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Modification of ISFETs with a monolayer of latex beads for specific detection of proteins

G.A.J. Besselink *, R.B.M. Schasfoort, P. Bergveld

MESA+ Research Institute, University of Twente, P.O. Box 217, Drienerlolaan 5, 7522 AE Enschede, The Netherlands

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Abstract

The so-called ion-step method is a novel potentiometric approach that can detect protein adsorbed onto the gate area of modified ion-sensitive field-effect transistors (ISFETs). In this report, a generic technology is described for immobilization of peptides and proteins to the ISFET gate in order to confer specific binding properties to the ISFET. For this, the surface of the ISFET was covered with a *monolayer* of Amino beads (diameter, 0.9 μm) followed by immobilization of protein ligands onto these beads. Amino beads are latex spheres that contain primary amino groups at the outer surface. Preactivation of the latex-bound amino groups with glutaraldehyde, and consecutive incubation with polylysine resulted in covalent immobilization of this polyamine, as revealed by ion stepping measurements. For ImmunoFET applications, human serum albumin (HSA) was immobilized onto the Amino bead-covered ISFETs, by passive adsorption but also by covalent coupling. Resulting devices were used for qualitative detection of α-HSA antibodies by means of the ion step method. The binding of antibody was very specific and fast (most of the binding was accomplished in 15 min) with signal yields up to 17 mV. Efforts to increase the antibody-binding capacity of the solid phase on the ISFET exploiting amino group activation (with glutaraldehyde or other homobifunctional cross linkers) before HSA coupling, did not improve signal yield. The bead technology described in this report is an easy, generic method for coating the ISFET with a solid phase that, using the ion-step method, can be applied to immunosensing.

Keywords: Biosensor; ISFET; ImmunoFET; Latex beads; Antibodies

1. Introduction

Many types of biosensors exist that are capable of detecting biomolecular interactions, amongst which immunosensors are becoming increasingly important (Luppa et al., 2001). A number of authors report on measuring protein adsorption by equilibrium potentiometry, but in many measurements the observed responses were poor. One obvious exception is the work of Feng et al. (2000), which describes direct potentiometric detection of human immunoglobulin G (down to 0.2 ng/ml). Studies concerning protein adsorption to bare devices, such as ion-sensitive field-effect transistors (ISFETs), were started from the expectation that proteins should modulate the static ISFET response, considering the fact that proteins carry electrical charge. However, such an operational mechanism is unlikely to occur because counterions shield the charged protein molecules, thus resulting in the absence of an electric field beyond a distance determined by the Debye length of the sample solution (Schasfoort et al., 1990). An alternative potentiometric method for detection of adsorbed protein is the so-called ion-step method (Schasfoort et al., 1990a) that will be described below.

Several examples exist of indirect potentiometric protein detection by application of antibody/enzyme conjugates and subsequent detection of enzyme-based acidification for analytes as, for example, anti-(human IgG) (Campanella et al., 1999) and α_2-interferon (Sergeyeva et al., 1999). Addition of a specific enzyme substrate triggers a local change in pH at the surface of the transducer that results in an electrochemical signal. The light addressable potentiometric (LAP) sensor system has been used for the determination of specific

* Corresponding author. Tel.: +31-53-489-1051; fax: +31-53-489-1105.
E-mail address: g.a.j.besselink@tn.utwente.nl (G.A.J. Besselink).

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proteins, viruses and bacteria down to very low concentrations (<1 ng/ml) by sequential use of a sandwich immunoassay, membrane filtration capturing, binding of conjugate (consisting of urease and secondary antibody), and detection of urease amplified pH change (Lee et al., 2000).

Very few examples exist concerning direct detection of protein using potentiometric biosensors. The so-called ion-step method represents a novel measurement concept for potentiometric detection and quantification of adsorbed biomolecules in which modified ISFETs are used. The ion-step method was first introduced by Schasfoort et al. (1990a) and was further developed by Van Kerkhof et al. (1994) and Eijkel et al. (1997). The ion-step method is based on a dynamic measuring principle, whereas many other potentiometric methods are static and measure in a state of thermodynamic equilibrium. Predominant mechanism of the ion-step response measured by the ISFET devices described in this report, is the release or uptake of protons by the sensor-bound protein molecules. Proteins show different titration behavior in high compared with low-salt medium, which originates from a shift in proton dissociation equilibria of the different protein-derived functional groups. A detailed theoretical understanding of the ion-step response has been supplied by Eijkel et al. (1997a). To execute the ion step, the modified ISFET is placed in a flow-through system, and the electrolyte solution flowing over the ISFET gate is suddenly changed to a solution having a higher ion concentration than the initial one but with the same pH. Application of this ion step induces a transient potential change as measured by the ISFET (Van Kerkhof et al., 1994). The amplitude and the time of decay of this transient potential contain information concerning the fixed charge concentration on the ISFET surface, determined by the concentration of acidic and basic groups derived from side-chain and end-chain functional groups of immobilized protein molecules close to the ISFET surface (Eijkel et al., 1997a; Besselink and Bergveld, 1998). The ion-step measuring method has been demonstrated for the ISFET-based detection of charged macromolecules like the protein lysozyme (Schasfoort et al., 1990a; Eijkel et al., 1997, 1997a), anti-HSA (Schasfoort et al., 1990a), and heparin (Van Kerkhof et al., 1993).

In the past, ISFET modification has been realized by passive adsorption of solutes to the bare ISFET surface (e.g. of protamine: Van Kerkhof et al., 1995) or to a thick micro porous polystyrene membrane deposited on top of the ISFET gate (e.g. of lysozyme: Eijkel et al., 1997a). Disadvantages of the approaches mentioned before are the unequal adsorption levels anticipated for different types of ligands, the possible leakage away of ligand and, in case of the thick membrane, a restricted diffusion that necessitates the use of relatively long incubation times. In this report, results are described from ISFET modification with a monolayer of latex beads followed by immobilization of human serum albumin (HSA) to these beads. Employing the ion step method, the resulting ISFET devices were used for qualitative detection of anti-HSA antibodies.

2. Experimental

2.1. Materials

Amino beads (Polybead™, diameter 0.913 ± 0.024 μm) were obtained from Polysciences, Inc. (Warrington, PA). HSA (crystalline, $>99\%$), rabbit α-(HSA), rabbit α-(human fibrinogen) serum and poly-L-lysine hydro bromides (molecular weights of 4, 22 and 37 kDa) were all obtained from Sigma Chemical Co. (St. Louis, MO). Glutaraldehyde (50% in water), $NaBH_3CN$, 2-aminoethanol and Tween® 20 (zur Synthese) were purchased from Merck-Schuchardt (Hohenbrunn, Germany). Dimethylpimelimidate, dimethyladipimidate and disuccinylglutarate were obtained from Fluka (Buchs, Switzerland).

2.2. Methods

Ta_2O_5-ISFETs were fabricated in the $MESA^+$ cleanroom laboratory following the usual ISFET processing steps. The chips were mounted on a piece of printed circuit board and encapsulated with Hysol epoxy, leaving uncovered a circular area around the gate with a diameter of 1.7 mm and a depth of 150 μm. The ISFETs showed a sensitivity of -59 mV per pH. Before each experiment, the ISFET gate area was rubbed with a cotton bud soaked in detergent solution (1% Alconox™) for cleaning. Precoating with polylysine and application of Amino bead suspension resulted in ISFETs that were covered with a monolayer of beads (details of this newly developed method are explained in the first part of Section 3).

Different homobifunctional cross linkers were applied for preactivating the primary amino groups on the ISFET-bound Amino beads: glutaraldehyde (GA; 5% in 0.2 M sodium phosphate, pH 7.8), dimethyladipimidate, dimethylpimelimidate (20 mM in 40 mM $K_2B_4O_7$, pH 8.5) and disuccinimidyl glutarate (10 mM in a mixture consisting of 10% DMSO and 90% 40 mM $K_2B_4O_7$, pH 8.5). After preactivation for 15 min, the monolayer was rinsed extensively in the respective buffer (minimal 2 h total washing time, during which the washing fluid was renewed three times). Next, preactivated (or non-activated) Amino bead-devices were incubated with polylysine or HSA (10 mg/ml in PBS; incubation time 15–30 min), followed by washing in PBS. In case of GA preactivation, ISFETs with

immobilized protein were treated with $NaBH_3CN$ (20 mg/ml in 0.2 M sodium phosphate, pH 7.8; for 10 min) for chemical reduction (and thus stabilization) of the newly formed Schiff's base bond between protein and GA-activated substrate. Next, residual activated groups were blocked by treatment for 10 min with 0.25 M 2-aminoethanol.

Success and magnitude of ISFET-modification with polyamines was assessed by means of the ion-step method. Stability of binding of immobilized polylysine was tested after washing for 5 min with 5% Tween® 20. Ion stepping was performed as described elsewhere (Besselink and Bergveld, 1998). ISFETs, modified with HSA, were incubated for 15 or 30 min with Rabbit α-HSA antiserum (pure or diluted up to 1:3 with PBS). Subsequently, ion-step responses of ISFETs were determined again, from which the differential amplitude (i.e. the difference between the ion-step response amplitude after and before incubation with sample) was calculated. In part of the experiments, the formed antibody/HSA complex was cross linked directly after incubation with antiserum, by treatment with dimethylpimelimidate (10 mg/ml in 0.2 M sodium phosphate, pH 7.8). The treatment with dimethylpimelimidate (a chemical cross linker) was included in order to stabilize the antibody–antigen complex.

3. Results

3.1. Preparation of Amino bead-covered ISFETs

After applying Amino beads onto polylysine (PLL)-precoated ISFETs, spontaneous adhesion of the latex beads occurred. A coating procedure was developed for the ISFETs resulting in a bead monolayer (Fig. 1). The molecular weight of the PLL used for precoating should not be too small. ISFETs containing a precoating consisting of polylysine with a molecular weight of 4 kDa (= PLL4), showed good adhesion of latex beads. However, the mechanical stability of the resulting bead monolayer on the ISFET was poor, as became dramatically clear when these bead-covered ISFETs were placed in the wall-jet cell of the ion stepping set-up. ISFETs coated with larger PLLs (such as PLL22 or PLL37) showed good adherence of beads and also good mechanical stability of the bead monolayer structure: multiple ion stepping (20–40 measurements per experiment) did not lead to lesions in the monolayer coverage. The PLL-concentration of the precoating solution must be chosen ≥ 0.05 mg/ml (in PBS; 10 μl per device) with incubation times of 2 min or longer. After PLL precoating and washing of the ISFET, 2 μl of a suspension of Amino beads (10% in demineralized water) was applied onto the ISFET gate.

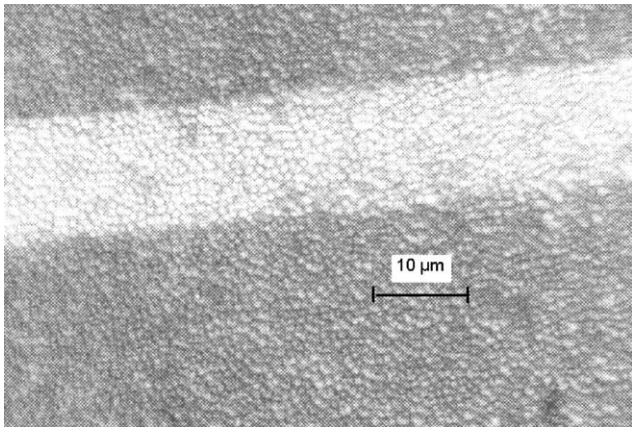

Fig. 1. Photomicrograph of part of an Amino bead-covered ISFET surface (magnification 320 ×; the light-colored structure is the gate area). **Note**: no sharp image could be obtained at this magnification and with this kind of preparation because depth of field was insufficient.

3.2. Modification of Amino bead-covered ISFETs with protein

Bead-covered ISFETs can be used for covalent coupling of peptides and proteins as was exemplified with the coupling of polylysine. Polylysine was used because success and magnitude of ISFET-modification with polylysine can be assessed easily with the ion step method. This is because the ion step response will change consequently to immobilization of positive charge, originating from the protonated side chains of immobilized polylysine.

Polylysines exhibit a high binding tendency toward the surface of Amino beads. Bead-covered ISFETs that were incubated with polylysine showed a markedly different ion step response compared with the response before incubation (compare curves 1 and 4 in Fig. 2).

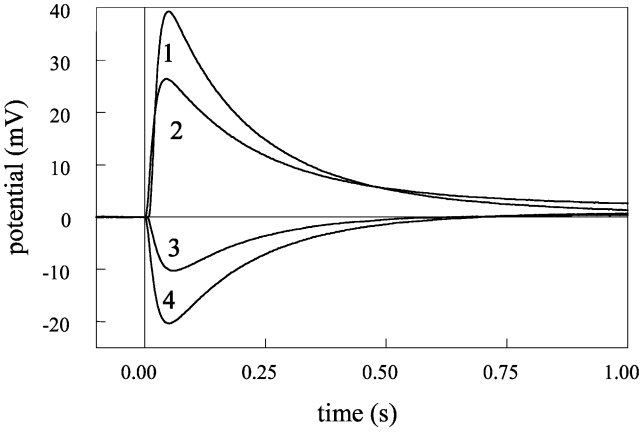

Fig. 2. Ion step responses of Amino bead-covered ISFETs before (curve 1) and after incubation with PLL (curve 4) and washing with Tween® 20 solution (curve 2, non-activated; and curve 3, GA preactivated). Ion stepping was performed at pH 7.42.

Adsorption of PLL to amino beads proceeded very rapidly. At 100 μg/ml of PLL 37, adsorption was completed within 10 s of incubation (using static conditions, no stirring) while at 25 μg/ml of the polylysine, saturation was reached after about 1 min. Physically adsorbed polylysine was desorbed from the surface of amino beads in a quantitative way by washing with Tween® 20, as deduced from the near-complete reversal to the starting situation (curve 2 in Fig. 2), indicating the non-covalent nature of the attachment of PLL to its substrate. However, the Amino bead surface contains primary amino groups and this functionality can be activated by treatment with bifunctional cross linkers such as glutaraldehyde (GA). Amino bead-covered ISFETs activated with GA before PLL addition, showed a different behavior compared with non-activated devices: bound PLL was largely retained, despite the washing with Tween® 20 (curve 3 in Fig. 2). This observation strongly indicates the covalent nature of the attachment of PLL to GA-treated Amino bead-covered ISFETs. It should be emphasized that incubation of GA-activated bead-covered ISFETs with PLL must be followed by a reaction with $NaBH_3CN$ in order to stabilize the newly formed bonds between polyamine and the Amino bead substrate. If the reaction with $NaBH_3CN$ was omitted, the covalent anchoring of PLL was substantially less as shown by the reversal of the curve to positive values (results not shown). It is well known that reaction of glutaraldehyde with primary amines gives rise to so-called Shiff's bases, which are hydrolytically labile but can be rendered more stable by reduction with $NaBH_3CN$.

3.3. Anti-HSA binding to HSA-coated Amino bead-covered ISFETs

Incubation of ISFETs with polylysine is a feasible way for proving success of ISFET modification by observing the change in response amplitude. However, polylysine is not a very useful ligand for immunosensing purposes. Therefore, HSA was immobilized as ligand and qualitative detection of α-HSA antibodies was attempted for assessing ImmunoFET feasibility. In first instance HSA was immobilized by physical adsorption only, i.e. no preactivation of Amino beads was carried out. ISFETs coated with HSA showed a changed ion-step response upon incubation with Rabbit α-HSA antiserum (Fig. 3; Table 1A) and this effect was very specific as incubation with Rabbit α-(**bovine** serum albumin) (BSA) antiserum (Fig. 3) and Rabbit α-(human **fibrinogen**) antiserum (Table 1A) did not appear to have effect. Maximal binding of α-HSA to HSA-coated ISFET was attained within about 15 min (data not shown).

Ion stepping was performed at various pH values in order to check the optimal ion stepping pH for α-HSA detection. The maximal change in amplitude was found to be about 16–17 mV, at an ion stepping pH of 4 (Table 1B). From the results it also appeared that (dimethylpimelimidate) cross linking of HSA/α-HSA complex, before ion stepping at pH 4.0, led to a substantially higher signal compared with the case when dimethylpimelimidate was not used (Table 1, compare treatments II and III). The formation and sustaining of the antibody/antigen complex is pH-sensitive. Crosslinking with dimethylpimelimidate is one way to prevent complex break-up when measurement has to take place at more extreme pH values.

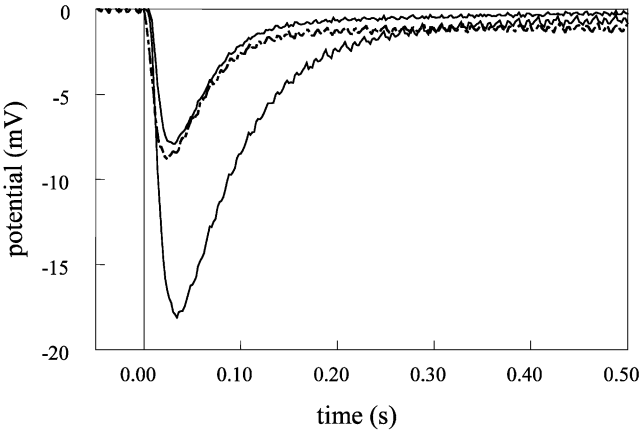

Fig. 3. Ion step responses of HSA-coated ISFET before (upper solid curve) and after incubation (for 15 min) with undiluted α-HSA (lower solid curve) and α-BSA (dashed curve). Ion stepping was performed at pH 4.02. Further details are stated in Sections 2.1 and 2.2.

Preactivation of amino beads with cross linkers such as GA, dimethylpimelimidate, dimethyladipimidate or disuccinimidylglutarate, before addition of HSA, did not lead to better results concerning ultimate detection of α-HSA as compared with physical adsorption alone (illustrated in Table 1B for GA only: compare treatments III and IV).

4. Discussion

Former research in our group has resulted in a laboratory set-up for an ISFET-based biosensor, in which the so-called ion-step method is used (Besselink and Bergveld, 1998; Van Kerkhof et al., 1994). Advantage can be made of the small planar performance of the ISFET and the fast response to local pH changes. In the past, ISFETs were used as heparin sensor or protein sensor after covering the device with a polystyrene/agarose membrane with a thickness of more than 8 μm. Protein was immobilized to the outer surface of the constituent latex beads by passive adsorption. The disadvantage of this 'thick membrane approach' is the need for relatively long incubation times (during modification and also during detection). In this report, a new

Table 1
Effect of incubation with antisera on ISFET response amplitude

Treatment	pH	A, before	A, +αFg	A, +αHSA	ΔA
(*A*)					
I (10)	4.02	−8.63±2.82	−8.78±2.63	-	−0.15
II (10)	4.02	−8.35±3.73	–	−18.13±2.82	−9.78

Treatment	pH	A, before	A, after	ΔA
(*B*)				
III	4.02	−4.78±1.42 (9)	−21.52±1.33 (7)	−16.74
III	7.42	25.76±2.35 (9)	19.67±1.45 (7)	−6.09
III	10.80	N.D.	21.44 ± 0.98 (7)	N.D.
IV	4.02	−8.71±2.23 (9)	−22.98±1.10 (9)	−14.27
IV	7.42	21.16±3.93 (9)	14.97±3.09 (9)	−6.19
IV	10.80	11.85±2.32 (9)	23.69±2.49 (9)	+11.84

Ion step response amplitudes (A; in mV) determined on HSA-treated Amino bead-covered ISFETs (mean±S.D., number of ISFETs per series indicated in parentheses) before and after incubation with antisera. Also indicated is the differential amplitude (ΔA), i.e. the difference in amplitude after and before the incubation with antiserum. In case of treatments I–III no preactivation was included while for treatment IV preactivation was carried out with glutaraldehyde. Incubation with antiserum (diluted 1:3 with PBS) was performed for 15 min, and was followed by crosslinking with dimethylpimelimidate for treatments III and IV but not for treatments I and II.

approach is introduced namely the covering of ISFETs with a monolayer of Amino beads (∅ 0.9 μm). Subsequently, these bead-covered ISFETs were used as a solid phase for immobilization of antibodies or antigens. These procedures were tried out as part of a generic method for obtaining immunoFETs. One obvious advantage of this procedure is the relatively short assay time (15 min or less) needed for maximal detection of the 150 kDa antibody analyte, to be compared with incubation times of 1–3 h needed to attain full saturation of the thick polystyrene/agarose membrane with the 15 kDa heparin analyte (Van Kerkhof et al., 1993). Another strong quality of the system, described in this report, is the high specificity of the antibody determination by HSA-coated Amino bead-covered ISFETs even when undiluted serum is used as matrix. The pH at which ion stepping was performed was expected to be a very determining parameter concerning the magnitude of the differential response attributed to immobilized antibody as the former influences the protein charge density. At an ion-stepping pH < 5 notably the protein-derived carboxylate groups contribute to ion-step-induced proton release (Eijkel et al., 1997a). This information might explain the relatively poor signal with ion stepping at pH 7.4 and the better signal when ion stepping was performed at pH 4.0. The maximal differential amplitude found after incubation of HSA-coated Amino bead-covered ISFETs with α-HSA antiserum was about 17 mV. The differential amplitudes found after binding of polylysine to bead-covered ISFETs were much larger (up to 60 mV). This difference is not surprising as polylysine is a synthetic protein that possesses a much higher number of protonable groups compared with antibodies and other naturally occurring proteins.

The reason that we tried to develop an immobilization method other than physical adsorption is twofold:

1) Application of modified ISFETs in a (jet) flow measurement set-up and long-term liquid storage of modified devices may result in leakage of ligand. Covalent immobilization of ligand protein has to prevent this.
2) We want to develop a more generic immobilization technology that can be applied for immobilizing ligand proteins that do not adsorb spontaneously or that lose biospecific activity upon passive adsorption.

Our ImmunoFET approach is very suited for detection of polyclonal antibodies. The affinity couple described in this report consisted of HSA/α-HSA wherein the antigen HSA was immobilized to the Amino bead-covered ISFETs and the α-HSA antibody was used as sample. This system clearly has advantages over a couple consisting of immobilized antibody and antigen-containing sample which can be reasoned as follows: each antibody molecule possesses two antigen-binding sites and the orientation of the immobilized antibody with respect to the underlying surface determines if the antigen-binding sites are accessible toward antigen approaching from the medium. In case of improper orientation, immobilized antibody cannot bind antigen or can bind antigen with difficulty only, especially in the case of larger antigens. Immobilized antigen combined with polyclonal serum does not have that disadvantage. Each antigen molecule carries multiple antigenic determinants to which an array of antibodies, having different specificities, can bind. Even when a substantial number of antigenic determinants are not approachable

from the medium, due to the immobilization of the antigen, sufficient determinants will remain for interaction with antibodies that do have the appropriate specificity. Severe protein denaturation, evoked by the substrate, may also impair the interaction between antibody and antigen. HSA is known to be a rather sturdy type of protein that is not very susceptible to denaturation but other proteins may behave differently. Nevertheless, based on the results described in this report, despite the theoretical disadvantages of the method, physical adsorption should not be ruled out as choice of method for immobilization of antigen proteins.

In this report, bead technology has been described for coating the ISFET with a solid phase for immunochemical reaction. Specific detection of α-HSA has proven to be feasible in a qualitative way. Using the ion-step approach, potentiometric devices might be realized that can be applied in immunosensing. Although the technology described in this paper is promising, the current technology is not yet robust, reliable and sensitive enough for a breakthrough in the market especially in terms of liquid handling. The technology, therefore, demands better controlled micro fluidics. For the future, the new lab-on-a-chip technology with injection of the higher salt concentration and transport to the detection area, including the construction of micro fabricated channels for transportation of sample and regeneration liquids, is expected to improve the performance of the system significantly.

Acknowledgements

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