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Short communication

Towards a biosensor immunoassay of protein-bound isopeptides in human plasma

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ABSTRACT

This study demonstrates that synthetic isopeptides formed on BSA can be quantitatively analyzed by a surface plasmon resonance-based biosensor method. A monoclonal IgM antibody 81D4, that reacts with the synthetic isopeptide and also with the natural isopeptide cross-link in D-dimer (but not with its non-cross-linked fibrin monomer), was covalently immobilized to a carboxymethylated dextran surface, a CM5 surface. Its immunocapturing efficiency was found to be good. The affinity of the interaction between the monoclonal 81D4 and the synthetic isopeptide was estimated to approximately 4×10^{-7} M. Good reactivity was also observed when human plasma spiked with this isopeptide was used as test solution. Cross-linked D-dimer in the plasma of patients is a marker of disseminated intravascular coagulation (DIC) which occurs late in sepsis. This biosensor method has the potential to be developed into a rapid sensitive assay for measuring the level of natural isopeptide cross-links in proteins in the plasma of patients with a suspected diagnosis of sepsis.

1. Introduction

The diagnosis of sepsis, in the absence of a clinical history of infection, is based primarily on clinical symptoms and in such cases mortality rates, in spite of efficient procedures for correcting vascular, cardiac, pulmonary and renal abnormalities, are still of the order of 40–50% [1]. One hall-mark of sepsis is disseminated intravascular coagulation (DIC) but it occurs late in the pathology. It is a syndrome characterised by systemic intravascular activation of coagulation, leading to extensive deposition of insoluble fibrin cross-linked by isopeptide bonds formed by the action of activated plasma transglutaminase, FXIII [2]. The cross-linked fibrin is subsequently degraded by plasmin to yield products called D-dimers. An elevated plasma level of D-dimer is a well-documented characteristic for the prognosis of thrombosis [3]. Hence, in suspected cases of sepsis without overt infection, the direct detection of bound isopeptides prior to the subsequent action of plasmin could constitute a marker of sepsis, which may be detectable earlier than increases in D-dimer.

One antibody that appears to be suitable for detecting isopeptide cross-links is the 81D4 mouse monoclonal antibody. It reacts with isopeptide cross-links formed enzymically by the action of cellular or plasma transglutaminase between the γ carboxamide of glutamine residues on one protein chain and the ε amino groups of lysines on another homo or hetero protein chain. It reacts neither with free isopeptides nor with bound isopeptides that have free primary amino groups but does react with these same bound isopeptides when their amino groups are blocked or when they are present as a cross-link between two protein chains, see Fig. 1 [4]. This antibody, therefore, has a clear potential in immunoassays to detect isopeptide cross-linked proteins in individuals at risk for thrombogenesis.

With regard to the immunoassays, both direct and indirect methods can be envisaged. One direct approach consists in using a surface plasmon resonance (SPR)-based affinity biosensor which measures the association of a free molecule (analyte) with an immobilized ligand. The response is recorded as an SPR signal measured in resonance units (RUs) over time in a sensorgram [5]. In principle, the binding of the analyte to the ligand immobilized on the surface gives rise to a local change in refractive index, which is directly proportional to the change in mass at the surface. Typically 1000 RU approximates the binding of 1 ng/mm^2 protein [6]. SPR-based biosensors are widely used to determine the kinetics and affinity of analyte–ligand interactions. No labelling of the interacting molecules is required and the binding is monitored in real-time, thus providing an answer within minutes.

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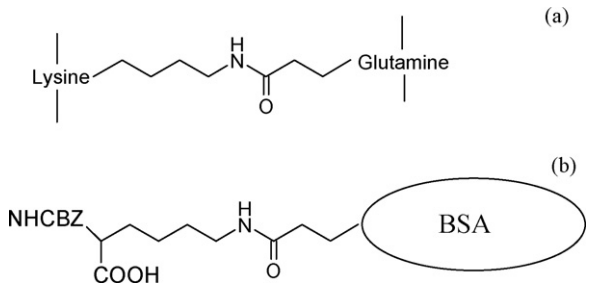

Fig. 1. Schematic structure of (a) the natural isopeptide cross-link between the γ carboxamide of a glutamine residue on one protein chain and the ε amino groups of a lysine on an adjacent protein and (b) the synthetic isopeptide formed on BSA. CBZ, carbobenzyloxy.

In the indirect approach a second fibrin-specific detection antibody is required, hence there is the risk of cross-reactivity with soluble (non-cross-linked) fibrin. In a biosensor-based assay using the 81D4 as a capture antibody the need for the second fibrin-specific antibody is obviated. Another inherent advantage of the biosensor-based assay is the use of a carboxymethylated dextran surface, a CM5 surface, for the immobilization of the ligand. Such a surface offers a large interaction area and hence an enhanced sensitivity compared to a procedure based on immobilization at a flat surface [7,8].

This first study describes a direct assay where the covalently immobilized monoclonal anti-isopeptide antibody 81D4 was used to capture an isopeptide (synthesized on BSA) from buffer or plasma and to determine the affinity constant of the interaction.

2. Materials and methods

2.1. Chemicals

BSA-isopeptide and the monoclonal antibody 81D4, an IgM, were gifts from Covalab, France, and a negative control, mouse IgM was purchased from DakoCytomation, Denmark. Sensor chip CM5, Amine coupling kit (*N*-hydroxysuccinimide, (NHS), *N*-ethyl-*N*-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC), ethanolamine (1 M, pH 8.5) and HBS buffer (degassed 10 mM HEPES buffer saline, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P-20) were obtained from Biacore, Sweden. Deionized distilled water of Millipore grade was used to prepare the 10 mM citrate–phosphate buffer of pH 4. Human plasma from healthy blood donors was obtained from the Sahlgrenska University Hospital in Göteborg, Sweden.

2.2. Surface plasmon resonance experiments

The SPR experiments were carried out at 23 °C on a Biacore 2000. All solutions were degassed and filtered (0.2 μm) prior to use.

2.2.1. Immobilization of 81D4 and control IgM antibodies

The ligand 81D4, a monoclonal IgM antibody, was covalently immobilized onto the commercial sensor chips covered by carboxymethylated dextran (CM5 sensor chips) using the amine coupling procedure [9]. Throughout the immobilization the flow rate was set to 5 μl/min. A 10-mM citrate–phosphate buffer of pH 4 was used as flow buffer during the immobilization and for dilution of 81D4. Briefly, CM5 surfaces were activated by 35 μl EDC/NHS and 81D4 (15 μl of 400 μg/ml) was immobilized generating a response level of approximately 12,000 RU. A control surface was generated by immobilizing 50 μl of 100 μg/ml negative control mouse IgM creating a response level of 6300 RU. The pH of the control IgM was adjusted to 4.5 with 1 M HCl immediately prior to immobilization. After immobilization the remaining reactive groups were deactivated by injection of 1 M ethanolamine.

2.2.2. BSA-isopeptide–81D4 interaction studies

The binding experiments were carried out at a flow rate of 10 μl/min using HBS buffer pH 7.4 as running buffer. Injections of the BSA-isopeptide conjugate were performed over surfaces previously derivatized with 81D4 or with control IgM antibodies. In between injections, the ligand surfaces were regenerated by a 0.5-min pulse of 6 M HCl diluted to pH 3.5, which was found to be a suitable condition for removing analyte bound to immobilized antibody. Calibration curves were obtained from the dose response of BSA-isopeptide in HBS buffer and in 1% plasma diluted with HBS buffer.

3. Results and discussion

3.1. Immobilization of 81D4

Efficient immobilization of the ligand molecule with retention of its biological activity is a prerequisite for successful biosensor analysis. A carboxymethylated dextran matrix, which constitutes a hydrogel, is often used for this purpose. Proteins covalently attached to such a hydrogel are known to retain their biological activity over long periods of time. Using an efficient coupling procedure that gives a high density of immobilized antibodies, a biosensor immunoassay with high sensitivity can be obtained.

A series of experiments were undertaken to optimize the immobilization of the 81D4 antibody to the carboxyl-functional surface. The parameters studied were buffer composition, buffer pH and concentration of the antibody. Best results in terms of immobilization efficiency were obtained with a 100-mM citrate–phosphate buffer of pH 4 and an antibody concentration of 400 μg/ml. Under these conditions this monoclonal IgM antibody gave a response level of 12,000–15,000 RU, corresponding to a binding of 12–15 ng/mm^2.

3.2. Isopeptide conjugate binding

The affinity binding of the BSA-isopeptide, in concentrations ranging from approximately 23 nM to 3 μM, to the immobilized anti-isopeptide 81D4 antibody was monitored. Fig. 2 shows the response from the binding of 1.5 μM analyte to surface-bound 81D4 and subsequent regeneration by a solution of 3 μM HCl, pH 3.5.

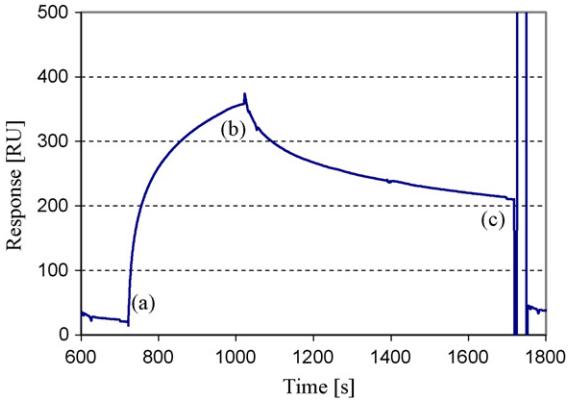

Fig. 2. Sensorgram obtained after injection of (a) 1.5 μM BSA-isopeptide in HBS buffer leading to capture by 81D4 followed by (b) a slow dissociation in HBS running buffer and a subsequent (c) regeneration by a 30-s pulse of 3 μM HCl, pH 3.5.

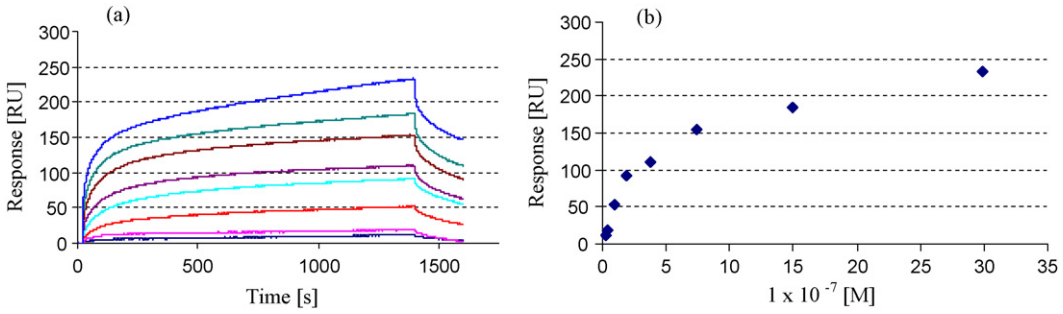

Fig. 3. (a) Sensorgram obtained from injections of isopeptide conjugate in HBS buffer. The curves represent concentrations from 23 nM (bottom) to 3 μM (top) and are presented as the response after subtraction of the response from a surface with a control IgM. (b) A plot of the equilibrium analyte response of isopeptide conjugate as a function of analyte concentration.

The optimal regeneration solution should efficiently wash away the affinity-bound isopeptide while leaving the ligand in place with preserved binding capacity. There is usually a delicate balance between desorption efficiency and retention of the capture activity of the coupled antibody. The choice of a 3-μM HCl solution of pH 3.5 as the optimum regeneration solution was the result of a series of experiments using different aqueous compositions and different pH values. However, the compromise pH of 3.5 was not low enough to fully desorb all the bound analyte, hence a small accumulation of material was observed. Nevertheless, the regeneration was good enough for the surfaces to be used repeatedly and for the affinity of the interaction to be estimated.

Fig. 3a shows the interaction of the isopeptide conjugate to bound anti-isopeptide antibody at concentrations of 23 nM to 3 μM. One of the concentrations was run in duplicate (23 nM) and the average response was taken. The anti-isopeptide antibody was immobilized in the second flow cell on the sensor chip, while the control antibody was bound in the first flow cell. The sensorgrams shown were obtained after subtraction of the reference signal (flow cell 1) from the response in the flow cell with immobilized 81D4 (flow cell 2). A control of 100 μg/ml BSA in HBS buffer was injected over the anti-isopeptide antibody. However, BSA showed no detectable binding to the antibody (data not shown).

The association and dissociation equilibrium constants K_A and K_D, respectively, describe a system at equilibrium. K_A is the inverse of K_D and $K_D = (k_d/k_a)$, where k_a and k_d are the association and dissociation rate constants, respectively. The equilibrium constants and the affinity of an interaction can be determined from the analyte equilibrium response as a function of analyte concentration if the system reaches equilibrium at a reasonable time. Hence, the dissociation equilibrium constant, K_D, was estimated by affinity analysis from the plot of the equilibrium response as a function of concentration, see Fig. 3b, taking the concentration that gives 50% of the maximum response. The affinity of the interaction between the monoclonal 81D4 and the synthetic isopeptide was found to be approximately 4×10^{-7} M, i.e. of moderate strength (Fig. 2b).

The theoretical value of the maximum response can be calculated using the expression:

$$\text{response}_{\max} = \left(\frac{\text{MW}_{\text{BSA-isopeptide}}}{\text{MW}_{\text{IgM}}} \right) \times \text{response}_{\text{IgM}} \times V_{\text{IgM}}$$

where V_{IgM} is the theoretical number of available binding sites [5], $MW_{BSA\text{-}isopeptide} = 67$ kDa and $MW_{IgM} = 950$ kDa.

The estimated maximum binding of the isopeptide conjugate (~280) was considerably lower than the theoretical value (~4200) assuming all binding sites on the IgM antibody to be available and immobilization level of about 12,000 RU 81D4. However, this likely not the case in this type of assay with immobilized IgM. The 81D4 IgM used here is purified, however, it is possible that there is concomitant covalent immobilization of non-IgM species which may have contributed to the ligand RU value. Further, in this type of assay with immobilized IgM the constraints associated with the immobilization of one of the immunoactive components may introduce effects that cause deviations from the binding kinetics between antibody and antigen in solution [10].

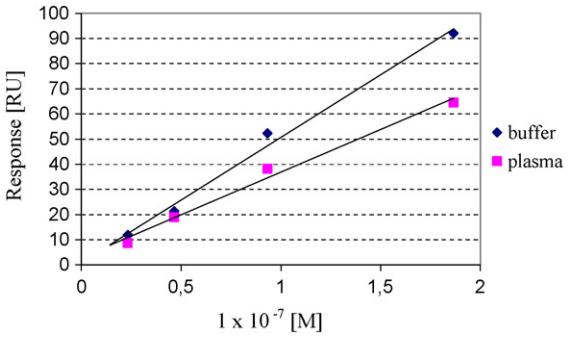

Fig. 4. Dose–response curves for the isopeptide conjugate in HBS buffer and for 1% human plasma spiked with the isopeptide conjugate.

Fig. 4 shows results from the dose–response experiments for the BSA-isopeptide in buffer and in 1% plasma in the linear region (23–186 nM). The sensitivity limit under the conditions used was about 20 nM both for the buffer and for the 1% plasma solution. As can be seen, the dose response for the isopeptide was steeper for the buffer solution than for the human plasma. The difference in slope may be associated with a slight interaction between the synthetic isopeptide and plasma proteins in solution. The data show that there was no significant non-specific adsorption of plasma components to the 81D4 immobilized surface since mouse IgM was used as negative control in both experiments and that the values plotted for both the buffer and the plasma experiments represent the differences between test and control. It is also very satisfactory that when added in minute amounts to human plasma, there is a proper correlation between the amount of isopeptide added in solution and the bioassay response. No cross-reactivity with the plasma components was detected. The results show that the principle of using surface plasmon resonance to monitor the immunocapture of protein-bound isopeptides from solution phase by a surface-bound antibody can be used as a basis for developing assays to determine whether these molecules could be early markers of sepsis.

4. Conclusions

This study shows that protein-bound isopeptides diluted in buffer and in human plasma can be measured directly by a SPR biosensor coated covalently with the capture anti-isopeptide

antibody 81D4. The immobilized 81D4 binds specifically to the isopeptide with an apparent affinity constant of 4×10^{-7} M. Non-specific binding of BSA to 81D4 was not detected. Although the maximum binding of the isopeptide was considerably lower than the theoretical value, the assay was able to detect the isopeptide moiety with linear dose response in buffer and in plasma over the low concentration range 23–186 nM. An SPR-based biosensor has the potential to measure directly traces of protein-bound isopeptides in the plasma of patients.

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