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Several approaches for vitamin D determination by surface plasmon resonance and electrochemical affinity biosensors

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ABSTRACT

This work has been focused on the development of novel optical (Surface Plasmon Resonance) and electrochemical based biosensors for the determination of 25-OH vitamin D (25OHD) which is an important factor involved in avoiding both skeletal damage and a variety of pathological conditions, and to evaluate their potential use in clinical practice.

Different approaches to the determination of vitamin D using affinity based biosensors, are described herein; firstly, an immunosensor based on SPR transduction was realized for direct determination of vitamin D, obtaining a LOD of 2 μg/ml which unfortunately is too far from the needs in clinical analysis. In order to enhance the sensitivity, the vitamin D was modified with gold nanoparticles (AuNPs): the binding of 25OHD with AuNPs determines the amplification of SPR signal, allowing to lower the LOD down to 1 μg/ml, doubling the sensitivity. An alternative SPR method, based on the indirect determination of vitamin D by means of Vitamin D Binding Protein (VDBP), led to a further sensitivity increase reaching a LOD of 45 ng/ml which is really close to the fixed accomplishment.

Finally, an electrochemical transduced biosensor has been realized, based on the reaction of vitamin D with 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione (FMTAD): once derivatized, the determination of 25OHD was possible in the range 20–200 ng/ml with a LOD of 10 ng/ml. The latter proposed system fits the requirement of determining vitamin D in a concentration range which is of significance for clinical applications; moreover, since a screen printed electrode has been used, this opens the possibility to miniaturize the sensor and developing a portable and easy-to-automate point-of-care testing device. The proposed devices provide an improvement with respect to traditional methods that are time and reagents consuming and require radioactive compounds, pretreatment procedures and expensive instrumentation.

1. Introduction

The vitamin D deficiency is a condition of great significance from health point of view; although it is widely diffused among the populations of different countries of the world, this problem is commonly unknown (Holick, 2007; Dusso et al., 2005; Malabanan et al., 1998; Chapuy et al., 1997; Holick et al., 2005). Several clinical and experimental data show that vitamin D is an important factor involved in avoiding both skeletal damage and a variety of pathological conditions (Lips, 2001; Holick, 2006): in this sense, hypovitaminosis D is now considered a key element in the pathogenesis of many multifactor diseases (Holick, 2006; Bischoff-Ferrari et al., 2006; Schwalfenberg, 2007).

On the basis of clinical studies it can be stated that values of 25OHD between 20 and 32 ng/ml (50–80 nmol/l) express a relative vitamin insufficiency (Heaney et al., 2003). On the other hand, levels greater than or equal to 32 ng/ml (80 nmol/l) correspond to a state of sufficiency, since at that concentration the intestinal absorption of calcium is optimal and the incidence of fractures is low (Dawson-Hughes et al., 2005).

Although the dosage of vitamin D is widespread today, most likely related to a better understanding of the problems associated with the deficiency of this vitamin, a method that fits optimally and precisely the clinical needs has not yet found. Conversely, the results obtained by the methods of determination used in laboratories are often unreliable, often resulting in

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a fallacious classification of the vitamin D status (Singh, 2008). Three methods are currently used for the determination of vitamin D: HPLC-MS, radioimmunoassay (RIA) and non-isotopic automated determination (chemiluminescence) (Jafri et al., 2011; Snellman et al., 2010).

The most used method in clinical laboratories is the RIA assay, which is very specific because of the use of direct antibody against the vitamin D, but has weaknesses as the failure to distinguish between forms of vitamin which must be determined, the presence of radioactive material which involves problems of storage and waste disposal and a high cost for the analysis; the HPLC-MS method, instead, is the reference method (Roth et al., 2008; Lensmeyer et al., 2006) being the one with the highest sensitivity, really it is able to reach the determination of concentrations of the order of femtomols and it is the only method able to discriminate between the two forms of vitamin (Adamec et al., 2011; Netzel et al., 2011; Stepman et al., 2011), however, the required high skill and the length of analysis time, together with very high cost of instrumentation, make it unsuitable for routine use. Finally, the chemiluminescent method although it is very useful in clinical practice having as points of strength the ability to analyze the sample without prior extraction and the easy automation of the continuous analysis through the use of an autosampler, it has the main drawback of not detecting vitamin D_2 which is still used in clinical practice as vitamin D supplementation; in addition, the interference with the lipids, particularly cholesterol, and bilirubin can lead to false results.

In this work, an approach based on the biosensor's technology has been attempted to the fast, simple and cheap determination of vitamin D that is, to the best of our knowledge, the first example of vitamin D determination by means of a biosensor so far. To this end, two different transduction methods have been employed: firstly, an immunosensor based on Surface Plasmon Resonance (SPR) transduction was realized for either direct determination of 25-hydroxyvitamin D (25OHD) using vitamin D antibody (Ab-25OHD) as biological component or indirect determination of vitamin D by means of Vitamin D Binding Protein (VDBP). Secondly, also in the direction of developing a miniaturized biosensor for "in situ" analysis, a gold screen printed electrode (SPE) modified immunosensor based on 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione (FMTAD), was developed: this custom made compound is able to bind 25OHD through a Diels–Alder reaction between the conjugated diene of vitamin and triazoline-3,5-dionic group of FMTAD acting as dienophile thus allowing a voltammetric transduction thanks to the properties of electroactive ferrocene group.

2. Materials and Methods

2.1. Materials and apparatuses

The pure standards ($\geq$98% by HPLC) of 25-hydroxyvitamin D_3 (25OHD), O-(2-aminoethyl)-O′-(2-maleimidoetil) ethylene glycol trifluoroacetate (AMEG) ($\geq$90% by HPLC), 11-mercaptoundecanoic acid (MUA) (95%), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) (commercial grade), N-hydroxysuccinimide (NHS) (98%), tetrachloroauric acid (99.999%), were purchased from Sigma-Aldrich (St. Louis, MO, USA). The monoclonal antibody anti-25OHD (expressed in mouse) and the Vitamin D Binding Protein (95%) SDS-PAGE purified were purchased from Gentaur (Brussels, Belgium). All other reagents were of analytical grade.

The standard solutions of 25OHD were prepared in ethanol $\geq$99.8% from Fluka (Seelze, Germany). All solutions were prepared using ultrapure deionized water (resistance: 18.2 MOhm cm at 25 °C; TOC $<$10 g/ml) obtained from an instrument Direct-Q UV3 Millipore (France).

The gold disks SensorDisc Au bare gold for SPR analysis were purchased from Xantec Byoanalitics (Duesseldorf, Germany), the screen printed electrodes (SPE) Au 220BT (Au working electrode and counter electrode, Ag/AgCl reference electrode) for electrochemical analysis were purchased from Dropsens (Oviedo, Spain). The SPR measurements were performed by Autolab Springle SPR of EcoChemie (Utrecht, The Netherlands). The electrochemical analysis were performed using a potentiostat μ-Autolab type III Metrohm (Herisau, Switzerland), interfaced to a personal computer running GPES manager software (version 4.9, Metrohm) for both instrument drive and data collection and elaboration.

2.2. SPR measurements

The planar gold SPR disks were extensively cleaned in a freshly prepared piranha solution (3:1 H_2SO_4 98%:H_2O_2 30%). After 1 h, the disks were thoroughly rinsed with water, dried in a stream of nitrogen gas and immediately incubated overnight in a 2 mM of 11-mercaptoundecanoic acid (MUA) in ethanol. After self-assembled monolayer (SAM) formation, the disks were washed with ethanol and water and dried with nitrogen gas. The carboxyl functions on the SAM layer were activated with a mixture containing 0.5 mM N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) and 0.1 mM N-hydroxysuccinimide (NHS) in water. After removing the EDC/NHS mixture and rinsing the disk, acetate buffer containing a solution of 25OHD antibody 50 μg/ml was pumped over the sensors surface for 20 min to achieve a covalent cross-linking by amino reactive groups of antibodies with the aldehyde terminals, followed by a deactivation step of non-reacted activated groups with ethanolamine to reduce the non-specific adsorption and a treatment with 10 mM glycine pH 2.5 to eliminate non specifically adsorbed antibody molecules (Frasconi et al., 2009). Alternatively, when the sensing surface was modified with 25OHD instead of Ab–25OHD, the above described procedure was slightly modified as following: after activation of the surface with EDC/NHS mixture and rinsing the disk, acetate buffer containing a solution of AMEG 0.52 mM was flowed over the sensors surface for 25 min to achieve a covalent cross-linking by amino reactive groups of AMEG with the aldehyde terminals. After successive deactivation with ethanolamine, the surface modification was completed by immobilizing 25OHD: this was achieved by adding a solution of 25OHD (1.4 mM in acetate buffer) that links to the surface thanks to a Diels–Alder reaction with AMEG.

For the measurements carried out in presence of gold nanoparticles (AuNPs), thiol-shelled AuNPs were synthesized by a modification of the Brust–Schiffrin method (Frasconi et al., 2010) then functionalized with 25OHD as described in detail in Appendix A.

Experimental measurements were carried out using 0.01 M phosphate-buffered saline (PBS) pH 7.4 with 0.1 M NaCl as coupling buffer; the regeneration of the surface was obtained by injection of a pH 2.5 solution of 0.5 M NaCl.

2.3. Electrochemical measurements

The synthesis of 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione (FMTAD) was carried out by a modification of the method proposed by Shimizu et al. (1991), which allows to synthesize the FMTAD with a three-step synthesis starting from ferrocene acetic acid (see Appendix A).

The electrochemical biosensor was prepared by a procedure similar to that one used for the optical biosensor with SPR; briefly, the gold surface of the SPE has been functionalized by means of a SAM of mercaptopropionic acid (MPA), obtained by immersion for 12 h in a 2 mM thiol solution in ethanol. The carboxylic groups

were activated with a EDC/NHS mixture deposited on the electrode surface, followed by 20 μl of a solution 0.03 mg/ml of Ab–25OHD in 0.1 M acetate buffer at pH 5.5.

The samples of the adduct 25OHD–FMTAD were prepared by previous incubation of 5 μl of the 25OHD standard 1 mg/ml with 10 μl of FMTAD 5 mM in THF for 30 min and subsequent dilution in PBS 0.01 M pH 7.4 up to the final volume of 1 ml to obtain the mother solution, different aliquots of which was further diluted with the same PBS to obtain solutions in the range 10–200 ng/ml. In order to prevent possible artifacts, the contribution to signal due to nonspecific adsorption of unreacted FMTAD was checked by performing electrochemical measurements for increasing concentrations of FMTAD in absence of 25OHD: no redox signal was detected for FMTAD concentration down to 0.065 mM.

The electrochemical measurements were carried out in PBS 0.01 M pH 7.4 by means of either CV or DPV after having allowed the 25OHD–FMTAD adduct to react for 30 min with the immobilized antibody.

3. Results and discussion

Three different approaches have been considered: (i) the direct detection of 25OHD (either free or bound to AuNP) by SPR transduction using an Ab–25OHD modified surface; (ii) the indirect detection of 25OHD employing a competition analysis by SPR transduction using VDBP in solution and 25OHD-modified sensor surface; (iii) the detection of FMTAD-derivatized 25OHD by electrochemical transduction using an Ab–25OHD modified surface.

3.1. SPR-based immunosensor for direct detection of 25OHD

The first adopted experimental approach was to realize an optical immunosensor for the direct determination of 25OHD by employing SPR as transducer, based on the antigen–antibody interaction, using the specific antibody for 25OHD (Ab–25OHD). The immunosensor thus obtained was tested for the direct determination of different 25OHD concentrations: as showed in Fig. 1, typical association and dissociation SPR curves were registered for different 25OHD concentrations (solid lines) demonstrating the ability of the sensor to bind the 25OHD in the decade 5–50 μg/ml with a sensitivity of 2.8 m°ml/μg (see inset of Fig. 1); despite the obtained results are very interesting since this represent one of the first approach to detect Vitamin D via a direct SPR biosensor, however the analytical performances are too poor for proposing the method as is in clinical application. In fact a LOD of approx. 2 μg/ml was detected, which is too high to apply the method in the plasma determination of 25OHD concentration (reference values are of the order of ng/ml (Dawson-Hughes et al., 2005)). A possible explanation of such high value found for the detection limit can be attributed to the low molecular weight of 25OHD: in fact, in the absence of any improvement, the SPR technique is limited in determining low molecular weight compounds, generating low SPR signal and poor sensitivity (Mitchell, 2010). With the aim to overcome this limitation of the transducer, 25OHD was functionalized with suitable particles in order to increase its molecular weight hence amplifying the produced signal, thereby decreasing the LOD: to this purpose gold nanoparticles (AuNPs) have been used, whose physico-chemical characteristics have often proved to be particularly useful in enhancing signal of SPR-based biosensors (Frasconi et al., 2010). To this end thiol-shelled gold nanoparticles were synthesized by a modification of Brust-Schiffrin method then functionalized with 25OHD as described in detail in Appendix A.

In Fig. 1 it can be observed that the binding of AuNPs to 25OHD causes an amplification of the SPR signal (dashed line (c′)) with respect to the signal detected in absence of AuNPs for the same 25OHD concentration (solid line (c)): the improvement is due, at the same time, to an increase of the mass and to the coupling of the carrier wave on the gold surface plasmon sensor with that of the plasmonic effect of AuNPs as extensively documented in literature (Daniel and Astruc, 2004; Sardar et al., 2009). The SPR signal obtained for several 25OHD concentrations after AuNPs

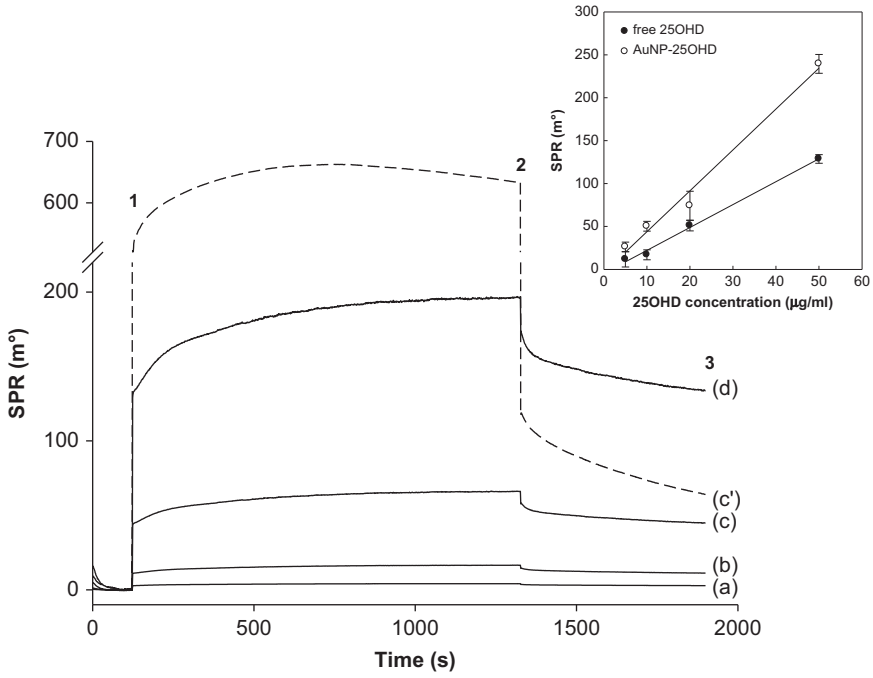


Fig. 1. Sensorgrams of Ab–25OHD modified surface in 0.01 M PBS pH 7.4 with 0.1 M NaCl, in presence of different concentrations of 25OHD either free in solution (solid lines) or bound to AuNP (dashed line); curves (a), (b), (c and c′) and (d) refer to different 25OHD concentration values: 5, 10, 20 and 50 μg/ml, respectively. Inset: calibration plot of SPR signal as a function of 25OHD adduct concentration either free in solution (black dots) or bound to AuNP (white dots) in the linearity range (5–50 μg/ml).

functionalization, demonstrate a significant improvement of either sensitivity and LOD: really, as it can be observed in the inset of Fig. 1, the obtained sensitivity (4.8 m°ml/μg) is about twice the sensitivity registered in absence of AuNPs, and the LOD is lowered to 1 μg/ml which is still far to the range requested for the application to real sample analysis. Nevertheless, as shown in Fig. 1, although the AuNPs functionalization of 25OHD determines a very high SPR signal variation during the association phase (step 1–2), a rapid signal decrease is observed during the dissociation (step 2–3): this leads to poor analytical performances of the method since the significant signal related to the 25OHD concentration is taken as the difference of steady state signal of step 2–3 with respect to baseline. Hence, while the huge signal variation of step 1–2 is caused by the perturbation of bulk solution composition mainly due to presence of AuNPs, the small difference between steady state signal after dissociation of step 3–4 and the baseline can be attributed to the amount of functionalized 25OHD that binds to the surface, that is less than expected. A reasonable explanation can be found taking into account the steric limitations: in fact the very large size of AuNPs compared to the small size of 25OHD do not favor the binding between the vitamin D and its antibody.

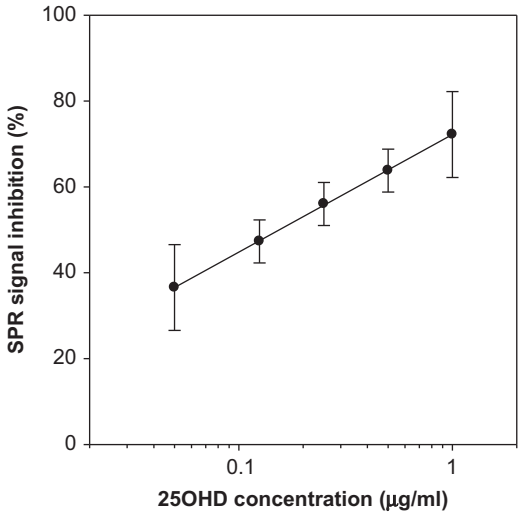


Fig. 2. Calibration plot obtained for different 25OHD concentrations in the range 0.05–1.0 μg/ml, by the inhibition of SPR signal of a 25OHD modified surface in 0.01 M PBS pH 7.4 with 0.1 M NaCl, in presence of VDBP 80 μg/ml.

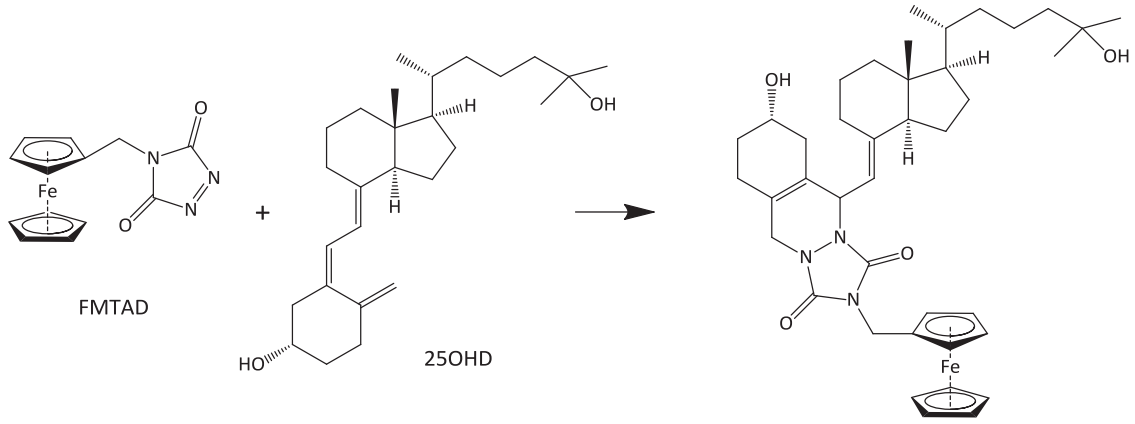
3.2. SPR-based VDBP sensor for indirect detection of 25OHD

This approach is based on a competition scheme of analysis: to this end, the 25OHD was immobilized onto the SPR sensing surface while the Vitamin D Binding Protein (VDBP) was present in the bulk solution at fixed concentration and partly binds to immobilized 25OHD generating an intense SPR signal variation due to its high molecular weight (around 52 KDa); the analysis of 25OHD is carried out under a competition scheme by adding 25OHD to the solution thus displacing the bound VDBP.

First of all, the ability of immobilized 25OHD to bind VDBP free in solution was assessed by registering several sensorgrams for different VDBP concentrations; it was observed that the experimental signal increases until a plateau is attained for concentration values higher than 80 μg/ml: this concentration value was adopted for the competition analysis. The sensorgrams obtained at a fixed concentration (80 μg/ml) of VDBP in absence and in presence of different 25OHD concentrations show an overall competition effect both during the association and the dissociation phase indicating that either immobilized and free in solution 25OHD compete for binding VDBP. The percentage of signal inhibition was calculated and plotted as a function of 25OHD concentration in solution; the obtained calibration plot is reported in Fig. 2. The method displays a linear dependence with concentration logarithm in the range 0.05–1.0 μg/ml with a sensitivity 72.2% / log(μg/ml) and a LOD of 0.045 μg/ml that represents an interesting improvement with respect to the LOD revealed in direct assay which was about fiftyfold higher. Nevertheless, despite this improvement of analytical performances, also this affinity sensors described so far based on SPR transduction seem to be unsuitable to practical application considering that the clinical requirement is to establish whether or not the plasma concentration of 25-hydroxyvitamin D exceeds 30 ng/ml that is a value significantly lower with respect to LODs of proposed sensors.

3.3. Electrochemical-based immunosensor for detection of FMTAD-derivatized 25OHD

In order to achieve analytical devices for the rapid determination of 25OHD on the field, such as portable type Point-of-Care testing devices, the possibility of an electrochemical transduction for the immunochemical determination of vitamin D was also evaluated. The 25OHD does not have an electroactive chemical nature allowing a direct determination by voltammetric techniques; therefore, vitamin D has been functionalized with an appropriate electroactive molecule that, on the one hand, binds the 25OHD and, secondly, retains the ability of being oxidized and reduced in order to carry out the electrochemical analysis. After modification, the 25OHD



Scheme 1. Diels–Alder reaction between FMTAD and 25OHD.

should still be able to react with its antibody immobilized onto the sensing surface. To this purpose the 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione (FMTAD) has been synthesized as described in Appendix A: as displayed in the Scheme 1 this molecule is capable of binding the 25OHD through a Diels–Alder reaction between the conjugated diene of vitamin D and the triazolin-3,5-dionic group acting as dienophile thus allowing the voltammetric detection thanks to the properties of ferrocene (FC) moiety.

Before employing the electrochemical transduction, the possibility of using the Diels–Alder reaction between 25OHD and FMTAD for vitamin D detection was firstly checked by SPR; the results obtained (shown in Appendix A) confirm that even after the modification with FMTAD, the 25OHD retained its ability to bind to vitamin D antibody, thus allowing to employ the electrochemical transduction of the considered immunoreaction without the drawbacks of the steric limitations displayed by AuNPs.

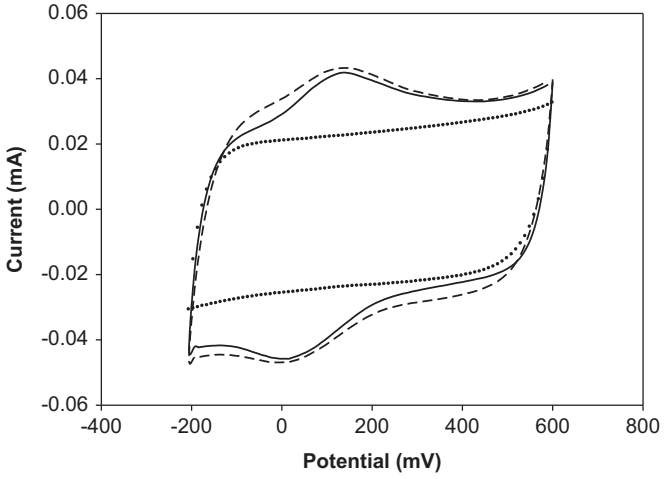


Fig. 3. Cyclic voltammograms of Ab–25OHD modified SPE in PBS 0.01 M pH 7.4, in the range −200 to 600 mV vs Ag/AgCl at scan rate 100 mV/s; blank (dotted line) and in presence of 25OHD–FMTAD 100 ng/ml, first scan (solid line) and after 100 scan (dashed line).

In Fig. 3 the cyclic voltammograms of an Au screen printed electrode modified with a self assembled monolayer binding the 25OHD antibody in the presence and in the absence of 25OHD–FMTAD adduct, are reported. It is readily evident that the FC retains its redox properties showing a typical reversible behavior with an $E^{\circ\prime}$ of 70 mV vs Ag/AgCl. Moreover, considering that after one hundred cycles the voltammetric signal is practically unchanged it can be affirmed that the 25OHD–FMTAD adduct appears stably docked to the Ab–25OHD. As further clue of this interaction stability, the linear dependence of current peak intensities with the potential scan rate, indicating the absence of diffusion as expected from an immobilized system, was observed (data shown in Appendix A).

Nevertheless, both from the signals registered in presence of 25OHD–FMTAD and from blank signal in Fig. 3, it is particularly evident a huge contribution of capacitive current that is not surprising taking into account that the presence of immobilized antibody onto the electrode surface leads to a wide double layer and consequently to a greater contribution of capacitive current. Since this could reduce the analytical performance of the system, in order to enhance faradic response reducing capacitive contribution, the following electrochemical measurement were performed by differential pulse voltammetry instead of cyclic voltammetry.

In Fig. 4 the experimental response using DPV obtained for different 25OHD–FMTAD adduct concentrations, is reported; considering that the 25OHD/FMTAD ratio in the adduct is 1:1, a linear response (reported in the inset of Fig. 4) is observed in the range 20–200 ng/ml of 25OHD with a sensitivity 0.020 μA ml/ng and a LOD of 10 ng/ml (calculated as three times the noise signal) which is well adequate for application of this method to vitamin D detection in real samples, taking into account that as already mentioned a 25OHD concentration in plasma of 32 ng/ml is considered the threshold of hypovitaminosis. This result has been obtained using an Ab–25OHD 0.03 mg/ml solution in the immobilization procedure

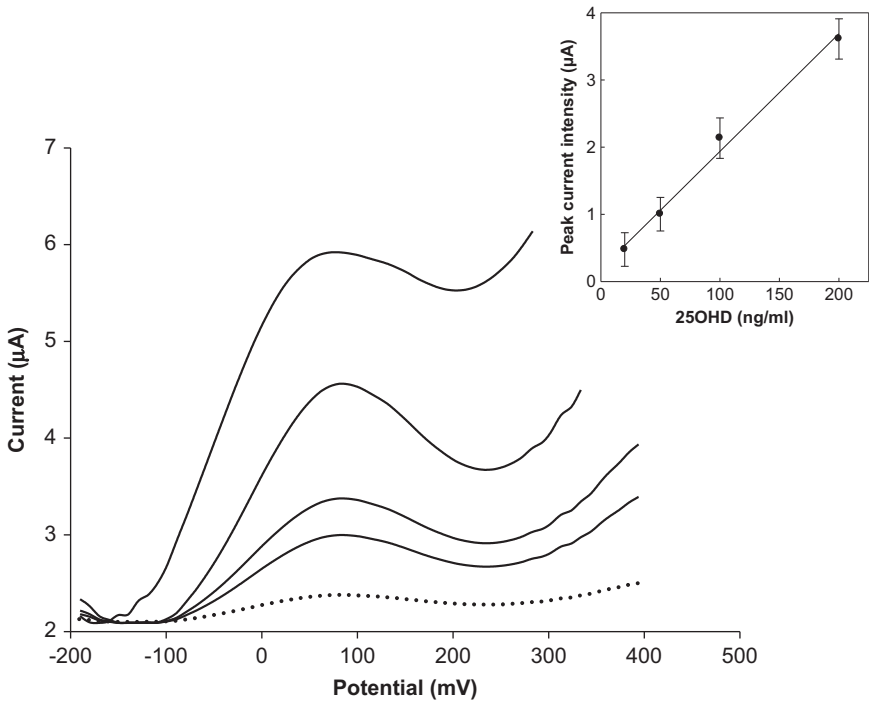


Fig. 4. Differential pulse voltammograms of Ab–25OHD modified SPE in PBS 0.01 M pH 7.4, in the range −200 to 400 mV vs Ag/AgCl, in absence (dotted line) and in presence (solid lines) of different 25OHD–FMTAD adduct concentration in the range 20–200 ng/ml. Inset: calibration plot of voltammetric signal as a function of 25OHD–FMTAD adduct concentration in the linearity range (20–200 ng/ml).

Table 1
Analytical performances of the different affinity sensors considered (VDBP: Vitamin D Binding Protein; FMTAD: 4-ferrocenilmetil-1,2,4-triazolin-3,5-dione).

Immobilized recognition element	Transduction	Type of recognition	Linearity range	Sensitivity	LOD
25OHD Antibody	SPR	Direct interaction with 25OHD	5–50 μg/ml	2.8 m°ml/μg	2 μg/ml
25OHD Antibody	SPR	Direct interaction with AuNP-functionalized 25OHD	5–50 μg/ml	4.8 m°ml/μg	1 μg/ml
25OHD	SPR	Competition with VDBP in solution	0.05–1.0 μg/ml	72.2% / log(μg/ml)	45 ng/ml
25OHD Antibody	DPV	Direct interaction with FMTAD-derivatized 25OHD	20–200 ng/ml	0.020 μA ml/ng	10 ng/ml

for modifying electrode surface as described in experimental section; this concentration of antibody was optimized by checking the DPV instrumental response, in presence of 0.25 μM 25OHD–FMTAD adduct in solution, of different electrodes prepared by varying the concentration of Ab–25OHD solution used in the immobilization procedure in the interval 0.01–0.1 mg/ml. The results (shown in Appendix A) indicate a maximum current value for antibody concentration of 0.03 mg/ml that, as stated above, was the concentration used along the experiments.

Finally, the possibility of regenerating the Ab-25OHD modified electrode was checked by inserting the immunosensor in a flow cell that made possible to carry out several measurements in stopped-flow conditions with the same electrode since the surface can be easily regenerated between a measure and the subsequent one, by flowing a 0.5 M NaCl solution at pH 2.5 for 10 min; after the regeneration the DPV signal coincides with the blank, the peak current intensity dropping to zero. Then, a further addition of derivatized 25OHD restores the peak current with a good repeatability (RSD ≤ 8) for at least 10 cycles of regeneration.

4. Conclusions

Different approaches to the determination of vitamin D using affinity based biosensors, are described herein; comparison is made considering that a potential application of developed biosensor to clinical practice requires a thorough determination of 25-hydroxyvitamin D at ng/ml levels, being 32 ng/ml the agreed threshold level of hypovitaminosis (Dawson-Hughes et al., 2005).

In Table 1, the proof-of-concepts of followed approaches and the analytical performance thereof, are summarized. Firstly, an immunosensor based on SPR transduction was realized for direct determination of vitamin D, obtaining a LOD of 2 μg/ml which unfortunately is too far from the needs in clinical analysis. In order to enhance the sensitivity, the vitamin D was modified with gold nanoparticles (AuNPs): the binding of 25OHD with AuNPs determines the amplification of SPR signal, allowing to lower the LOD down to 1 μg/ml, doubling the sensitivity. An alternative SPR method, based on the indirect determination of vitamin D by means of Vitamin D Binding Protein (VDBP), leaded to a further sensitivity increase reaching a LOD of 45 ng/ml which is really close to the fixed accomplishment.

Finally, an electrochemical transduced biosensor has been realized, based on the reaction of vitamin D with 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione (FMTAD) allowing the voltammetric detection of derivatized 25OHD thanks to the properties of electroactive ferrocene group: the determination of 25OHD was possible in the range 20–200 ng/ml with a LOD of 10 ng/ml. The latter proposed system fits the requirement of determining vitamin D in a concentration range which is of significance for clinical applications; moreover, the used electrode is a screen printed one thus opening the possibility to miniaturize the sensor and developing a portable and easy-to-automate point-of-care testing device.

Basing on these findings we are going to test the use of this electrochemical modified biosensor for the analysis of vitamin D in serum samples, either in single and differential modality measurements, in order to reduce the matrix effect.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.07.077.

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