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Potentiometric enzyme immunoassay using miniaturized anion-selective electrodes for detection

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Abstract

An enzyme-linked immunosorbent assay (ELISA) for prostate specific antigen (PSA) detection in human serum was developed based on the potentiometric detection of 6,8-difluoro-4-methylumbelliferone (DiFMU). The assays were carried out in anti-human PSA capture-antibody modified microtiter plates (150 μ l volume). After incubation in the PSA containing serum samples, β -galactosidase-labeled PSA tracer antibody was added. The β -galactosidase label catalyzed the hydrolysis of 6,8-difluoro-4-methylumbelliferyl- β -D-galactopyranoside (DiFMUG) and the resulting DiFMU[−] anion was detected by potentiometric microelectrodes with anion-exchanger membrane. The selectivity of the anion-exchanger electrode is governed by the lipophilicity of the anions in the sample. Since DiFMU[−] is much more lipophilic ($\log P = 1.83$) than any of the inorganic anions normally present in the working buffers and occurs in its anionic form at the physiological pH ($pK_a = 4.19$), it was chosen as the species to be detected. The potentiometric ELISA-based method detects PSA in serum with a linear concentration range of 0.1–50 ng/mL. These results confirm the applicability of potentiometric detection in diagnostic PSA assays. Owing to simple methodology and low cost, potentiometric immunoassays seem to offer a feasible alternative to the development of *in vitro* diagnostic platforms.

Introduction

The introduction of enzyme immunoassays in the early 1970s^{1,2} made possible to move immunodiagnostics from specialized radioisotope laboratories to general chemistry laboratories to be applied also by unspecialized users and in field applications. Various detection methodologies were implemented to match requirements of sensitivity, selectivity, and massively parallel determinations. Optical methods are clearly dominating the field, however, electrochemical detection³ has also been shown to provide benefits in terms of increased sensitivity,^{4–6} cost-effective mass production, and miniaturization. Very early, extremely sensitive voltammetric techniques were shown to be the electrochemical methods of choice in enzyme immunoassays. Still, some of the inherent characteristics of voltammetric sensing such as limited selectivity, susceptibility of the electrode to fouling and, unless ultramicroelectrodes are used, strong dependence of the current signal on mass transport conditions, render difficult its use in conventional setups. While most of these problems were solved using a variety of innovative approaches,⁷ the implementation of more robust detection

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methodologies would have its niche among immunosensors especially in field applications. Ion-selective potentiometry is not affected by the above-mentioned issues and provides an even simpler measurement method with widely available instrumentation and well-established techniques for miniaturization and low-cost fabrication.⁸ The latter advantages are well documented by the extensive use of ion-selective sensors in commercial point-of-care and automatic blood-gas analyzers. These electrolyte analyzers are based on potentiometric detection, hence, their compatibility with potentiometric immunoassays could lead to more versatile *in vitro* diagnostic instruments. Therefore, the limited number of potentiometric immunoassays reported up to now is rather surprising, although research in this field started in the 1970ies already, with pioneering contributions by the group of Rechnitz.^{9–11} Early efforts were mainly focused on using Severinghouse-type potentiometric gas sensors such as ammonia or CO₂ electrodes in conjunction with urease-,^{12,13} asparaginase-,¹³ adenosine deaminase-,^{13,14} and chloroperoxidase-labeled¹⁵ immunoreagents. Another research direction aimed at developing enzymatic reaction schemes and generating ions detectable with the most established all-solid-state ion sensors such as iodide-¹⁶ and fluoride-¹⁷ selective electrodes. Horseradish peroxidase (HRP) used as a label of immunoreagents was shown to catalyze the oxidation of iodide to iodine¹⁸ and the rupture of C–F bonds while, more recently, alkaline phosphatase (ALP) was reported to catalyze the hydrolysis of C–P bonds,¹⁹ inducing changes in the concentration of the relevant anions, I[–] and F[–].

The direct potentiometric detection of a participant in the immunoreaction was realized in the so-called ionophore modulation immunoassay²⁰ and by using polycation-selective electrodes for competitive homogeneous assays.²¹ In the first case,²⁰ a K⁺-selective ionophore was covalently linked to the steroidal cardiac drug digoxin, and the presence of digoxin antibodies in the sample was found to alter the emf of the electrode. In the second study,²¹ the potentiometric response of a synthetic polycation–analyte substrate is suppressed when binding to the antibody occurs. Further variations of direct potentiometric detection were proposed but the commercial unavailability of the reagents, and in some cases, the unidentified response mechanism²² could impose limitations to their applicability.

Recent improvements in the lower detection limits of polymer membrane electrodes allowing the measurement of subfemtomole amounts of analyte²³ motivated to revisit potentiometry as a detection method in immunoassays. Thus, a miniaturized Ag⁺-selective electrode was successfully used as a transducer for sandwich immunoassays in connection with the capture and silver enhancement of gold nanoparticle tracers.²⁴ The silver ions released by the oxidative dissolution of silver were detected by a Ag⁺-selective electrode in close analogy to a previously proposed voltammetric assay.²⁵ Following the same principle, immunoassays were built on the detection of Cd²⁺ released from CdSe quantum-dot-labeled tracer antibodies.²⁶ In these studies, it became apparent that to detect cations in bioassays might be rendered difficult owing to their contingent adsorption and/or complexation by biomolecules decreasing their activity. This might be one of the reasons why even in simple model systems only ppm detection limits could be achieved for mouse IgG detection. Therefore, we here explore the feasibility of designing enzymatic schemes for ELISAs based on the generation and detection of organic anions. In general, ionophores for anions have much poorer selectivity than those for cations which, at first sight, may constitute a serious impediment in developing potentiometric transducers based on anion detection. However, if sufficiently lipophilic anions are generated by an enzymatic reaction, a simple anion-exchanger electrode could be used for their detection. This approach is beneficial also in terms of fabrication costs since it eliminates the need for an expensive ionophore. According to our knowledge, the only use of ion-exchanger electrodes (albeit for organic cations) as transducers have been reported by Umezawa's group.²⁷ In their elegant approach, limited exclusively to lipid antigens, tetrapropylammonium ions (TPA⁺) were trapped in the aqueous inner reservoir of liposomes partly constructed from the lipid

antigen. Upon immune lysis by interaction with the specific antibody, the released TPA^+ was detected by a cation-exchanger electrode.

It seems that till now little follow-up work was done on most of the early potentiometric immunoassay approaches, which might be due, partly, to the limited availability of commercial reagents needed for unconventional ELISA systems as well as to the poor detection limit and dynamic range of the early potentiometric ion or gas sensors. Therefore, we have focused our work on the three enzyme labels widely used in ELISA, i.e., alkaline phosphatase, horseradish peroxidase, and galactosidase and commercially available substrates. The feasibility of the potentiometric detection and its optimization was demonstrated by determining the prostate specific antigen (PSA) from serum samples.

Experimental

Reagents

The membrane components and solvents were tridodecylmethylammonium nitrate (TDMA- NO_3), 2-nitrophenyl octyl ether (*o*NPOE), bis(2-ethylhexyl) sebacate (DOS), poly(vinyl chloride) (PVC), and tetrahydrofuran (THF) of Selectophore[®] grade from Fluka (Buchs, Switzerland). SuperBlock (TBS) Blocking Buffer Dry Blend, Protein-Free (TBS) Blocking Buffer, and neutravidin[®] were obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). 6,8-difluoro-4-methylumbelliferyl- β -D-galactopyranoside (DiFMUG), 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU) from Invitrogen (Carlsbad, CA, USA), and 4-nitrophenyl- β -D-galactopyranoside (pNPG) from Sigma-Aldrich Inc. (St. Louis, MO, USA). The capture (anti-human PSA SPRN-1) and tracer (anti-human PSA SPRN-5) antibodies (Ab) were purchased from Medix Biochemica (Kauniainen, Finland). The latter was biotinylated by the Institute of Isotopes Co., Ltd. (Budapest, Hungary), which also provided the prostate-specific antigen (PSA) standards, controls, serum samples, and washing solutions. Galactosidase conjugates, β -galactosidase-biotin (GAL-biot) labeled from *Escherichia coli* and streptavidin-galactosidase (Str-GAL) were from Sigma and Thermo Fisher Scientific, respectively.

Electrodes and potentiometric setup

The typical composition of ion-selective membranes used in this study was PVC (40 wt.%), plasticizer (60 wt.%), and TDMA- NO_3 (10 mmol/kg). After dissolving all components in THF, master membranes were cast in glass rings (30 mm). Miniaturized electrodes were fabricated using 10- μL pipette tips. To improve the adhesion of the ion-selective membrane to the inner pipette wall, the pipette tips were first cleaned by repeatedly dipping them into THF and draining out the solvent.

Membrane segments were cut out from the master membrane and dissolved in THF (1:13, w/w). The pipette tips were dipped into this membrane solution until the column of the liquid reached 4–5 mm. Then, the pipettes were placed in a holder and the THF was left to evaporate overnight yielding solvent polymeric membranes of ca. 300–500 μm thickness situated at the very end of the pipette tip. The pipette was filled with a solution containing 1 mM phosphate buffer (PB), 1 mM MgSO_4 , 10^{-2} M KCl, and 10^{-5} M DiFMU. An inner reference electrode was also prepared from a 10- μL pipette tip whose narrower opening was obstructed by a porous plug made from a hydrophobic polypropylene membrane (Celgard[®]). After filling it with 10 mM KCl and inserting a Ag/AgCl wire, the other opening of the pipette was sealed with Parafilm[®]. This pipette tip was then placed into the pipette tip containing the sensing membrane and secured with Parafilm[®]. A small orifice was made in the side of the latter tip to avoid overpressure during the assembly, which could damage the ion-selective membrane. To complete the emf cell, a miniature reference electrode was fabricated exactly as described

above for the inner reference electrode. The potentiometric response was measured with a high-input impedance ($10^{15} \Omega$) 16-channel pH meter (Lawson Labs, Inc., Malvern, PA, USA). Calibrations were made using 150- μ L samples confined to the wells of a microtiter plate. A special electrode holder was designed to accommodate the reference electrode and concentrically up to five ion sensors, which could be used simultaneously in a single well (Figure 1). Since DiFMU concentrations throughout this study never exceeded 1 mM, the ionic strength of all solutions being < 10 mM, all calculations were done using concentrations instead of activities.

Immunoassays

Sandwich immunoassays were performed on commercial ELISA plates (Immuno Module F8 Maxisorp Loose, No. 469949, Nunc, Roskilde, Denmark) at room temperature. All solutions were prepared with the working buffer, i.e., 1 mM PB containing 1 mM MgSO_4 at pH 7.7. First, the microtiter plates were modified with the capture antibody: 100 μ L of capture anti-human PSA antibody (5 μ g/mL) in the working buffer was placed in the polystyrene microwells, which were then sealed and incubated at 4 $^{\circ}\text{C}$ overnight. The wells were rinsed with 400- μ L aliquots of washing buffer three times, whereupon the surface was blocked using 400 μ L of blocking buffer (i.e., either SuperBlock, Protein-Free, or BSA (20 μ g/mL) solution). After drying, the modified plates were stored at 4 $^{\circ}\text{C}$ if not used immediately.

The assay started by adding 100 μ L of different concentrations of human PSA (hPSA) serum solutions into the wells and incubating for 1 h. Following this step, a solution (100 μ L) of biotin-conjugated tracer Ab (5 μ g/mL) in the working buffer was injected and incubated for 1 h. After the formation of the sandwich immunocomplex was completed, a solution (100 μ L) of streptavidin conjugated β -galactosidase (5 μ g/mL) in the working buffer also containing Protein-Free blocking buffer (1%, v/v) was applied for 30 min. After removing this solution and rinsing the wells, freshly prepared 0.5 mM DIFMUG substrate in the working buffer (150 μ L) was added and incubated at room temperature for another 30 min. Finally, the enzymatic reaction was stopped with 2 mM CuSO_4 (15 μ L) and the released DiFMU was detected potentiometrically.

Results and discussion

A reaction scheme showing the formation of organic anions can be designed for each of the most commonly used enzyme labels in immunoassays, i.e., horseradish peroxidase (HRP), alkaline phosphatase (ALP), and galactosidase (GAL). However, the anions should comply with the following criteria for optimal potentiometric practice:

The resulting organic anion should be lipophilic enough to be selectively determined by solvent polymeric anion-exchanger-based electrodes, while its solubility in aqueous solutions and that of the substrate from which it originates, should reach about millimolar concentrations;

the product should be in anionic form, preferably singly charged, at the pH of the measurement, i.e., at the optimal pH of the enzyme catalysis. If a significant fraction of the compound is in neutral form, then, its extraction into the membrane can lead to a non-Nernstian response²⁸ and a poor detection limit. For most of the organic anions, this translates into having a pK_a smaller by at least 2 logarithmic units than the working pH;

for practical usefulness, both the enzyme substrate and the generated organic anion should be commercially available;

both the substrate and the resulting anion should have low toxicity and chemical stability;

the enzyme substrate should provide a high turnover rate;

the organic anion to be detected should preferably be formed directly in a one-step enzymatic reaction.

Typical reaction schemes of the three widely used enzyme labels that generate organic anions are shown in Fig. 2. While HRP provides limited flexibility in terms of anion-generating substrates, there is a plethora of commercially available substrates for ALP and GAL enzymes. Remarkably, the detected products generated by these two hydrolyzing enzymes are practically the same since the artificial substrates differ only in having either an inorganic phosphate group or a β -D-galactopyranoside to be cleaved. The use of ALP was, however, immediately ruled out since the substrate also is an anion and there is little difference in the lipophilicities of the substrate and the product. In addition, the substrate is in large excess with respect to the resulting product. To overcome this problem, alternatively, tris(4-nitrophenyl) phosphate or nitrophenyl phosphates with alkylated, nonionizable phosphate esters could, in principle, be used as substrates. However, while the former compound is unstable at basic pH, the latter compound class comprehends notorious neurotoxins. Therefore, all arguments seem to advocate the use of β -galactosidase conjugates, and this choice is further enforced by the rather good solubility of the galactopyranoside conjugates in aqueous solution. The β -galactosidase is less popular than HRP and ALP for ELISA, mainly due to the much higher molecular weight (464 kDa). However, on the other hand, it has a series of attractive properties such as a high turnover number, good compatibility with an extremely large range of buffers and stabilizers, and unlike other commonly used enzyme labels, it is not present in mammalian tissues.

One disadvantage of using GAL as label is the severe constraint with respect to the pK_a of the generated organic acid. Unlike ALP, which has an optimal pH range of 9–10 where most of the weak acids are ionized, GAL has its pH optimum between 7–8. Therefore, the enzymatically generated product should have a pK_a of < 5–6 and still be lipophilic enough to ensure good selectivity. Screening the many of commercially available artificial substrates based on the above mentioned criteria basically results in only one galactosidase substrate, i.e., 6,8-difluoro-4-methylumbelliferyl- β -D-galactopyranoside (DiFMUG), which yields a hydrolysis product, 6,8-difluoro-7-hydroxy-4-methylcoumarin, having a pK_a of 4.9 and a calculated log P of 1.83. However, it should be mentioned that the criterion regarding the pK_a is of importance in continuous kinetic measurements only. If endpoint detection is pursued, then, a much larger number of substrates become available because the enzymatic reaction can be stopped by injecting highly concentrated NaOH. Thus, the endpoint pH of the solution is ca. 10, which allows the use of, e.g., nitrophenyl derivatives of pK_a around 7.

The multi-step approach used in heterogeneous immunoassays provides practically ideal conditions for the last step involved, which allows the use of simple anion-exchanger-based membranes for potentiometric detection. The only interference to be expected could arise from the inorganic anions of the buffer solution needed to ensure optimal enzyme activity by adjusting the optimal pH and Mg^{2+} level as well to generate the anionic form of DiFMU. Screening the potentiometric selectivity behavior of some of the commonly encountered inorganic anions and other potential organic anions for detection by the separate solution method at 1-mM level demonstrates that DiFMU, indeed, is the best choice. Not only that the selectivity coefficient improved by roughly a half an order of magnitude with respect to the 4-methylumbelliferone anion (MU^-) and *p*-nitrophenolate (PNP^-) but also selectivities exceeding -4 logarithmic units were observed for Cl^- and OH^- (Table 1). Interestingly, little difference was found in the selectivities when using plasticizers of markedly diverse dielectric constants (*o*NPOE and DOS), except OH^- that the *o*NPOE-plasticized membranes exhibited a significantly better selectivity for. A compromise between the selectivity and suitability of the buffer solution in terms of stability and optimal enzyme function resulted in the use of a 1 mM phosphate buffer containing 1 mM $MgSO_4$ at pH 7.7. This solution provided a theoretical

detection limit of 0.1 μM DiFMU, which was confirmed by calibration curves obtained in microtiter plates using sample volumes of 150 μL and a reading time of < 10 min (Fig. 3).

The practically identical calibration curves (data not shown) recorded in conventional sample volumes (5 mL) and in the microtiter plates suggest that no adsorption or contamination of the samples occurs during measurements, which further enforces the choice of using anionic marker species for potentiometric detection. Since during end-point-type immunoassays, DiFMU detections are performed after adding a proper stopping reagent, calibrations were done both in the presence of 1 mM NaOH and 2 mM CuSO_4 . The use of Cu^{2+} , which is a well known inhibitor of the GAL enzyme, proved to be more advantageous in many respects. Its counter anion is better discriminated than OH^- , while the addition of NaOH in certain cases led to sub-Nernstian response slopes. In contrast, the addition of Cu^{2+} resulted in a linear super-Nernstian response for DiFMU, which considerably increased the sensitivity of the determination. At the present stage, the mechanism causing this super-Nernstian response is yet unknown; however, it has proved to be reproducible.

Prostate-specific antigen (PSA) is a 33-kDa single-chain glycoprotein that is released at very low concentrations in the blood of healthy males, where it occurs in both the free and complexed forms. There is some controversy in the clinical literature regarding PSA screening; however, in clinical practice, a biopsy is requested in case of elevated PSA levels (> 3 ng/mL). The determination of human PSA was performed in a sandwich assay with a capture Ab having an affinity constant of 1.0×10^{10} L/mol.²⁹ The PSA bound to the capture Ab was then potentiometrically detected by using successive incubation in biotinylated tracer Ab, streptavidin- β -galactosidase conjugate, and, finally, DiFMUG.

All optimization steps in terms of concentrations of the capture antibody, tracer antibody, and streptavidin-galactosidase conjugate were done by using potentiometric DiFMU detection. The generated anion was detected sequentially after the addition of the stopping reagent in each individual well by 2–5 electrodes placed concentrically around the reference electrode (Fig. 1a, b). Thus, the results plotted in Fig. 4 are average values from a set of electrodes and three replicate measurements in different microwells.

Based on the optimization experiments, the lowest concentration of the bioreagents providing maximal, or close to maximal, response (tracer Ab) was used from here on, i.e., 5 $\mu\text{g/mL}$ for the assay components. The assays were also optimized in terms of reducing nonspecific adsorption, and a number of blocking agents (SuperBlock and Protein-Free blocking buffers as well as 20 μg of BSA/mL in 1 mM PB with 1 mM MgSO_4 , pH 7.7) were screened. The selection criteria involved both minimizing the potential response for the blank sample (0 ng of PSA/mL) and maximizing the potential range of the assay (defined as the potential difference between a sample of 50 ng of PSA/mL and the blank). While in terms of nonspecific adsorption, little difference was observed among the different blockers, the potential range increased spectacularly in the order of Protein-Free Blocking Buffer, SuperBlock Blocking Buffer, and the BSA-containing working buffer. Thus, a two-fold extension of the potential range was gained by switching from Protein-Free Blocking Buffer to the solution of 20 μg of BSA/mL.

The PSA calibrations performed with a human serum background were compared to the conventional optical detection-based ELISA kits using HRP labeling (Fig. 5). In accordance with the logarithmic concentration dependence of the potential, semilog calibration curves proved to be linear in the range of 0.1 to 50 ng of h PSA/mL.

Error propagation calculations (after fitting the experimental data with dose-response function) show that the uncertainty of the optical measurements in terms of concentration in the middle of the measuring range is 28.1 % while the potentiometric assay has a relative error of 8.9 %.

There was no significant difference at 95% confidence level between the results of the two methods for h PSA spiked serum samples.

Conclusions

The PSA ELISAs described here using the potentiometric detection of an enzymatically generated anionic marker have proved to be a viable alternative to those based on optical absorbance. The procedure in the present format cannot compete in terms of analysis time with the highly parallel microtiter plate readers using optical detection. However, it is by far superior to the recently published nanoparticle-based potentiometric immunoassays^{24,26} and also provides a sufficiently low detection limit ($\leq 0.1 \text{ ng mL}^{-1}$), which complies with the requirements of *in vitro* diagnostic PSA assays. It must be noted that at this stage, the detection limit is determined by the selectivity of the ion sensor. Therefore, considerable improvements in the detection are expected if the selectivity of the basic sensors is further enhanced, which is topic of current investigations.

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Fig. 1.
(A) Photograph and (B) scheme of the electrode arrangement that allows simultaneous multielectrode recordings in one microtiter well (ISE: ion-selective electrode).

Fig. 2.
Schemes of enzyme-catalyzed reactions generating ionizable products.

Fig. 3. Calibration curves for DiFMU⁻: (a) in 1 mM PB with 1 mM MgSO₄ at pH 7.7, (b) in the same buffer but with a content of 2 mM CuSO₄.

Fig. 4. Optimization of the amount of (A) capture antibody (50 ng/ml PSA, 5 $\mu\text{g/ml}$ tracer antibody, 20 $\mu\text{g/ml}$ Str-GAL), (B) tracer antibody (5 $\mu\text{g/ml}$ capture antibody, 50 ng/ml PSA, 20 $\mu\text{g/ml}$ Str-GAL), and (C) streptavidin-galactosidase conjugate (5 $\mu\text{g/ml}$ capture antibody, 50 ng/ml PSA, 5 $\mu\text{g/ml}$ tracer antibody) for h PSA detection in potentiometric ELISA format.

Fig. 5. Calibration curves for PSA in human serum background using ELISA assays with (A) optical absorbance and (B) potentiometric detection.

Table 1

Potentiometric selectivity coefficients ($\log K_{ij}^{\text{pot}}$) of various membranes for detecting DiFMU (for acronyms, cf. text).

Plasticizer	DOS	<i>o</i> NPOE	<i>o</i> NPOE
TDMA-NO ₃ , mmol/kg	20	20	10
SO ₄ ²⁻	-4.9	-5.5	-5.9
OH ⁻	-4.0	-5.0	-4.5
Cl ⁻	-3.6	-4.0	-3.9
NO ₃ ⁻	-1.9	-1.8	-1.8
MU ^{-*}	-0.6	-0.6	-0.5
PNP ^{-**}	-0.5	-0.5	-0.5

The potentiometric selectivity coefficients were determined by the separate solution method at 1 mM level. In case of MU and PNP the pH was adjusted with concentrated NaOH (* pH 9.0; **pH 10.0) to ensure the dominance of the anionic form.