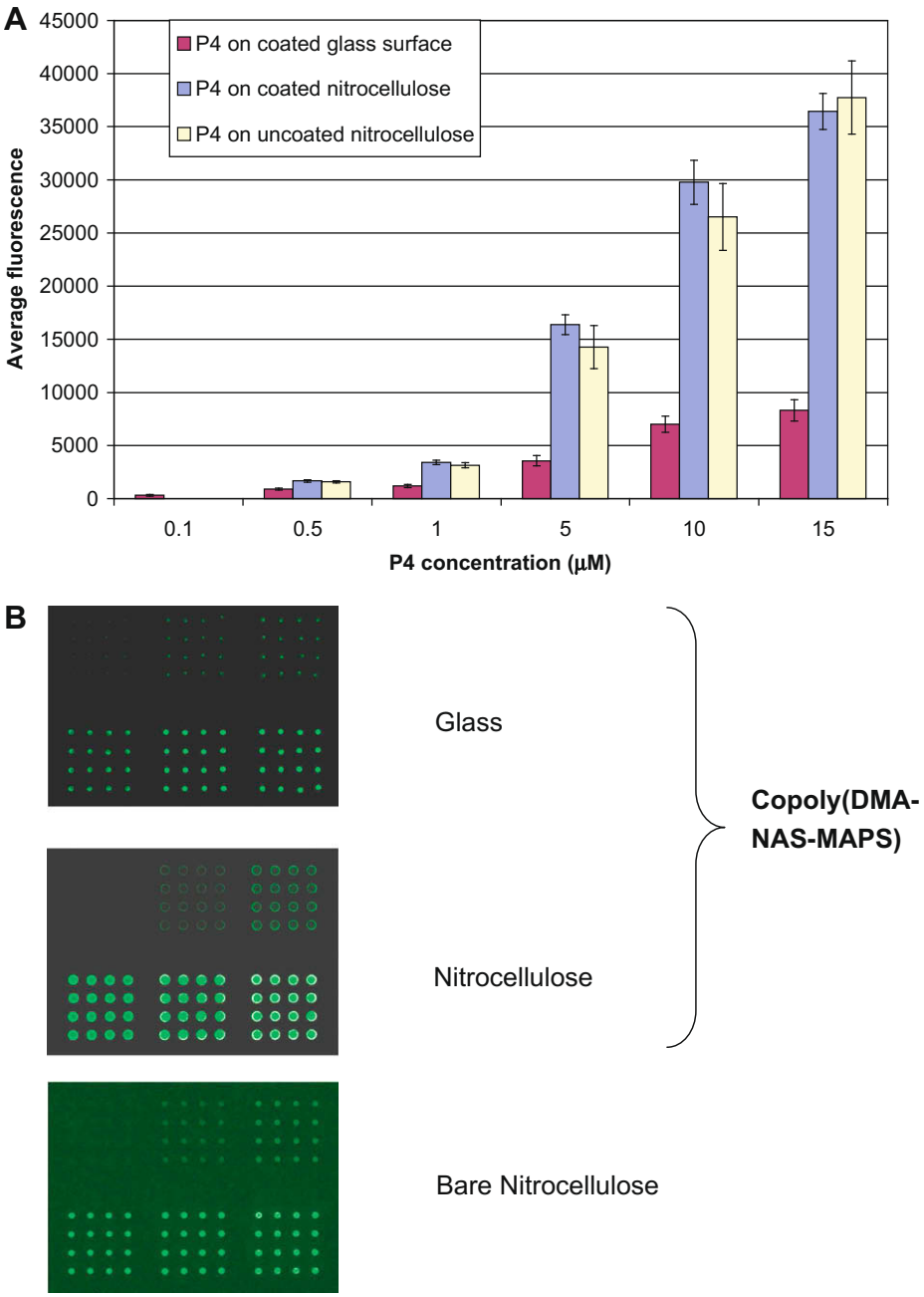

Fig. 1. (A) DNA binding test on copoly(DMA–NAS–MAPS)-coated glass and nitrocellulose and on uncoated nitrocellulose. Amino-modified fluorescent oligonucleotide (P4) at concentrations ranging from 0.1 to 15 μM was deposited on the slides. After overnight binding in a humid chamber and washing of unbound DNA, the average fluorescence of the spots was calculated for each concentration. The standard deviation is indicated by error bars. (B) Images of the DNA arrays on copoly(DMA–NAS–MAPS)-coated glass and nitrocellulose and on bare nitrocellulose. Arrays were analyzed with a fluorescence scanner at 22% laser power and 64% Photomultiplier tube gain.

slides is lower than that provided by uncoated nitrocellulose; the larger spots in coated nitrocellulose are due to the increase in surface hydrophilicity favored by the copolymer. A similar DNA binding test was carried out using an amino-modified oligonucleotide labeled with biotin (P5) to perform colorimetric detection through the use of NBT/BCIP and alkaline phosphatase conjugated to streptavidin. The results of this experiment performed on copoly(DMA–NAS–MAPS)-coated and bare nitrocellulose are shown in Fig. 2. In Fig. 2A, five oligonucleotide concentrations ranging from 0.05 to 1.5 μM are clearly visible with a high signal-to-noise ratio. In Fig. 2B, the results of the same experiment carried out on bare nitrocellulose are shown; due to the high background, it was not possible to detect any oligonucleotide concentration. The mixture of reactives required by the colorimetric assay did not spread on the surface and forms a stain due to the high substrate hydrophobicity.

The modification of nitrocellulose surface obtained by chemisorption of copoly(DMA–NAS–MAPS) did not alter the three-dimensional structure of the material, thereby keeping its superior probe binding capacity. Furthermore, it provided a lower background when using either fluorescence- or NBT/BCIP-based colorimetric detection.

DNA–DNA hybridization test

A set of experiments was performed to evaluate the fluorescence signal produced in a test experiment of oligonucleotide hybridization. This test is of great importance because the major

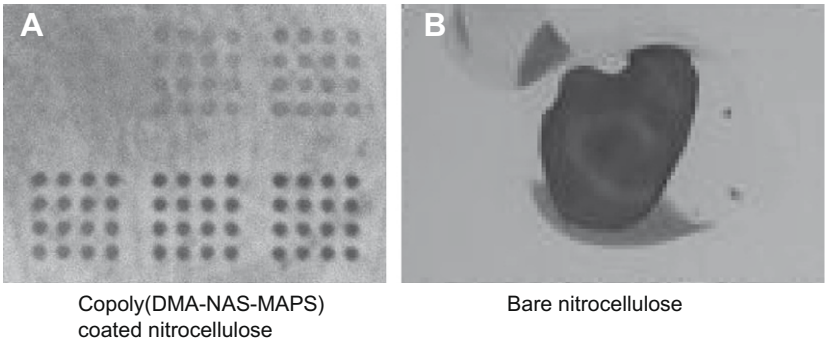

Fig. 2. (A) Results of the DNA binding test on copoly(DMA–NAS–MAPS)-coated nitrocellulose. Amino-modified biotinylated oligonucleotide (P5) at concentrations ranging from 0.1 to 15 μM was deposited on the slides. After overnight binding in a humid chamber and washing of unbound DNA, colorimetric detection was performed through the use of NBT/BCIP and alkaline phosphatase conjugated to streptavidin. (B) Results of the DNA binding test carried out as described above on bare nitrocellulose.

concern in oligonucleotide arrays on nitrocellulose is (i) the strength of probe binding, (ii) the accessibility of the probe for hybridization, and (iii) the specificity obtained in the interaction. A 22-mer probe oligonucleotide (P1) at 10 μM concentration was spotted and hybridized with the complementary Cy3-labeled oligonucleotide (P2) in a total volume of 25 μl at 1 μM concentration. The hybridization was performed at 65 °C for 2 h as described in Materials and methods. The results obtained with copoly(DMA–NAS–MAPS)-coated and bare nitrocellulose, as well as on a glass slide coated with the same copolymer, are shown in Fig. 3. In the upper panel of the figure, the fluorescence efficiency is reported as the average value of the fluorescence signal obtained on an array of 81 replicates; in the lower panel, a portion of the arrays is shown. The fluorescence intensity obtained after hybridization was 10 times higher on the coated nitrocellulose than on the bare one and also much higher than that on coated glass. No detectable nonspecific hybridization signals were seen with the noncomplementary control oligonucleotide probe (data not shown). We believe that the copoly(DMA–NAS–MAPS) coating confers to the nitrocellulose the ability to covalently bind amino-modified DNA through the active ester (NAS), still keeping the superior binding capacity of its three-dimensional structure. The fluorescence intensity of the hybridization test is, therefore, higher on coated nitrocellulose than on uncoated nitrocellulose. In stringency conditions, bare nitrocellulose is unable to retain the probes adsorbed to its fibers. Instead, when coated with copoly(DMA–NAS–MAPS), it provides hybridization efficiency also higher than glass because it binds a much higher amount of oligonucleotide probe.

A similar experiment was performed on coated and uncoated nitrocellulose using P1 as the oligonucleotide probe and a biotin-labeled complementary oligonucleotide target (P3) for performing an NBT/BCIP-based colorimetric detection. Similar to the binding experiments described in the previous paragraph, we were unable

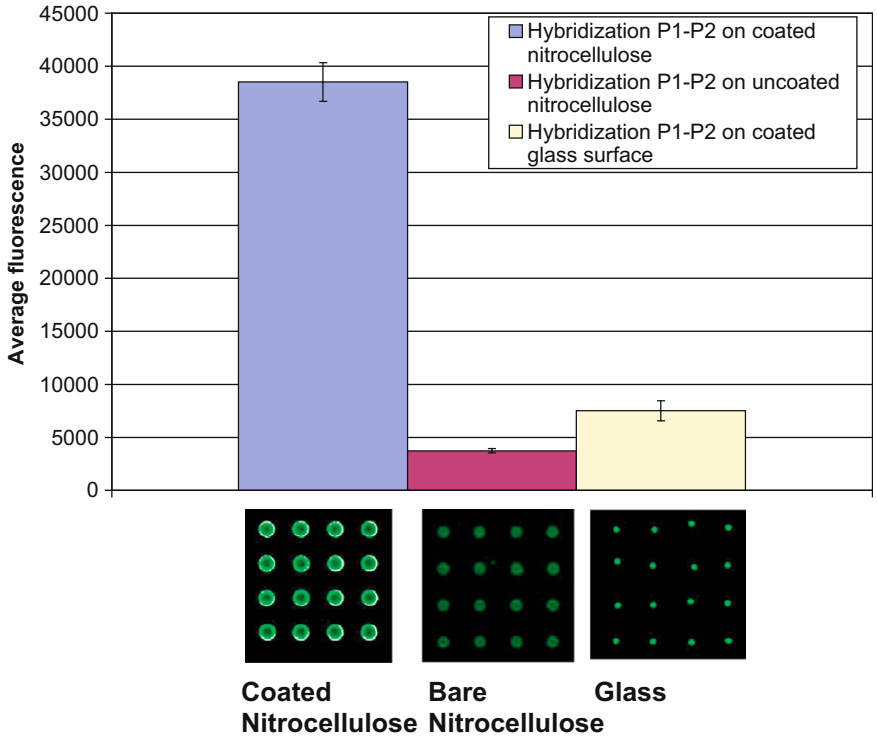

Fig. 3. Results of a model DNA–DNA hybridization test using oligonucleotides P1 and P2. (Upper panel) Average fluorescence of 81 replicated spots on copoly(DMA–NAS–MAPS)-coated nitrocellulose and glass slides as well as bare nitrocellulose. (Lower panel) Images of a portion of the arrays on copoly(DMA–NAS–MAPS)-coated nitrocellulose and glass slides as well as bare nitrocellulose. Arrays were analyzed with a fluorescence scanner at 22% laser power and 64% Photomultiplier tube gain.

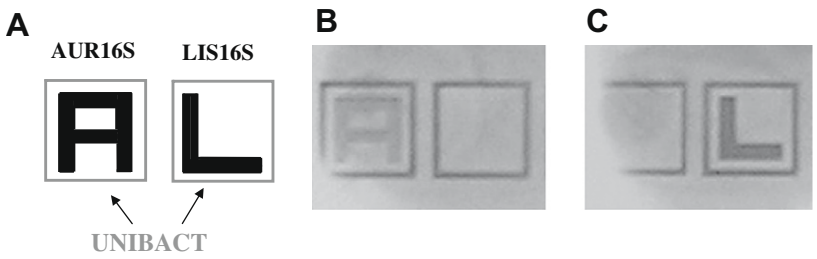

Fig. 4. (A) Spotting scheme on coated nitrocellulose slides. A short pitch (200 μm) between the spots was set to favor the merging of contiguous spots and to generate naked eye visible letters on the slides. AUR16S was arrayed on the supports in the shape of an "A", LIS16S was arrayed in the shape of an "L", and UNIBACT was arrayed as a square frame around the specific bacterial probes. (B) Results of the genotyping experiment performed with an *S. aureus* DNA sample. (C) Results of the genotyping experiment performed with a *Listeria* DNA sample.

to detect the probe by hybridization on the bare nitrocellulose due to the high background, whereas we successfully detected it on copoly(DMA–NAS–MAPS)-coated nitrocellulose.

Microbial genotyping

Detecting the presence of pathogenic microorganisms in water or food is important for ensuring environmental quality and public health safety. Several microbial pathogens (*Campylobacter*, *Listeria*, *Salmonella*, *Shigella*, and *Vibrio*) account for a large number of microbial foodborne illnesses. Classical diagnostic methods, including culture and biochemical characterization, share the shortcoming that only one of a few kinds of bacteria can be identified in a complete cycle of experiments [11]. The DNA microarray technology meets the demand of parallel detection of many pathogens in one experiment. Primers for the PCR amplification, a universal bacterial probe, and probes specific for the identification of *S. aureus* and *Listeria* were synthesized according to Ref. [11] with details provided in Table 1. The PCR primers are designed on the conserved regions 8 and 10 of 16S ribosomal DNA (rDNA) and amplify a common region from genomic DNA of *S. aureus* and from *L. monocytogenes*. The forward primer is biotin-labeled and generates amplicons of approximately 370 bp. The oligonucleotide probes AUR16 and LIS16S (Table 1) were designed to be complementary to variable regions within the common amplicon, thereby specifically addressing *S. aureus* and *Listeria*, respectively. AUR16S and LIS16S were arrayed on copoly(DMA–NAS–MAPS)-coated nitrocellulose together with UNIBACT, a universal probe for bacteria as the positive control (see Fig. 4A for the spotting scheme). Taking advantage of the flexibility of the piezoelectric spotter, we devised a pattern of spotted probe for an easy interpretation of the results: AUR16S was arrayed on the supports in the shape of an "A", LIS16S was arrayed in the shape of an "L", and UNIBACT was arrayed as a square frame around the specific bacterial probes. A short pitch (200 μm) between the spots was set to favor the merging of contiguous spots and to generate naked eye visible letters on the slides. The biotinylated amplicons generated by PCR amplification of bacterial DNA samples, without any purification step, were denatured at 95 °C for 10 min and immediately deposited on the arrayed copoly(DMA–NAS–MAPS) slides to hybridize with the complementary sequences for 2 h at 50 °C. After the washing steps, the NBT/BCIP-based colorimetric detection protocol was carried out to develop black signals on the slides. Fig. 4B shows the typical results obtained after hybridization with a sample of PCR from *S. aureus* genomic DNA, and Fig. 4C shows the results from a *Listeria* DNA sample. The signal of the positive control (UNIBACT) is clearly visible on both slides and ensures, in the case of negative results, that the PCR reaction was performed correctly. The presence of DNA from *S. aureus* or *Listeria* is detected with optimal specificity, providing clearly interpretable results visible to the naked eye without the need for any scanner, imager, or detection software.

Conclusions

This article has described a method to allow nitrocellulose to covalently bind DNA molecules by coating with copoly(DMA–NAS–MAPS). The coated nitrocellulose was used to develop naked eye visible DNA microarrays for pathogen detection, opening the opportunity to implement this material in analytical microdevices where low cost and simplicity of fabrication and use are relevant.

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