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Eggshell membrane: A natural substrate for immobilization and detection of DNA

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ABSTRACT

Chemically modified eggshell membranes (ESM) have been explored as potentially novel platforms for immobilization of oligonucleotides and subsequent detection of target DNA. The fibrous network of the native ESM as well those functionalized with acetic acid or *n*-butyl acetate has been examined by field-emission scanning electron microscopy (FESEM). The formation of surface functional moieties has been confirmed by Fourier-transform infrared spectroscopy (FTIR). DNA molecules, with an end terminal $-NH_2$ group (at 5′ end) have been immobilized on the chemically modified ESM surface. The effect of surface modification on the DNA immobilization efficiency has been investigated using fluorescence microscopy and atomic force microscopy (AFM). The above studies concurrently suggest that functionalization of ESM with *n*-butyl acetate causes a better homogeneity of the DNA probes on the membrane surface. On-chip hybridization of the target DNA with the surface bound capture probes has been performed on the functionalized membranes. It is observed that *n*-butyl acetate modification of ESM pushes the limit of detection (LOD) of the DNA sensors by at least an order of magnitude compared to the other modification method.

1. Introduction

Since the successful launch of Affymetrix GeneChip® in 1994, DNA microarray technology has revolutionized disease diagnosis, drug discovery and toxicological research [1–3]. A microarray contains thousands of microscopic DNA spots attached to a solid surface, where each spot represents a specific gene. Conventionally, glass and silicon have been used for microarray fabrication because of the favorable physical properties and established surface functionalization schemes [4]. However, a strong interaction between DNA and silicon oxide surface has been found to induce conformational changes in the deposited DNA [5–6]. Further, it was shown that epoxide-coated glass substrates (uncharged) are preferred to amine-functionalized glass (charged) for immobilization of short DNA probes [7]. Alternative to glass and silicon, thermoplastic materials such as cyclic olefin copolymers, poly(methylmethacrylate), and polycarbonate have been demonstrated as low-cost array substrates [8–10]. Though plastic substrates facilitate integration of microarrays with microfluidics, the chemically inert and highly hydrophobic surfaces make the immobilization of DNA probes challenging. Interestingly, when an electrochemically active substrate like pencil graphite electrode was used for immobilization of double-stranded DNA molecules, flavonoid compounds or therapeutic molecules could be successfully detected [11,12].Thus the substrate surface appears to play a deterministic role in efficient immobilization of biomolecules and the subsequent assays.

Naturally occurring substances such as silk, collagen, bamboo inner shell membrane and eggshell membrane (ESM) have been tested as alternative platforms for the immobilization of proteins [13–17]. An enhanced stability of the immobilized proteins was observed on the biomaterials, which in turn increased the shelf-life of the respective biosensors. Large specific surface area and presence of native functional groups favor such biotic materials as immobilization support. Some of these natural substances, however, suffer from limitations like low stability and susceptibility toward microbial attack. Fortunately, ESM has been found stable and long-lived supporting matrix for enzyme immobilization [17]. Eggshell membrane comprises an interwoven lattice network of stable and water-insoluble protein fibers with large specific surface area. The said attribute is beneficial for high loading of bioaffinitive probes like enzymes or nucleic acids. Choi and his coworkers used ESM as an enzyme immobilization platform to fabricate a variety of electrochemical biosensors [15–18]. Li et al. immobilized glucose oxidase and horseradish peroxidase on eggshell membrane to develop a chemiluminescence flow-through sensor for glucose detection [19]. Further, Tang et al. used eggshell membrane as immobilization platform for determination of human IgG with a detection limit 15 ng/ml [20]. Though ESM has been used successfully as an effective immobilization matrix for proteins, to our knowledge, there is no report on immobilization of nucleic acids on this naturally occurring membrane.

In this article, we report on the chemical modification of eggshell membrane to explore its potential for being utilized as an efficient

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platform for immobilization of DNA. We have made a comparative study of two surface functionalization schemes and demonstrated how they influence the homogeneity of the surface immobilized oligonucleotides. The grafting of reactive moieties and subsequent immobilization of amine-terminated DNA molecules on the ESM surface were confirmed by FTIR. Fluorescence microscopy and AFM have been deployed to evaluate the surface homogeneity of DNA molecules on the untreated or the chemically modified ESM surface. Finally, we have studied the efficacy of eggshell membrane as a platform for detection of DNA hybridization. Though state-of-the-art DNA analysis is often carried out in a microarray format, the present study was performed on macroscopic analog of the DNA spots on a microarray. The macroscopic features facilitate comprehensive analysis of the DNA-loaded membrane surfaces with a range of widely used analytical techniques without sacrificing sensitivity or resolution.

2. Materials and methods

2.1. Materials

Glacial acetic acid (100%) was purchased from Merck Millipore, Germany and *n*–butyl acetate (extra pure grade) was obtained from S D Fine–Chem Limited, India. Phosphate buffer saline (10× concentrate, pH 7.4, DNAse free), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 98%) and N-hydroxysuccinimide (NHS, 98%) were purchased from Sigma-Aldrich, Germany. All chemicals were used without further purification and the solutions were prepared using nuclease-free water (molecular biology grade), obtained from Sigma-Aldrich, Germany. Oligonucleotides (HPLC purified) with complementary sequences were purchased from Eurofins Genomics, Germany (Table 1).

2.2. Membrane extraction and characterization

Eggshells were collected from fresh chicken eggs after the albumen and yolk had been removed and thoroughly washed with deionized (DI) water. Eggshells were incubated with 2% acetic acid (ESM-AA) or 5% *n*-butyl acetate (ESM-BA) for 30 min at room temperature. Post incubation, membranes were thoroughly washed with DI water under continuous stirring for 20 min to remove any trace amount of solvent, which may interfere with DNA immobilization. The membranes, extracted from the chemically treated eggshells, were dried overnight at room temperature and stored in a refrigerator at 4 °C for subsequent immobilization of DNA. As a reference, naturally occurring ESM was peeled off mechanically from eggshell (ESM-Peel) and was used for DNA immobilization. Morphology analysis of the ESM surfaces was performed using field-emission scanning electron microscopy (FESEM, model: Supra 35 VP, Carl Zeiss, Germany). The presence of reactive moieties on the functionalized ESM surface and the covalent immobilization of amine-terminated DNA molecules on the said surface were confirmed by Fourier-transform infrared spectroscopy (FTIR) with an attenuated total reflectance (ATR) system from Thermo Nicolet Corporation, WI, USA (model number: NEXUS-870).

Table 1
Sequence information of the oligoneucleotides.

Annotation	Oligonucleotide sequence
NH_2-DNA (capture probe)	5′-[NH_2]-C6-TTT TTT GCC GCC GCC GCC GCC GCC-3′
FAM-DNA (fluorophore-tagged target DNA)	5′-[FAM]-C6-TTT TTT GGC GGC GGC GGC GGC GGC-3′

2.3. DNA immobilization on ESM

Processed ESM samples were cut into square shapes having dimensions of 1 cm × 1 cm and fixed onto clean glass slides using a latex-free biocompatible adhesive 'Duo', manufactured by American International Industries, CA, USA. Activation of the carboxylic acid groups on the ESM-AA samples were carried out by immersing the samples in a mixture containing 1:1 volume ratio of 0.2 M EDC and 0.05 M NHS. ESM-Peel and ESM-BA samples were used without any further modification. Each of the 5′ amine-terminated single-strand DNA (ssDNA), termed as NH_2-DNA, and its complementary strand having a fluorophore at its 5′ end (FAM-DNA) was suspended in 1× phosphate buffer saline (PBS) to make stock solutions of 100 μM. For optical studies, equimolar concentrations of NH_2-DNA and FAM-DNA were mixed in a vial and left overnight for hybridization. Each hybridized DNA molecule contained a reactive primary amine at one end, for binding on the modified ESM surface, and fluorophore at the other end. For on-chip hybridization or non-specific binding studies, amine or fluorophore-tagged ssDNA molecules were used in isolation without mixing. For direct visualization of DNA immobilization on ESM surface, 1 μl of 5 μM pre-hybridized DNA solution was spotted in replicates onto the surface of the native (ESM-Peel) and chemically modified membranes. The spotted arrays were then incubated in a nuclease free, sterile and humidified chamber at room temperature for 6 h in dark to ensure proper binding of DNA onto the ESM surface. After incubation, all the samples were thoroughly rinsed in 1× PBS for 15 min under continuous shaking at 50 rpm in the dark. Subsequently, the samples were studied under fluorescent microscope (model: DM 1000 LED, Leica, Germany) using a 495 nm filter. For estimation of surface coverage of the immobilized DNA molecules, atomic force microscopy (AFM, model: Multiview 3000, Nanonix Imaging Limited, Israel) and FESEM studies were performed.

2.4. On-chip hybridization

For this set of experiments, 10 μM of NH_2-DNA (capture probe) suspension in 1× PBS was spotted in quadruplicate on the ESM-AA and ESM-BA samples. After overnight incubation with capture probes and thorough rinsing with PBS, the ESM samples were ready for target detection. Stock solution of fluorophore-tagged target oligos (FAM-DNA) was diluted to 5, 1, 0.1, and 0.01 μM concentrations, respectively in 1× PBS. A droplet (1 μl) of each concentration was spotted on the aforesaid quadruplicate spots on ESM-AA and ESM-BA samples. After hybridization for 6 h at room temperature under a nuclease free, sterile and humidified conditions in dark, unbound and non-specifically bound target DNA molecules were washed off by thorough rinsing in PBS. The ESM samples were observed under the fluorescence microscope (as above) and images of the fluorescence spots were captured at 5× magnification by a computer-interfaced CCD camera. The intensity of the fluorescence spots were quantitatively analyzed using Image-J (NIH) software [21]. For each sample, the signal was calculated as the mean intensity of three fluorescence spots after subtracting the background.

3. Results and discussion

The choice of acetic acid and *n*-butyl acetate for the modification of the eggshell membranes was based on the fact that the methyl group of these molecules readily penetrates into the ESM and occupy positions just beneath the lipid head groups [22]. The affinity of the methyl groups toward the hydrophobic lipid chains in the phospholipid bilayer facilitates such process. Once a layer of the respective modifier is formed through cell penetration, the ESM surface is grafted with carboxylic acid or acetate functional moieties, which can have facile conjugation with the amine-terminated DNA molecules (Fig. 1).

Surface morphology of the native and chemically modified ESM was examined using scanning electron microscopy. The FESEM micrographs