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New immobilisation method for oligonucleotides on electrodes enables highly-sensitive, electrochemical label-free gene sensing

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ABSTRACT

We present a versatile and facile procedure for the immobilisation of bioprobe molecules to an electrochemical sensing element. We eliminate lengthy preparation procedures for direct functionalisation of electrode surfaces by pre-attaching probe molecules to carboxylic acid bearing termonomers of pyrrole phenylenes or thiophene phenylenes. We demonstrate that these conjugates can be electrodeposited at low potentials to form nano-scale porous, electroactive conducting polymer films, exposing the bioprobe and retaining activity and specificity for binding, exemplified here with DNA sensors. The electrochemical reaction impedance for $Fe(CN)_6^{3-/4-}$ on oligonucleotide-modified electrodes showed remarkable (down to aM) detection sensitivity for target DNA sequences present in solution. Cross-sensitivity to non-complementary target sequences is small and multi-target arrays are easily made. There is no need for labelling of either probe or target oligonucleotide.

1. Introduction

DNA measurement is the 'gold standard' for diagnosis of infectious diseases, genetic mutations, forensics, food and environmental quality analysis. Current technologies rely on hybridisation of probe DNA sequences, attached onto solid surfaces, to the complementary, fluorescently or radioactively labelled target. Direct electrical transduction of hybridisation promises to avoid the time, expense and complexity of probe or target labelling (Aydemir et al., 2015; Drummond et al., 2003; Kannan et al., 2011; Korri-Youssoufi et al., 1997; Peng et al., 2005; Thompson et al., 2003). We extended the approach by employing such sensing films in real-time, label-free detection of a gene amplification during polymerase chain reaction (Aydemir et al., 2015). The polypyrrole copolymers utilised are easily grown by electropolymerisation. However, robust, yet simple surface modification is a major barrier: probe entrapment (Komarova et al., 2005; Wang et al., 1999), affinity interactions (Bidan et al., 2004; Dupont-Filliard et al., 2001; Rodrígez et al., 2002) and covalent immobilisation (Godillot et al., 1996; Lassalle et al., 2001b; Livache et al., 1994a; Schuhmann et al., 1993, 1990) have issues of limited tagging efficiency, limited accessibility of probe sequences to targets, leaching of probe molecules and speed (Aydemir et al., 2016). Particularly, chemical functionalisation has proved robust but is performed after electrodeposition. It is slow and thus not suitable for low-cost industrial-scale fabrication. Here, we present a novel approach, more amenable to fabrication and mass manufacture of electrically conducting polymer (ECP) sensors, featuring covalently pre-functionalising an ECP *monomer* with various oligonucleotide probes prior to electropolymerisation onto the electrode. A published approach to a pre-functionalised ECP monomer (Livache et al., 1994b) was based on the attachment of ONs to the nitrogen moiety of pyrroles (Py) via phosphoramidite chemistry. The obtained Py-ON matrix was then electrochemically addressed onto the electrodes for DNA sensing and hybridisation event monitored via either photocurrent signals (Livache et al., 1994b), fluorescent microscopy (Livache et al., 1998), surface plasmon resonance (Livache et al., 2003) (SPR) and differential pulse voltammetry (Wang et al., 2004) (DPV). This methodology, despite addressing the issues of post-functionalisation, still suffer from involving a cumbersome and lengthy phosphoramidite chemistry, that the attachment of ON to the nitrogen disturbs the conjugation of the polypyrrole backbone thus decreasing the conductivity of the sensing film, and the procedure requires specialised instrumentation for the synthesis of ON-Py matrix.

We have recently synthesised and extensively characterised a number of novel ECP 'termonomers' based on thiophene phenylenes and their (co)polymers (Chan et al., 2015). Here we extend this class of compound with new thiophene phenylene (ThP) and pyrrole phenylene (PyP) carrying carboxylic acid moieties (Fig. 1) and present fabrication of DNA sensors based on the new termonomers. Utilising these

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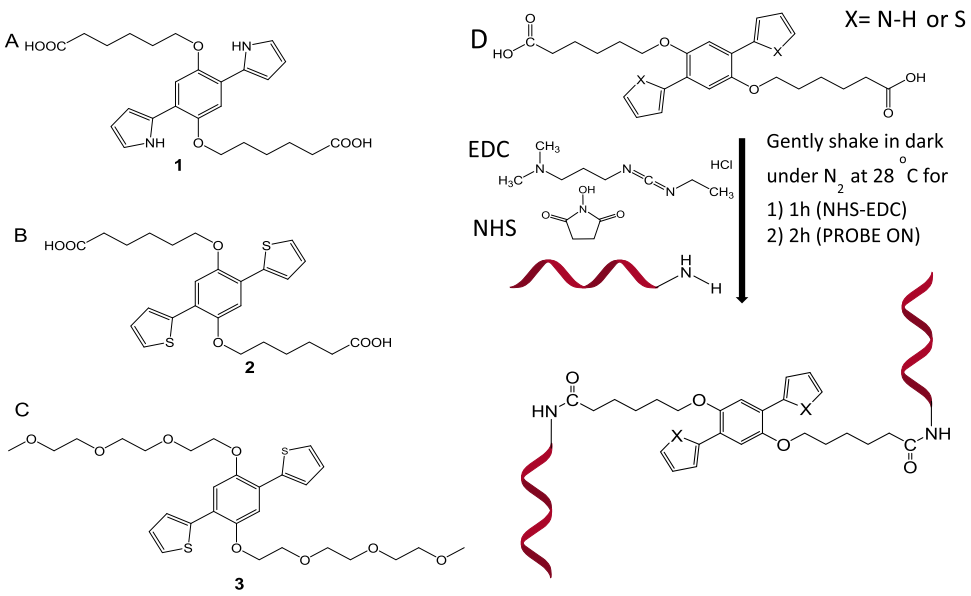

Fig. 1. Chemical structures of A) PyPhCOOH (1), B) ThPhCOOH (2), C) ThPhEG (3), D) Reaction scheme of termonomer and ON coupling (X = NH or S).

termonomers we demonstrate a direct and robust deposition of probe oligonucleotides on electrodes that occurs simultaneously with the creation of the sensing element. The methodology consists of: (i) synthesising novel functionalised ECP termonomers (pyrrole and thiophene derivatives); (ii) pre-attaching various oligonucleotides (ON) probes to these termonomers by means of facile carbodiimide chemistry – thus creating a 'library' of ON-carrying pyrroles and thiophenes; (iii) fabricating ECP sensor films electrochemically in a fraction of a second and at low polymerisation potentials that are fully benign to the ON-probe. As monomers employed in this study possess functional groups on the middle benzene ring rather than on the side pyrroles or thiophenes, the ON substitution does not hinder α-α coupling during the oxidative polymerisation of the termonomers. These monomers present a number of additional advantages for preparation of DNA sensing films: the conjugates are easily prepared and purified; they electropolymerise rapidly at low potentials, such that the attached ON is not oxidatively damaged; the probe density is easily adjusted by copolymerisation of ethyleglycol-functionalised termonomer that also improves hydrophilicity.

Here, we demonstrate a highly sensitive and selective, label-free, target ON sequence detection and simultaneous multi-target detection via electrochemical read-out, by the sensing films based on the polymers of these termonomers. We suggest that this versatile procedure can be adopted to a wide range of biological probes, such as proteins, aptamers, and enzymes, providing an avenue for a universal immobilisation methodology.

1.1. Chemicals

Dimethylformamide (DMF), tetrahydrofuran (THF), phosphate buffered saline (PBS) pellets, sodium paratoluene sulfonate (NaTos), potassium ferricyanide ($K_3[Fe(CN)_6]$), potassium ferrocyanide $K_4[Fe(CN)_6]$, N-hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and pyrrole (Py) were purchased from Sigma Aldrich. (6,6′-((2,5-Di(1H-pyrrol-2-yl)-1,4-phenylene)bis(oxy)) dihexanoic acid (PyPhCOOH) and 6,6′-((2,5-di(thiophen-2-yl)-1,4-phenylene)bis(oxy))dihexanoic acid (ThPhCOOH) were synthesised as described below and 2,2′-(2,5-bis(2-(2-(2-methoxyethoxy) ethoxy)ethoxy)-1,4-phenylene) dithiophene (ThPhEG) was synthesised according to ref (Chan et al., 2015). 24 base sequences of oligonucleotides (ONs) of chronic lymphocytic leukemia (PBGD), bladder cancer (FGFR 3) and non-Hodgkin lymphoma (Non-Hodgkin) were purchased from Alpha DNA; sequences are provided in Table S.1.

1.2. Electrodes

1.6 mm diameter gold disk (MF-2014), 3 mm diameter glassy carbon (GC) (MF-2012), standard Ag/AgCl (MF-2052) and platinum (Pt) wire electrodes were purchased from BASI. Leak-free Ag/AgCl (ET072) electrode was purchased from Warner Instruments. Au and GC electrodes were employed as working electrodes (WE) whereas standard and leak-free Ag/AgCl electrodes were used as the reference electrodes (RE) and Pt wire was used as the counter electrode (CE). Both Au and GC electrodes were polished before use by 0.5 μm alumina slurry and ultra-sonicated in ethanol and deionized water (Milli-Q) for 5 min each.

1.3. Synthesis of PyPhCOOH and ThPhCOOH

Detailed syntheses of both PyPhCOOH and ThPhCOOH are provided in the Supporting information, S.2).

1.4. Termonomer-ON coupling procedure

All the solvents were degassed for 10 min, using N_2, prior to use. 100 μl of the monomer (either PyPhCOOH **1** or ThPhCOOH **2**) stock solution (200 μM in tetrahydrofuran (THF)) was pipetted into a plastic 1.5 ml eppendorf tube. 100 μl of PBS (pH 6.5) containing EDC (20 mM) and NHS (10 mM) was then added to the eppendorf tube. The solution was gently shaken, under N_2 in the dark, for 1 h. An additional 100 μl of THF and 100 μl of 1 mM NH_2-ON solution in PBS (pH: 7.5) were then added to the eppendorf tube and mixed for another 2 h, at 28 °C, under N_2, in the dark. The final solution contained 250 μM of ON, 50 μM of the monomer, 5 mM of EDC and 2.5 mM NHS in a total of 400 μl of 1:1 THF/PBS (pH: 7). For FTIR and sensitivity experiments, PyPhCOOH **1** was grafted with 24 base sequence Non-Hodgkin probe and ThPhCOOh **2** was grafted with 24 base sequence PBGD probe. For sensing selectivity measurements both monomers were attached with Non-Hodgkin probe sequence. After the ON attachment, THF was removed under vacuum and the remaining aqueous solution was centrifuged at 12,500 rpm for 10 min. The supernatant solution (containing unreacted NHS, EDC and unbound