A bienzymatic amperometric immunosensor exploiting supramolecular construction for ultrasensitive protein detection†

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Abstract

Self-assembly of a ferrocene-appended polymer bearing an antigen fragment and lactate oxidase on a cyclodextrin-modified surface provides a highly sensitive, easy-to-operate and self-sufficient immunosensor.

In recent years, self-assembly has emerged as a promising method for the fabrication of structured and functional thin films on solid substrates. This method, initially developed for the preparation of electrostatically confined multilayer assemblies, has been successfully extended to various other driving forces such as host–guest interactions.[1,2] Supramolecularly organized structures involving cyclodextrin (CD) and different guest interactions have recently attracted a wide interest as surface modification tools for biomolecule immobilization[2,3] in the development of biosensors.[4,5] Thiolated CDs have been attached to gold surfaces via the well known S–Au bond to form self-assembled monolayers (SAM) permeable to electron transfer[6] and have been used as supports to detect small molecules[7] or to immobilize guest-appended polymers.[8] Among the many existing CD guests, ferrocene (Fc) is one of the most effective electron transfer mediators between electrode surfaces and the active site of many oxidase enzymes and is also able to form inclusion complexes with βCD with stability constants in the order of 10^3–10^4 M^{-1}.[9]

Recently we reported the attachment of bifunctional carboxymethylcellulose (CMC) carriers modified with adamantane (ADA) moieties and a capture protein on gold surfaces modified with thiolated cyclodextrin polymer **1** and demonstrated the feasibility of this

assembly in the amperometric detection of autoantibodies in real samples.[10] In this system, CD/ADA interactions serve to dock the polymer to the surface while detection is carried out using a peroxidase-labeled secondary antibody, thus requiring the addition of hydrogen peroxide and a mediator to generate the electrochemical signal.

In this communication, we report a mediator confined platform for detection of autoantibodies in which the electrochemically inactive ADA units are replaced by Fc which has the function of linking the CMC carrier bearing a capture protein (**2**, **3**) to the βCD modified surface. The target antibodies are then recognised by a peroxidase labelled secondary antibody that recognises the analyte forming a sandwich type immunocomplex and also uses the Fc units as an electron transfer mediator (Fig. 1i). In order to increase the sensitivity of the system an oxidase enzyme was co-attached to the Fc–CMC carrier (**3**). Several oxidases, such as lactate oxidase (LOx),[11] choline oxidase (ChOx)[12] and glucose oxidase (GOx),[13] which are able to in situ produce H_2O_2 by oxidation of substrate molecules existing in blood were explored with the aim of simplifying the detection system and increasing the sensitivity by avoiding diffusion of the substrate to the electrode surface (Fig. 1ii).

The morphology of the surface was studied by AFM and STM (Fig. 2). Round shaped structures of about 15–20 nm diameter are observed on the gold surface modified with **1β**. The shape and size of these structures is consistent with the presence of a cross-linked CD polymer composed of ∼12 CD molecules in a random pattern,[5] thus generating polymer molecules with a globular shape. The coverage of the surface is relatively regular, similar to that reported previously on SAMs of monomeric thiolated cyclodextrins,[14] indicating the formation of a rather compact monolayer of **1β** on gold. After deposition of **2** on this surface, the morphology changes noticeably, with filamentous structures of ∼1–2 nm cross-section with analogous shapes to those obtained after the deposition of native CMC on mica.[15] Evidence of the inclusion of Fc units in the βCD cavities was obtained using surface enhanced Raman spectroscopy (Fig. 2e) where a red-shift in the Fe–Cp bands was observed in the 200–400 cm^{-1} region, due to the elongation of the Fe–Cp bond upon inclusion in the βCD cavities.[16] The bands around 500 cm^{-1} present in the spectra of Au/**1β** and Au/**1β/2** have also been observed in monolayers of thiolated βCD but could not be unambiguously assigned.[17]

The role of Fc/CD supramolecular interactions in the immobilization of **3a** was studied by surface plasmon resonance (SPR) on gold chips modified with **1α**, **1β** and **1γ**. When a 1 mg mL^{-1} solution of **3a** was injected, only the channel modified with **1β** gave a significant response (725 RU), in contrast with the 49 RU and 75 RU obtained with **1α** and **1γ**, respectively. In addition, injection of a CMC polymer modified only with gliadin units (i.e. not carrying Fc moieties) on an Au/**1β** surface gave a non-significant response (35 RU). These results demonstrate that **3a** is immobilized via the formation of an inclusion complex with βCD cavities. The recognition ability of the **1β/3a** interface was demonstrated with a 435 RU response obtained after injection of 1 μg mL^{-1} of anti-gliadin antibody. The biosensor surface could be regenerated by application of three pulses of glycine, pH 2, to break the antigen–antibody interaction whilst not affecting the interaction of **3a** with the CD-modified support.

The amperometric responses of the system Au/**1β/3** were then studied by measuring the current levels obtained in the detection of 1 μg mL^{-1} of anti-gliadin antibody using a peroxidase-labelled secondary antibody (Fig. 3). The current levels decreased in the order **3a** ≫ **3b** > **3c**. On the other hand, ELISA tests carried out on **3a** and **3b** immobilized on Nunc plates gave similar responses in the detection of the anti-gliadin antibody as for polymer **2**, while **3c** showed a markedly lower response. Hence, the amperometric behavior could be explained considering a combination of pH, enzymatic activity and steric hindrance factors. The three enzymes have different optimum pH values (7.5 for ChOx,[12] 6.5 for LOx[11] and 5.5 for GOx[13]) with k_{cat} values varying in the order ChOx < Lox ≈ GOx.[19,20] Hence only the optimum pH of LOx matches well with that of HRP (pH_{opt} 6.0–6.5)[21] and thus the generation of H_2O_2, which triggers the electrochemical response, is maximized. This explains the low response obtained with ChOx. In the case of GOx, its optimum pH is close to that of HRP but the size of GOx (160 kDa) is about 80 times bigger than that of the capture protein (2 kDa) and about two times bigger than those of the CMC carrier (90 kDa) and LOx (80 kDa). Thus, in this case steric hindrance could block the access of the target antibody towards gliadin in the polymer, explaining the highest signal being generated by LOx.

Fig. 4 shows the calibration curves obtained for the detection of anti-gliadin antibody using systems i and ii in Fig. 1. For system ii (Au/**1β/3a**), assuming a linear behavior between 0–4 μg mL^{-1} the sensitivity of the system is 50.6 nA μg^{-1} mL^{-1}, with a detection limit of 70 ng mL^{-1}. This represents a 43-fold sensitivity increase with respect to system

i (Au/**1β/2**) which lacks the oxidase enzyme and depends on addition of H_2O_2. In addition, the replacement of Fc by ADA in polymer **2** (thus requiring the addition of an external mediator) resulted in an even higher decrease of the amperometric signal (trace iii in Fig. 4), as recently observed by our group in the construction of a DNA-based detection platform.[22] Hence, the combination of a surface-confined electron transfer mediator with the continuous in situ enzymatic generation of H_2O_2 by LOx in the presence of lactate is responsible for the remarkable improvement in sensitivity. A negligible loss in amperometric response was detected for system ii after storage at 4 °C for two weeks in the presence of a stabilizing buffer.

In conclusion, we have developed a novel strategy for immunosensor construction combining cyclodextrin/ferrocene host–guest chemistry with in situ enzymatic generation of substrate. Compared to other amperometric immunosensors reported for detection of anti-gliadin autoantibodies based on adamantane–cyclodextrin interactions[10] or gliadin-coated glass beads,[23] this approach resulted in an increase in device sensitivity by at least one order of magnitude. In addition, from an operational point of view, the approach described here affords an easy-to-operate platform compared to classical HRP-labeled sandwich type assays, which may be amenable for the development of packaged and disposable diagnostic devices as it avoids the need for storing unstable mediator/H_2O_2 mixtures[24] and requires only the sample introduction. Studies in this direction are currently being carried out.

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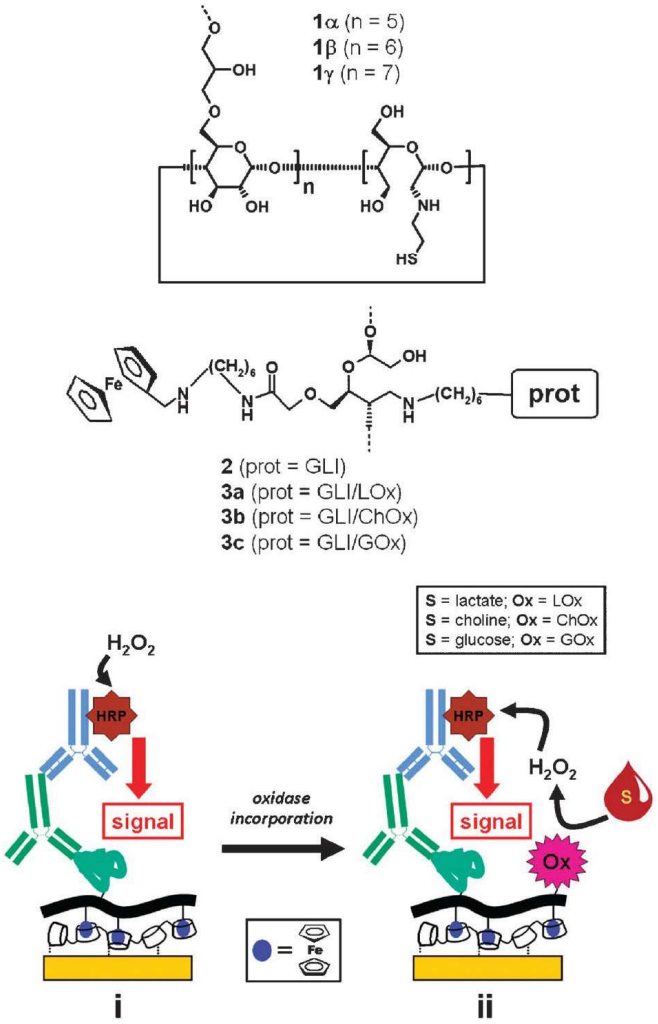

Fig. 1 Detection principles based on (i) self-assembly of **2** on CD-modified support and detection using HRP-labeled antibody upon addition of H_2O_2; (ii) self-assembly of **3** with in situ H_2O_2 generation by oxidase catalyzed oxidation of blood metabolites. GLI: gliadin.

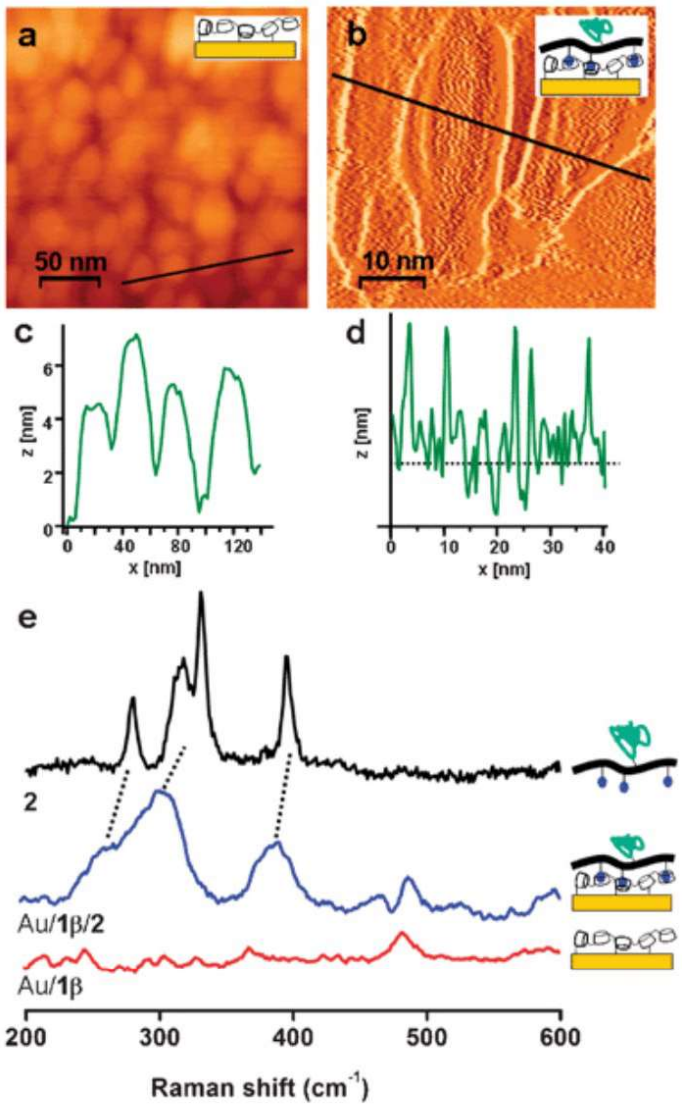

Fig. 2 (a) AFM images of **1β** deposited on gold, (b) STM image of **2** deposited on an Au/**1β** surface; (c) and (d) height profile along the lines indicated in (a) and (b), respectively.[18] (e) Surface enhanced Raman spectra in the 200–600 cm^{-1} region of Au/**1β**, **2** and Au/**1β/2**.

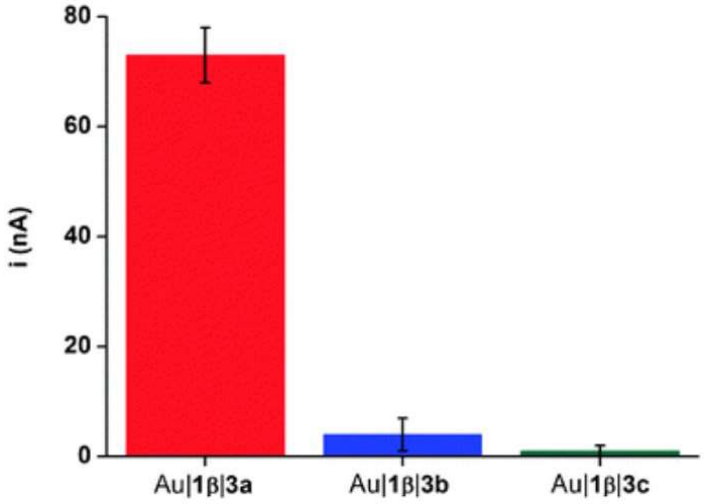

Fig. 3 Amperometric responses obtained for the system Au/**1β/3** with different oxidases. Conditions: E = 0.2 V vs. Ag/AgCl, supporting electrolyte: 0.1 M PBS + 0.15 M KCl (pH 6).

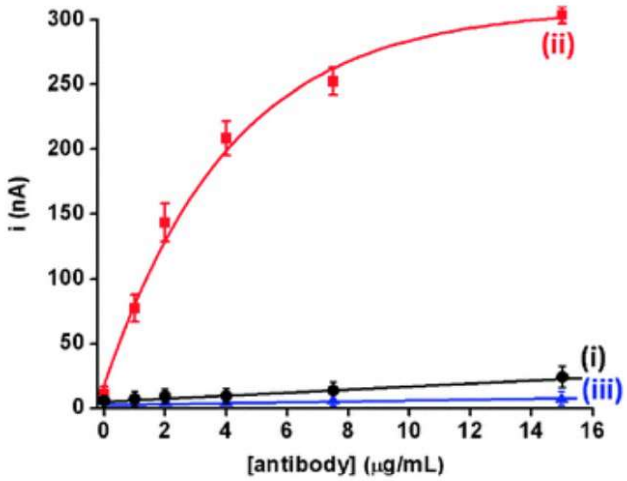

Fig. 4 Amperometric calibration curves for anti-gliadin antibody detection using systems i, ii and iii.