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A mixed film composed of oligonucleotides and poly(2-hydroxyethyl methacrylate) brushes to enhance selectivity for detection of single nucleotide polymorphisms

April K.Y. Wong [a,c], Denys O. Marushchak [b,c], Claudiu C. Gradinaru [b,c], Ulrich J. Krull [a,c,*]

[a] Department of Chemistry, University of Toronto, Toronto, Canada
[b] Department of Physics, Institute for Optical Sciences, University of Toronto, Toronto, Canada
[c] Department of Chemical and Physical Sciences, University of Toronto Mississauga, 3359 Mississauga Rd. N., Mississauga, ON, Canada L5L 1C6

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ABSTRACT

Preliminary studies of mixed films composed of oligonucleotides and poly(2-hydroxyethyl methacrylate) (PHEMA) have recently been shown to enhance the selectivity for detection of 3 base-pair mismatched (3 bpm) oligonucleotide targets. Evaluation of selectivity for detection of single nucleotide polymorphisms (SNP) using such mixed films has now been completed. The selectivity was quantitatively determined by considering the sharpness of melt curves and melting temperature differences (ΔT_m) for fully complementary targets and SNPs. Stringency conditions were investigated, and it was determined that the selectivity was maximized when a moderate ionic strength was used (0.1–0.6 M). Increases of ΔT_m when using mixed films were up to 3-fold larger compared to surfaces containing only immobilized oligonucleotide probes. Concurrently, increases in sharpness of melt curves for 1 bpm targets were observed to be up to 2-fold greater for mixed films. The co-immobilization of PHEMA resulted in a more homogeneous distribution of oligonucleotide probes on surfaces. Lifetime measurements of fluorescence emission from immobilized oligonucleotide probes labeled with Cy3 dye indicated the difference in microenvironment of immobilized oligonucleotides in the presence of PHEMA.

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1. Introduction

Single nucleotide polymorphisms (SNPs) are sites found in deoxyribonucleic acid (DNA) which contain a single base-pair mismatch [1]. In the human genome, a SNP occurs on average once in every 1000 bases [2]. As the most abundant human genetic variation, SNPs are genetic markers which are used to indicate the presence of genes that may be associated with certain diseases such as cancer, diabetes, Parkinson's and Alzheimer's [3–7]. Therefore, rapid, selective, and sensitive tools are needed for the identification and detection of SNPs. Since established molecular biology techniques which screen for SNPs involve amplification and separation steps that are time-consuming and laborious [8,9], a number of sensor and microarray detection strategies have been reported based on colourimetric [10,11], electrochemical [12–15], and optical [8,16–20] detection. Some of these involve large scale parallel analysis of samples for SNPs, and there is also interest in dedicated analysis to detect specific SNPs.

Since the difference in stability of a DNA duplex containing a fully complementary (FC) sequence compared to a SNP sequence is 4–5 °C for a 20-mer oligonucleotide in bulk solution [21,22], the design of nucleic acid biosensors must take account of factors that may reduce selectivity. Selectivity, once pH, ionic strength, and temperature are controlled, is affected by environmental factors that become dominant during interfacial hybridization and denaturation such as oligonucleotide distribution and density, the presence of nearest–neighbour interactions, and of interactions between probes and the surface [23]. Therefore, density is also a dynamic function of the degree of hybridization [23]. Ideally, to control density, one must control the structural environment around each oligonucleotide probe. This would then provide similar energetics for each probe molecule, resulting in improved selectivity for the detection of SNPs. Furthermore, interfacial melt curves tend to be broader, reflecting the diversity of energetics at an interface and providing even lower selectivity for SNP detection [24]. Thus, in order to achieve a higher selectivity between FC and SNP target populations, there must be a greater difference in melting temperature (ΔT_m) as well as a sharper melting transition. An

* Corresponding author at: Department of Chemistry, University of Toronto, Toronto, Canada. Tel.: +1 905 828 5437; fax: +1 905 569 4388.
E-mail addresses: Ulrich.krull@utoronto.ca, aca-krull@rogers.com (U.J. Krull).

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example of sharpening of melting transitions has previously been demonstrated by Taton et al. by implementation of gold nanoparticles [10].

Control of orientation and nearest–neighbour interactions of oligonucleotide probes and hybridized DNA has been shown to improve selectivity of hybridization at interfaces of electrochemical sensors. The application of electric fields on conductive substrates has been used to alter orientation of immobilized probes [25] to improve the discrimination of SNPs [14], and to enhance hybridization kinetics [26]. However, electrostatic "combing" is not possible with glass and silica-based substrates. A "matrix isolation" design was proposed by Piunno et al. which involved the co-immobilization of non-nucleic acid oligomers with oligonucleotide probes [23]. They have shown that a mixed film composed of oligonucleotide probes and ethylene glycol phosphate can lower the T_m by 5 °C and that SNP detection was still possible [23]. Another example of inclusion of non-nucleic acid oligomers was demonstrated by Boozer et al. [27]. The conformation of immobilized oligonucleotides was controlled by self-assembling thiolated oligoethylene glycol and oligonucleotides on a gold surface, resulting in improved hybridization efficiency [27].

We here report on the effectiveness of mixed films to increase the selectivity for the detection of SNPs. These mixed films were comprised of interspersed oligomer brushes with oligonucleotides immobilized on silica-based substrates, with the oligomers being grown by using surface-initiated atom transfer radical polymerization, as reported previously [28]. This report provides a detailed examination of conditions required to control the sharpness of the melting transitions and ΔT_m values to distinguish between SNP and FC targets for PHEMA-oligonucleotide coatings on glass surfaces. Fluorescence intensity measurements were used to reflect the extent of hybridization. Fluorescence lifetime data provided a further means to evaluate differences in the environments experienced by probe oligonucleotides in the PHEMA-oligonucleotides mixed films, and films composed only of oligonucleotides.

2. Experimental

2.1. Materials

Redistilled benzaldehyde (BZ, >99.5%), (3-aminopropyl)trimethoxysilane (APTMS, 97%), redistilled *N*,*N*′-diisopropylethylamine (99.5%), 2-bromoisobutyryl bromide (98%), *N*-hydroxysuccinimide (NHS), triethylamine (99%), 2-hydroxyethyl methacrylate (HEMA, 97%), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), Cu(I) chloride, Cu(II) bromide, 2,2′-dipyridyl (dpy), sodium bicarbonate, sodium sulfate, sodium carbonate, sodium chloride, sodium orthophosphate were from Sigma–Aldrich (Oakville, ON). (Succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate) (sulfo-SMCC) was from Fisher Canada (Nepean, ON). Ammonium hydroxide (30%), hydrochloric acid, and hydrogen peroxide (30%) were from EM Science (Gibbstown, NJ, USA). An inhibitor present in the HEMA was monomethyl ether hydroquinone MEHQ. HEMA was purified by using MEHQ inhibitor remover (Sigma–Aldrich, Mississauga, ON), which was packed in a column. All buffers were prepared in Millipore purified deionized water (Milli-Q water, 18 MΩ cm). All solvents including methanol (MeOH), dichloromethane (DCM), deuterated chloroform ($CDCl_3$), diethyl ether, dimethylformamide (DMF), dioxane, toluene, and silica gel for chromatography were from Sigma–Aldrich (Oakville, ON). Argon and nitrogen gas were from BOC Canada Limited (Oakville, Canada).

Table 1 lists the oligonucleotide sequences from Integrated DNA Technologies (Coralville, IA) which were HPLC purified by the manufacturer. They were dissolved in deionized water. A small aliquot of the disulfide form of each thiol-modified oligonucleotide were first reduced by TCEP and purified through a Sephadex G-25 DNA grade column from GE Healthcare (Baie d'Urfé, Québec, Canada).

2.2. Instrumentation

Fluorescence intensities originating from glass slides were collected with a Versarray Chipreader 5 μm confocal microscope system (Bio-Rad, Hercules, CA, USA) equipped with 532 and 635 nm laser sources and two detection channels.

The lifetime of the Cy3 emission was measured from labeled oligonucleotides (Sequence 2, Table 1) which were co-immobilized with and without PHEMA onto microscope glass cover slips (size 1.5, Corning). The experiments were performed on a custom-built hyperspectral confocal microscope described previously [29]. In brief, a femtosecond laser (Tsunami HP, Spectra Physics, USA) was tuned to 960 nm (FWHM = 13 nm) and was frequency-doubled in a nonlinear crystal to produce a narrow excitation spectrum centered at 480 nm. This linearly polarized beam passed through a 1.4 NA/100× plan-apochromat objective (Carl Zeiss, Canada) and illuminated the sample at intensities in the range of 100 W cm^{-2}. The emitted fluorescence was collected through the same objective and was spatially and spectrally filtered using a 50-μm pinhole and high-quality long-pass and band-pass filters (Semrock, Rochester, USA) to remove out-of-focus fluorescence and the Rayleigh scattering from the surface. Further, the fluorescence emission was divided into two components with polarization parallel and perpendicular to the polarization of the excitation beam using a broadband polarizing cube beamsplitter (Newport, Irvine, USA). Each beam was tightly focused onto an avalanche photodiode that featured a low dark noise, high sensitivity and picosecond timing (PD5CTC, Optoelectronic Components, Kirkland, Canada). Each time a photon was detected, the detector output an electric pulse that was registered by a counting module (PicoHarp300, PicoQuant Gmbh, Germany). The photon-by-photon data contained information about the emission rate, the excited-state lifetime, and anisotropy.

2.3. Preparation of oligonucleotide films and mixed films composed of oligonucleotide-PHEMA

The immobilization of both oligonucleotide and mixed films were described previously and the same initial surface derivatization steps could be applied to both types of surfaces [28]. Cleaned glass slides were first modified with a 1:1 mixture of (3-aminopropyl)trimethoxysilane (APTMS) and benzaldehyde

Table 1
Oligonucleotide sequences used.

Sequence #	Sequence	Notes
1	5′-SH-C6-ATT TTG TCT GAA ACC CTG T-3′	Thiol-modified SMN probe
2	5′-SH-C6-ATT TTG TCT GAA ACC CTG T-Cy3-3′	Thiol-modified Cy3-labeled SMN probe
3	5′- ATT TTG TCT GAA ACC CTG T-Cy3-3′	Cy3-labeled SMN probe
4	5′-Cy3-ACA GGG TTT CAG ACA AAA T-3′	Fully complementary target
5	5′-Cy3-ACA GGG TTT TAG ACA AAA T-3′	1 Base-pair mismatch target
6	5′-Cy3-ATA GGG TTT CGG ACA AAG T-3′	3 Base-pair mismatch target

Underlined bases indicate mismatches.

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