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Novel peptidylated surfaces for interference-free electrochemical detection of cardiac troponin I

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ABSTRACT

Novel peptidylated surfaces were designed to minimise interferences when electrochemically detecting cardiac troponin I in complex biological samples. Disulfide-cored peptide dendrons featuring carbomethoxy groups were self-assembled on gold electrodes. The carbomethoxy groups were deprotected to obtain carboxylic groups used to immobilise antibodies for cardiac troponin I marker. The chemisorption of two types of peptides, one containing triazole and the other with native peptide bonds, on a gold substrate was studied by quartz crystal microbalance (QCM), surface plasmon resonance (SPR) and X-ray photoelectron spectroscopy (XPS). Peptides formed ordered self-assembled monolayers, contributing to a more efficient display of the subsequently immobilised antibodies towards their binding to the antigen. As a result, electrochemical immunosensors prepared by self-assembly of peptides afforded higher sensitivities for cardiac troponin I than those prepared by the chemisorption of alkane thiolated compounds. Triazolic peptide-modified immunosensors showed extraordinary sensitivity towards cardiac troponin I [1.7 μA/(ng/mL) in phosphate buffer], but suffered from surface fouling in 10% serum. Modification with non-triazolic peptides gave rise to anti-fouling properties and still enabled the detection of cardiac troponin I at pg/mL concentrations in 10% serum without significant matrix effects.

1. Introduction

Important research advances have been made over the last decade related to the essential role of cardiac biomarkers as early indicators of cardiovascular diseases (Ghashghaei et al., 2016; Vasan, 2006). Cardiac biomarkers are being increasingly used for clinical studies due to the good correlation of their levels in the body fluids with a range of cardiac related conditions. Clinical practice is based on periodically measuring the concentration of a certain cardiac biomarker to establish its profile as an indication of the status of the patient or the patient's response to a given course of treatment.

Cardiac troponin I (cTnI) is a part of the troponin complex present in the cardiac muscle tissues. For more than a decade, cTnI has been known as a good biomarker of cardiac muscle tissue injury and used as standard marker in the early diagnosis of acute myocardial infarction. Rapid and accurate assessment of cTnI elevation is required for proper diagnosis at early stages of development, prognosis and monitoring of acute myocardial infarction (Fathil et al., 2015; Hoff et al., 2016). However, the clinically significant ranges are extremely low, with a recommended cut-off level of 0.01–0.1 ng/mL (Mohammed and Desmulliez, 2011) and hence, demand highly sensitive devices.

Electrochemical biosensors are characterised by their high sensitivity, low cost and easy miniaturisation, holding great promise for non-invasive cardiac biomarkers screening. Several electrochemical immunosensors have been developed with the capability to detect cTnI clinical concentrations (Periyakaruppan et al., 2013; Ahammad et al., 2011; Singal et al., 2014). However, to meet the requirements of clinical analysis, the sensor performance has to be tested in clinically relevant samples. Serum and plasma are the primary biochemically useful clinical samples for cardiac biomarkers analysis (Anderson, 2005). They include a complex mixture of biological compounds prone to nonspecifically bind on the surface of the sensor. This major problem has mainly been addressed in cTnI electrochemical detection by incorporating a blocking agent, such as bovine serum albumin, low-fat milk, gelatin or dendrimers (Guo et al., 2005; Kong et al., 2012;

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Shumkov et al., 2013; Akter et al., 2017). Biofouling is considered as one of the main limitations regarding the detection of cTnI levels in blood, especially when continuous monitoring is pursued to facilitate therapeutic guidance and assess the evolution of the patients' conditions. As a result, a tailored method is required to circumvent the problems that might arise from the analysis of complex biological samples. Electrochemical biosensors able to overcome these shortcomings are regarded as promising devices for point-of-care applications.

Non-fouling surfaces are critically important while developing biosensors for cardiac biomarkers. These surfaces have to show resistance to fouling caused by proteins present in the blood. Electrically neutral surfaces are required to minimise electrostatic interactions, while hydrogen-bond acceptors are preferred to hydrogen-bond donors in order to reduce the hydrogen bonding interactions with proteins. Research efforts have been devoted over years to develop low-fouling biomaterials, such as those that make use of poly(ethylene glycol) (PEG) (Liu et al., 2013), positively charged molecules (Gottenbos et al., 2001) or zwitterionic compounds (Chang et al., 2010) to modify surfaces.

Recently, peptides have been suggested as suitable modifiers to minimise fouling. Peptides can be designed to tailor their physical and chemical properties by displaying high density of selected peripheral functional groups (Eichler et al., 2011). The proximity among functional groups, and their capability to tailor the surface charge upon protonation or deprotonation, can exert a desired effect on the interaction with the environment and thus reduce undesired non-specific protein adsorption (Svenson and Tomalia, 2005).

Here, we explore the use of peptides as sensor modifiers to provide not only bioreceptor immobilisation, but also minimise surface fouling. We report the design and synthesis of triazolic and non-triazolic peptides, featuring disulfide groups and methyl esters. Disulfide bonds enable the formation of dative bonds on gold, forming thin films (Love et al., 2005; Seigel et al., 1997). The as-prepared self-assembled monolayers show high thermal, mechanical and chemical stability. Methyl esters can be hydrolysed to allow further immobilisation of bioreceptors. Triazolic peptides contain symmetrically oriented triazole rings that contribute to the self-assembly via the π-electron system, providing a flat orientation of the peptide dendrons on the gold surface. The self-assembled surfaces were probed using QCM, SPR and XPS. After alkaline hydrolysis, antibodies were covalently linked through their amine groups. Results were compared with controls where mercaptopropionic acid (MPA) was self-assembled instead of disulfide-cored peptides. This comparative analysis reveals the advantages of incorporating peptides onto gold surfaces for use in biosensor development.

2. Materials and methods

2.1. Synthesis and characterisation of disulfide-cored peptides

All solvents used in the reactions were dried prior to use. Reactions were monitored by thin layer chromatography (TLC). Silica gel G (Merck) was used for TLC and column chromatography was done on silica gel (100–200 mesh) using ethyl acetate/hexane as eluent. Melting points were recorded in a Fisher-Johns melting point apparatus. IR spectra were recorded on a Nicolet, Protégé 460 spectrometer as KBr pellets. ^{1}H NMR spectra were recorded on Brucker-DPX-300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) spectrometer using tetramethylsilane (^{1}H) as an internal standard. Coupling constants are in Hz and the ^{1}H NMR data are reported as s (singlet), d (doublet), br (broad), t (triplet), m (multiplet). High resolution mass spectra (HRMS) were recorded with micrOTOF-Q II using the ESI-technique. Optical rotations were measured with a Rudolph Research Analytical Autopol® V Polarimeter where concentrations are given in g/100 mL.

2.1.1. Synthesis of non-triazolic peptide **4** (Scheme 1)

N-hydroxysuccinimide (NHS, Sigma-Aldrich) (0.165 g, 1.43 mmol) and *N,N′*-dicyclohexylcarbodiimide (DCC) (0.295 g, 1.43 mmol) were added to an ice-cooled solution of *tert*-butyloxycarbonyl (Boc)-protected cystine **1** (0.300 g, 0.68 mmol) prepared in 50 mL of dry dichloromethane. The reaction mixture was stirred for 10 min, and afterwards glutamic acid methyl ester·HCl (0.302 g, 1.43 mmol) and triethylamine (0.2 mL, 1.43 mmol) were added and the solution was stirred for 24 h. The reaction mixture was filtered and the filtrate was washed with 0.2 N H_2SO_4, saturated aqueous $NaHCO_3$ and water. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to yield 0.431 g of **4** as white powder. Yield: 84%; Mp: 122–124 °C; [α] = + 34 (*c* 0.10, chloroform); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.46 (s, 18H), 2.00 (m, 2H), 2.31 (m, 2H), 2.47 (t, *J* = 7.2 Hz, 4H), 2.95 (m, 2H), 3.09 (m, 2H), 3.67 (s, 6H), 3.72 (s, 6H), 4.64 (m, 2H), 4.79–4.93 (br s, 2H), 5.53 (d, *J* = 9.3 Hz, 2H), 7.77 (d, *J* = 7.8 Hz, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 26.8, 28.3, 30.5, 33.9, 46.5, 51.8, 52.4, 54.2, 80.2, 155.7, 170.6, 171.7, 172.8; IR (KBr): 3340, 2983, 2949, 2921, 2853, 1737, 1663, 1524, 1442, 1379, 1309, 1267, 1208, 1170, 1114, 1021 cm^{-1}; HRMS calculated for $C_{30}H_{50}N_4NaO_{14}S_2$ *m/z* 777.2657, found *m/z* 777.2631.

2.1.2. Synthesis of triazolic peptide **5** (Scheme 1)

First, intermediate **2** had to be synthesised. NHS (0.261 g, 2.27 mmol) and DCC (0.468 g, 2.27 mmol) were added to an ice-cooled solution of **1** (0.500 mg, 1.13 mmol) prepared in 100 mL of dry dichloromethane. The reaction mixture was stirred for 10 min, and then propargylamine (0.145 mL, 2.27 mmol) was added and the mixture was stirred for 24 h. The reaction mixture was filtered and the filtrate was washed with 0.2 N H_2SO_4, saturated aqueous $NaHCO_3$ and water. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to yield 0.480 g of **2** as white solid. Yield: 86.4%; Mp: 170–172 °C; [α] = − 92 (*c* 0.10, methanol); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.48 (s, 18H), 2.18 (s, 2H), 2.90 (m, 4H), 4.05 (m, 4H), 4.92 (m, 2 H), 5.53 (d, *J* = 9.6 Hz, 2H), 8.09 (br s, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 28.5, 28.9, 47.4, 54.5, 71.6, 79.2, 80.4, 156.0, 170.1; IR (KBr) 3307, 2978, 2928, 2853, 1660, 1525, 1446, 1372, 1251, 1168 cm^{-1}; HRMS calculated for $C_{22}H_{34}N_4NaO_6S_2$ *m/z* 537.1812, found *m/z* 537.1799.

Further, to synthesise the triazolic peptide dendron, azide-functionalised glutamic acid methyl ester **3** was prepared. To an ice-cooled solution of azido acetic acid (0.114 g, 1.13 mmol) in dry dichloromethane, NHS (0.130 g, 1.13 mmol) followed by DCC (0.234 g, 1.13 mmol) were added and stirred for 10 min. Glutamic acid methyl ester·HCl (0.200 g, 0.94 mmol) and triethylamine (0.2 mL, 1.13 mmol) were added to this solution, and the reaction mixture was stirred for 24 h. The solution was filtered and the filtrate was washed with 0.2 N H_2SO_4, saturated aqueous $NaHCO_3$ and water. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to yield 0.210 g of **3** as colorless liquid. Yield: 75%; [α] = −22.5 (*c* = 0.48, methanol); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 2.15 (m, 2 H), 2.40 (m, 2 H), 3.70 (s, 3H), 3.77 (s, 3H), 4.02 (s, 2H), 4.63 (m, 1H), 7.05 (br s, 1H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 27.1, 30.0, 51.6, 51.9, 52.5, 52.7, 166.7, 171.7, 173.1; IR (KBr) 2928, 2857, 2669, 2327, 2099, 1744, 1657,1190 cm^{-1}; HRMS calculated for $C_9H_{14}N_4NaO_5$ *m/z* 281.0856, found *m/z* 281.0835.

Then, to proceed with the triazolic peptide dendron **5** synthesis, diisopropylethylamine (0.17 mL, 0.775 mmol), azide-functionalised glutamic acid methyl ester **3** (0.200 mg, 0.775 mmol) and CuI (0.014 g, 0.078 mmol) were added to an ice-cooled solution of **2** (0.200 g, 0.388 mmol) in 50 mL of dry acetonitrile under nitrogen atmosphere. The reaction mixture was stirred under nitrogen atmosphere for 18 h, evaporated afterwards and re-dissolved in ethyl acetate. It was then washed with 0.2 N H_2SO_4, NH_4Cl/NH_4OH (9:1) solution and water. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated to yield 0.330 g of **5**. Yield: 82.5%; Mp: 102–104 °C; [α] = + 24 (*c* 0.10, chloroform); ^{1}H NMR (CD_3OD, 300 MHz) δ 1.45 (s, 18H), 2.01 (m, 2H), 2.21 (m, 2H), 2.46 (t, *J* = 7.2 Hz, 4H), 2.91 (m, 2H), 3.18 (m, 2H), 3.68