Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 4770

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COMMUNICATION

Amperometric detection of antibodies in serum: performance of self-assembled cyclodextrin/cellulose polymer interfaces as antigen carriers†

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Received 25th March 2011, Accepted 12th May 2011
DOI: 10.1039/c1ob05473b

A bifunctionalised carboxymethyl-cellulose polymer bearing adamantane units and an antigenic fragment forms a highly stable interfacial complex with a βCD-containing surface. This allows the highly sensitive detection of antibodies using an amperometric immunosensor.

Among the many existing bioanalytical tools, electrochemical biosensors offer a series of advantages such as high sensitivity, portability, instrumental simplicity and multiplexing ability, making them ideal candidates for point-of-care applications.[1] Nanoscale design and functionalisation of transducer surfaces is vital for the effective and rapid assembly of biosensor devices and several methods have been reported for the attachment of the biorecognition elements on the electrode surface such as the formation of self-assembled monolayers (SAMs).[2] This technique has shown applicability, for example, in the detection of proteins in serum[3] or food extracts[4] but has the disadvantage of being limited to specific compounds such as thiols and noble metal substrates (*e.g.* gold).

Recent progress in the fabrication of highly organised molecular systems with the aid of supramolecular interactions has opened up new prospects for the control of molecular interactions and the design of novel functional materials and devices.[5,6] A particularly interesting approach to constructing organised structures on surfaces exploits natural or synthetic molecular receptors to construct self-assembled 2D[7] and 3D[8] nanoarchitectures on surfaces. However, despite the potential of host–guest chemistry for the supramolecular attachment of proteins (*e.g.* enzymes, antibodies) their exploitation has, to date, been limited. Recently, the interaction of adamantane (ADA) biomolecule conjugates with a surface modified with a monolayer of β-cyclodextrin (CD) has been employed to immobilize cytochrome c[9] and xanthine oxidase[10] on gold electrodes, with the electron-transfer and catalytic activities of both proteins being retained following electrode modification.

An alternative strategy takes advantage of the layer-by-layer technique, in which successive enzyme–adamantane conjugates are deposited on a CD-modified surface using CD-coated gold nanoparticles as the gluing element, finding application in the electrochemical detection of hydrogen peroxide.[11] Another approach involves the modification of gold or silicon surfaces with aminated CDs *via* a crosslinker onto which a second layer of dextran or polyethyleneglycol containing adamantane moieties is supramolecularly constructed through inclusion in the βCD cavities, as exemplified for the covalent attachment of rabbit IgG antibodies[12] and β-lactoglobulin.[13]

Here we report a novel technique for antibody sensing based on the interfacial self-assembly of adamantane-appended antigen carriers on βCD-modified gold surfaces (Fig. 1). In this approach, the initial modification of the surface with a layer of a hydrophilic CD polymer is expected to markedly reduce non-specific interactions, which is particularly critical in biosensor development. In addition, the use of a polymer backbone as antigen carrier allows optimisation of the density of the immobilised biorecognition elements *via* a controlled functionalisation with the antigen and

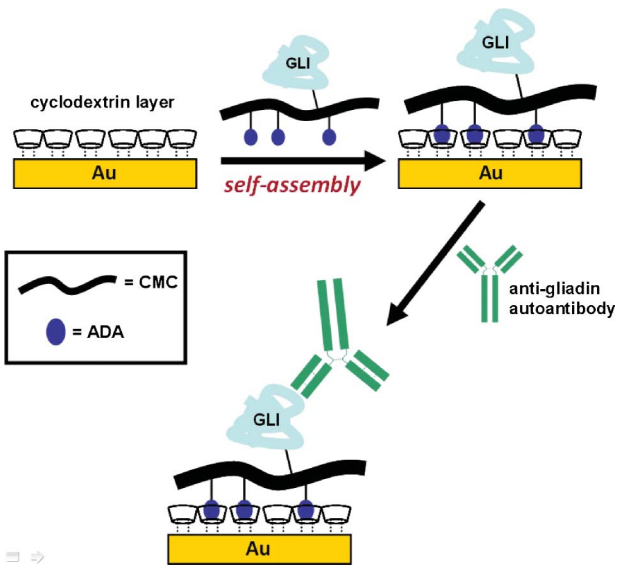

Fig. 1 Strategy employed for interfacial self-assembly of adamantane-appended antigen carriers on cyclodextrin-modified surfaces and autoantibody detection. GLI: gliadin, CMC: carboxymethylcellulose, ADA: adamantane.

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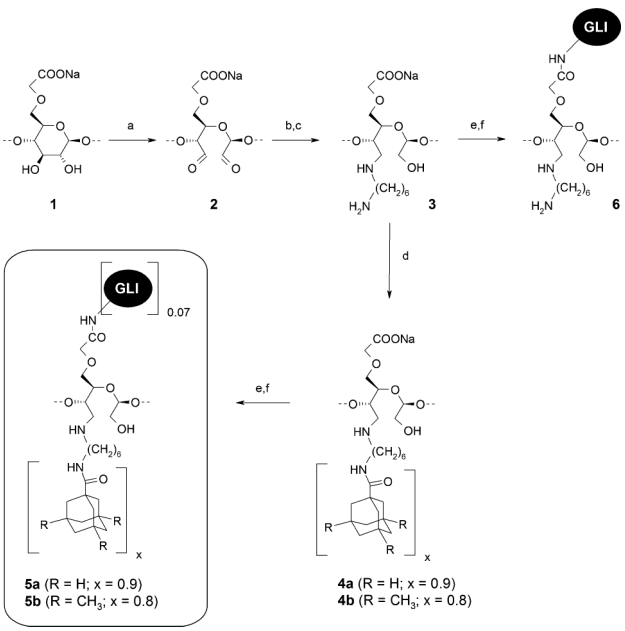
† Electronic supplementary information (ESI) available: Synthetic procedures and instrumentation, calibration curve, serum studies. See DOI: 10.1039/c1ob05473b

thus enhancing the sensitivity and detection limits of the device. As a model application we selected the electrochemical detection of anti-gliadin antibodies. Gliadin (GLI) is a glycoprotein present in wheat and other cereals as part of gluten, which is known to be toxic to genetically susceptible individuals affected by coeliac disease, resulting in the production of anti-gliadin autoantibodies.[14]

The sensing surface was first modified with a thiolated polymeric material derived from β-CD (βCDPSH).[7] For comparison, αCD (αCDPSH) and γCD (γCDPSH) polymers were prepared using the same strategy. The antigen carrier (Scheme 1, see ESI for details†) was prepared by periodate oxidation of carboxymethylcellulose (CMC, **1**) to give aldehyde **2**, which was reacted with 1,6-diaminohexane and further reduced with $NaBH_4$ to give **3**. This intermediate was coupled with EDC-activated adamantane-1-carboxylic acid to give **4a**. As a control, the bulkier 3,5,7-trimethyladamantane-1-carboxylic acid (TMADA) was also reacted in a similar way to give **4b**. Integration of the protons in the 0.5–1.2 ppm region of the ^{1}H-NMR spectra of **4a,b** with respect to the anomeric protons ($\delta = 4.9$–5.3 ppm) indicate an average of 0.9 and 0.8 adamantane residues per glucose unit, respectively. Digested gliadin was then conjugated to **4a,b** *via* EDC chemistry to give **5a,b**. UV measurements indicate that there are 0.3 mg of protein per mg of polymer, representing a 7% substitution of COOH groups by gliadin. For comparison purposes, amine **3** was also conjugated to gliadin to give polymer **6**, which carries no adamantane units.



Scheme 1 Preparation of the antigen carrier: (a) $NaIO_4$, (b) 1,6-diaminohexane, (c) $NaBH_4$, (d) EDC-activated ADA-COOH or TMADA-COOH, (e) EDC, (f) digested gliadin. GLI: gliadin fragment.

The interaction of **5a** with a βCDPSH-modified surface was studied by surface plasmon resonance (SPR, Fig. 2). Injection of successive amounts of **5a** resulted in a saturation of the SPR signal at 1300 RU (Fig. 2a), indicating its interfacial immobilisation. Displacement of bound **5a** could be partially achieved with injections of sodium adamantanecarboxylate (ADA-COONa) (Fig. 2b), which is expected to compete for the βCD binding sites, indicating a strong interaction between **5a** and the βCD layer

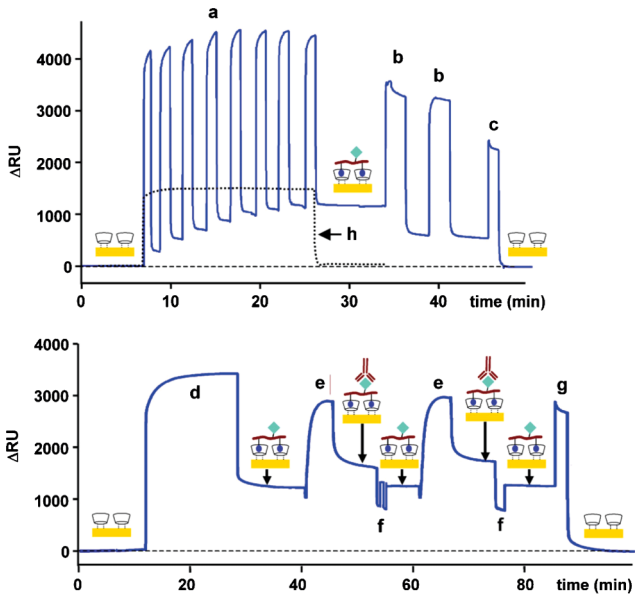


Fig. 2 SPR sensorgram for the interaction of **5a** with βCDPSH-surface after the injection of: (a) **5a**, (b) ADA-COONa (1 μM), (c) 0.1% SDS (βCDPSH surface regeneration), (d) **5a** (second injection), (e) 1 μg mL^{-1} anti-gliadin antibody, (f) 10 mM glycine buffer pH 2 (antigen regeneration), (g) 0.1% SDS, (h) **6**. Conditions: temperature: 20 °C; running buffer: 10 mM PBS pH 7.0; flow rate: 20 μL min^{-1}.

that could only be disrupted by the addition of 0.1% w/v sodium dodecylsulfate, SDS, (Fig. 2c). Interaction of the βCDPSH/**5a** surface with the anti-gliadin antibody gave an increase of 395 RU in the SPR response (Fig. 2e) and this immunocomplex association could be disrupted by an acidic buffer, whilst not altering the immobilised self-assembled gliadin functionalised support (Fig. 2f), leaving it available for a new interaction. After a second antibody association/dissociation process the βCD support was regenerated using 0.1% w/v SDS (Fig. 2g) rendering it reusable to capture another antigen carrier, thus demonstrating the reversibility of the βCD-modified surface and its efficiency as a support for immunosensor development.

The role of specific ADA/βCD interactions in the immobilisation of **5a** was further confirmed by observing very low SPR variations after the interaction of **5a** with an αCD-modified surface (5 RU) and of **5b** with the βCD surface (23 RU). Due to the relative structural complexity of the antigen carrier it can be expected that other types of interactions might occur between **5a** and the βCD-modified surface, such as hydrogen bonding to the amine, amide, and hydroxyl groups as well as non-specific hydrophobic interactions that do not involve inclusion of the ADA groups into the βCD cavities. To assess the contribution of these interactions, polymer **6**, which carries the GLI fragments but lacks the ADA units, was allowed to interact with the βCD-surface. In this case, the SPR response was 40 RU (Fig. 2h), which represents about 3% of the signal obtained for **5a**, indicating that the carbohydrate backbone and the polypeptide chains in **6** do not contribute significantly to unspecific interactions.

The CD/ADA stoichiometry for βCDPSH and γCDPSH with **5a** were calculated from the ratio of their corresponding SPR immobilisation levels and considering that **5a** has 0.9 ADA residues per glucose unit, afforded a ratio of $\beta CD_{7.8}$: ADA_1 and

Table 1 Interfacial association constants (K) for the interaction of **5a,b** with cyclodextrin-modified surfaces

	K (M^{-1})	
Support layer	**5a**	**5b**
αCDPSH	$(9 \pm 1) \times 10^{1}$	—
βCDPSH	$(2.5 \pm 0.4) \times 10^{10}$	$(6.1 \pm 0.3) \times 10^{5}$
γCDPSH	$(1.8 \pm 0.4) \times 10^{4}$	$(8.7 \pm 0.4) \times 10^{8}$

Table 2 Comparison of the anti-gliadin levels obtained by ELISA and with the supramolecular platform in two patients

	Antibody concentration (AU mL^{-1})	
Sample	ELISA	Sensor
1	16.5 ± 0.4	14.0 ± 0.5
2	22.4 ± 0.6	25.0 ± 0.4

$\gamma CD_{6.5}$: ADA_1 indicating that there is an excess of CD hosts on the surface available for the interaction with the ADA residues. The interfacial equilibrium constants for the complexation of **5a** and **5b** with the three different CD surfaces was determined by SPR using the Langmuir equation: $c/\Gamma = c/\Gamma_{max} + 1/K\Gamma_{max}$, where c and Γ are the bulk concentration (based on the molecular weight of CMC) and surface coverage by the polymers, K is the interfacial equilibrium constant and Γ_{max} is the maximum surface coverage of the polymers (Table 1). The value of the interfacial equilibrium constant for the βCDPSH/**5a** system is about 10^6 times higher than those obtained for the interaction of monomeric ADA derivatives with immobilised βCDs,[15] indicating a very strong and multivalent interaction of **5a** with the βCDPSH support layer and being consistent with the inability of adamantane-1-carboxylate to completely remove **5a** from the surface. The reverse trend in the K values observed with the γCD host indicates a better size compatibility with TMADA, while, as expected, αCD gave a very low K with **5a** (Table 1).

The developed Au/βCDPSH/**5a** surface was then tested for the amperometric detection of an anti-gliadin antibody using an anti-idiotypic-peroxidase conjugate as reporter in a standard sandwich type format. Fig. 3 shows a comparison of the specific (presence of antibody) and non-specific (absence of antibody) signals obtained. The non-specific signal (trace b) represented less than 8% of the specific signal in these conditions (trace a), indicating a very low tendency of the reporter conjugate to interact with the surface in the absence of target autoantibody. The role of the βCD support

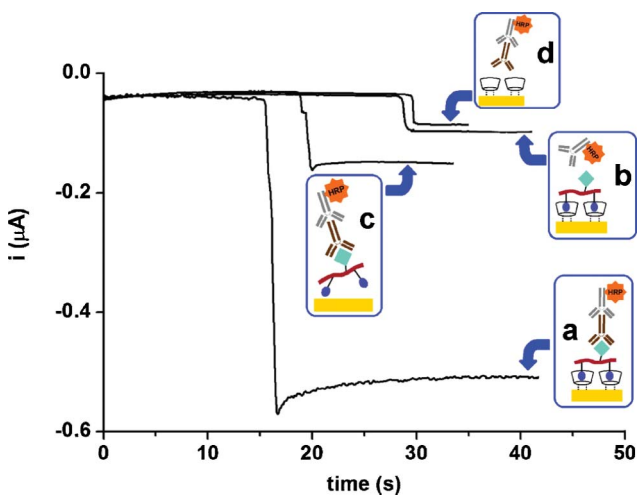


Fig. 3 Amperometric responses obtained for different systems: (a) full detection system, (b) absence of target antibody, (c) absence of βCD layer, (d) absence of **5a**. Conditions: [antibody] = 1 μg mL^{-1}, E = –0.2 V, supporting electrolyte: 0.1 M PBS + 0.15 M KCl (pH 6) containing 1 mM hydroquinone as mediator.

in assisting the immobilisation of the GLI fragments in **5a** is also evident by comparing the response obtained in the presence (trace a) and in the absence of βCD layer (trace c). In the latter case, only 22% or the original signal is observed, which can be attributed to some physical adsorption of **5a** on the gold surface. Finally, only 6% of the signal was recorded in the absence of **5a** (trace d) indicating excellent passivation properties of the hydrophilic CD polymer support, similar to that observed with other hydrophilic adsorbates such as polyethyleneglycol derivatives.[16]

The dependence of the amperometric signal on anti-gliadin antibody concentration was linear in the range 0–5000 ng mL^{-1}, with a sensitivity (taken as the slope of the linear fit) of 0.47 μA mL ng^{-1} and a limit of detection of 45 ± 3 ng mL^{-1} (n = 3, see ESI†). Compared with a commercial ELISA test (α-Gliatest S IgG Chromo from Eurospital SpA, Trieste, Italy), this value is about 4 times lower (200 ng mL^{-1}). In addition, preliminary experiments carried out in the presence of serum showed that the functionality of system is maintained with no noticeable effect of the matrix components, demonstrating its applicability to real samples (see ESI). Two serum samples of coeliac patients were thus analysed, and as can be seen in Table 2, the anti-gliadin levels of the two patients obtained by both methods showed excellent correlation, demonstrating the successful performance of the immunosensor and highlighting the effectiveness and applicability of the developed supramolecular platform to a real clinical scenario.

In conclusion, the highly stable interfacial self-assembly of a bifunctionalised polymer bearing adamantane units and an antigenic fragment onto a βCD-containing support has resulted in a versatile surface modification tool with a plethora of potential applications as demonstrated in the electrochemical detection of a coeliac disease related autoantibody. The surface also has the advantage of undergoing controlled regeneration, either by disrupting the immunocomplex in mild conditions or by simply restoring the βCD support, and has excellent anti-fouling properties. The detection limits obtained are better than that of a benchmark ELISA test and there is an excellent correlation of the antibody levels measured with the supramolecular immunosensor with respect to a standard ELISA kit. These results clearly demonstrate the potential for the widespread use of CD : CMC polymer interfaces in biosensors and biochips based on the compatibility and low non-specific adsorption of matrix proteins, the possibility of stripping and recycling of the CD surface and the flexibility of use as a scaffold for alternative architectures by self-assembly. These unique and interesting properties are currently being further explored and a generalisation of this strategy, including a study of the multiplexing ability (*i.e.* possibility to detect several analytes on the same platform), is currently under study using a panel of several coeliac disease autoantibody markers.

This work was supported by CD-MEDICS project (FP7-2008-ICT-216031) and Ministerio de Ciencia e Innovación, Spain, for a Ramón y Cajal Research Professorship and grant BIO2008-02841 to AF. We thank Dr H. J. Ellis (King's College London) for providing the samples used in this work.

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