An electrochemical sensor for quantitative analysis of Rhesus D antibodies in blood

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Abstract—The Hemolytic Disease of the Fetus and Newborn (HDFN), if untreated, can have severe consequences on baby's health, eventually causing death. Prevention and diagnosis of pregnancies at risk of HDFN consists in quantitative analysis in hospital every 2-4 weeks of blood group antibodies in maternal serum. The most important antibodies to be monitored are anti-Rhesus D (or anti-RhD) ones. Nowadays several portable systems for different healthcare applications have been developed towards decentralization of diagnosis thanks to the enormous advances in electrochemical biosensing. Home-monitoring of pregnancies at risk of HDFN could significantly improve patients' life quality. Here we demonstrate for the first time label-free quantitative detection of anti-RhD antibodies by means of electrochemical impedance spectroscopy (EIS), suitable for integration within a complete portable device. The specificity of the sensor was also proved.

Index terms— Hemolytic disease of the fetus and newborn, HDFN, Rh disease, electrochemical sensors, electrochemical impedance spectroscopy, blood typing

I. INTRODUCTION

The Hemolytic Disease of the Fetus and Newborn (HDFN) is a condition that, if untreated, can have severe consequences on the fetus/neonate health: 1 over 4 cases result in intense anemia and irreversible damages to the nervous systems (kernicterus); another 25 % of the cases in abnormal accumulation of fluid in 2 or more fetal compartments (hydrops foetalis) and eventually death [1]. Prevention and diagnosis include frequent analysis of maternal antibodies in serum [2]. In this regard, the realization of a portable device for monitoring of the immune response of the patient represents a significant step towards decentralization of medical care.

The HDFN can occur in the presence of a mismatch between the blood group of the mother and the fetus. Blood is generally classified according to the so-called ABO system into four different groups (A, B, AB, 0), depending on the presence or absence of the A and B antigens on the surface of the Red Blood Cells (RBCs, also called erythrocytes). Another important system that must be considered in blood typing is the Rhesus or Rh system [3]. So far, several different Rh antigens have been identified. However, the Rhesus D antigen is the most important one in terms of clinical relevance. If this antigen is present on the erythrocytes of the individual, his blood group is called Rh positive (Rh+), otherwise it is defined Rh negative (Rh-). A summary of the characteristics of the ABO and RhD systems is given in Figure 1. It can be observed that the absence of a particular antigen implies the

presence in blood serum of the corresponding antibodies that will cause agglutination in case of contact with incompatible RBCs. However, no anti-RhD antibodies are present in Rh- individuals serum until exposure to RhD antigens occurs. This process of antibodies production in response to alloantigens is called alloimmunization [4]. The HDFN can be associated to different blood systems and subsystems. The most critical one

Fig. 1. Chart of different blood groups showing the types of antigens present on each kind of red blood cell and the antibodies present in the corresponding serum.
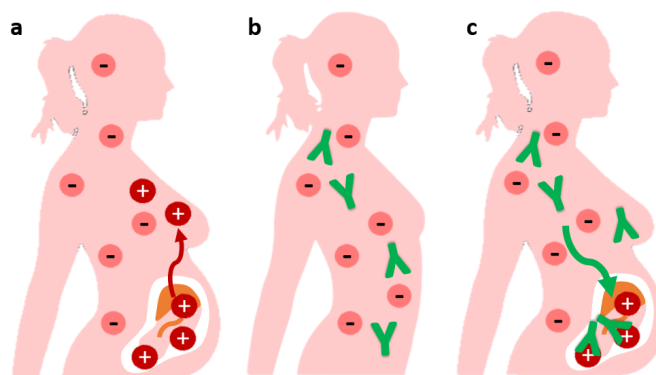

Fig. 2. Origin and development of the HDFN: a) the Rh- mother during pregnancy comes in contact with the blood of her Rh+ fetus; b) the mother will produce anti-RhD antibodies in response to fetal RhD antigens (alloimmunization); c) alloantibodies cross the placenta and attack fetal RBCs with severe consequences on fetus health.

is related to the RhD antigen occurring when the mother is Rh- and the fetus Rh+ [5]. This is normally referred to as "Rh disease" [2].

The origin and development of the HDFN is explained in more detail in Figure 2. When an Rh- mother come into contact with the nonself RhD antigens (typically via previous pregnancies as in the Figure or via transfusions), anti-RhD antibodies develop in woman blood serum. If able to cross the placenta, these antibodies attack the RBCs of the fetus causing anemia, hepatosplenomegaly (i.e. liver and spleen swelling), jaundice, permanent damages of the nervous system due to excess of bilirubin and eventually death [1].

Nowadays, HDFN due to Rhesus D alloimmunization attacks 0.4 over 1000 pregnant women and still represents the main cause of fetal anemia [1]. Screening programs to determine high-risk situations consists in monitoring the possible sensitization of the mother by analyzing her serum antibodies during pregnancy by means of the indirect Coombs test, also called Indirect Antiglobulin Test (IAT) [2]. This laboratory procedure exploits agglutination to reveal the presence in mother's serum of IgG alloantibodies that are likely to cross the placenta. In case of positive result, monthly checkups to quantitatively analyze maternal alloantibodies are required until the 18th week of gestation. Afterwards, the monitoring frequency should be doubled [1]. The amount of alloantibodies is typically determined by titration. The antibody titre is defined as the inverse of the maximum dilution factor at which it still possible to detect the antibodies in serum. If their amount increases significantly over time, it is possible to conclude that fetal RBCs are causing an immune response in mother's serum [1]. In this case, intrauterine or exchange transfusions in the newborn period can be necessary [5].

IATs tests oblige pregnant women at risk of HDFN to have frequent checkups in hospitals [1]. The development of a Lab-On-a-Chip (LOC) device able to send relevant data to doctors' device will certainly improve patients' life by allowing health monitoring at home. In this respect, electrochemical sensors represent a promising class of devices to realize portable and low cost diagnostic systems thanks to their easy miniaturization, small sample volume and easy integration into micro-fabrication processes for mass production [6]. In particular, EIS is a powerful electrochemical technique for detection of surface phenomena or of variations in bulk properties [7]. It is based on the analysis of the electrical impedance of the system, that is then properly fitted by means of an appropriate equivalent circuit to obtain quantitative information about circuit parameters [8]. The great potential of this method for biosensing applications is represented by the possibility of label-free detection, thus avoiding the need to modify the biomolecules with enzymes or fluorescent labels [9].

Physical adsorption of bioprobes on the electrodes surface offers a very convenient method for electrodes surface modification thanks to the minimum alteration of the biomolecules. Consequently, the risk of detection capabilities degradation is reduced. Moreover, they do not require several time-consuming steps, thus simplifying significantly the experimental protocol for the fabrication of the biosensor [10].

In the present paper, we propose the new idea of a portable electrochemical sensor for quantitative analysis of alloantibodies in mother's serum for monitoring pregnancies at high risk of HDFN. RhD peptides were physically adsorbed on the gold electrodes surface in order to form a probe layer for sensing of anti-RhD antibodies. Our preliminary results demonstrate the quantitative label-free detection of Rhesus D alloantibodies by means of electrochemical impedance spectroscopy (EIS).

II. Experimental

A. Materials

Dual Screen Printed Gold Electrodes (SPGEs) were purchased from Dropsens (Spain). Rabbit polyclonal IgG anti-RhD antibodies, rabbit polyclonal IgG isotope controls and Rhd peptides were purchased from Abcam (UK). All other chemicals were from Merck (Germany) and Sigma-Aldrich (US). 0.01 M Phosphate buffer saline (PBS, Sigma Aldrich, P4417) solution at pH 7.4 was prepared using ultrapure water.

B. Peptide immobilization and antibody incubation protocols

Electrochemical cleaning of all SPGEs was performed in a 0.5 M H_2SO_4 aqueous solution by multiple cyclic voltammetry scans between -0.2 and +1.5 V at a scan/rate of 50 mV/s until complete overlapping of two subsequent voltammetric curves [11]. The RhD peptides were physically adsorbed on the electrodes by incubation overnight at 4°C at dark. This step was followed by accurate washing in PBS to remove unbound peptides. The antibodies were incubated overnight at 4°C at dark with anti-RhD antibody solutions in PBS. A final rinsing step was performed to remove unbound antibodies residues.

C. Morphological and electrochemical measurements

All EIS measurements were performed using an Autolab potentiostat (Metrohm, Switzerland) controlled by a Nova 1.11 software (Metrohm, Switzerland). A sinusoidal AC signal with an amplitude of 10 mV was applied to the sample with a frequency range from 0.1 to 10^5 Hz. MEISP software by Khumo Chemical Laboratories was used for impedance fitting. This is based on complex non linear least-square fitting algorithm LEVM/LEVMW [12]. The software generates also values representing the accuracy of the fitting. In order to ensure a good fit, the χ^2 was always brought to values smaller than 10^{-3} and the standard deviation of each parameter to less than 5%.

III. Experimental results and discussion

The peptide concentration used for sensor surface modification was optimized to achieve the highest surface coverage. The presence of immobilized peptides onto the surface was detected by means of EIS measurements. The impedance response of electrochemical systems can be fitted by means of the Randles equivalent circuit (inset of Figure 3) [13]. With respect to the usual Randles model, here the normal capacitor was replaced by a constant phase element (CPE) to account for deviations from ideal behaviour typical of double layer

capacitors. [14]. The R_{ct} value can be used to detect surface modifications since these are expected to have a significant effect on the electron transfer capabilities at the electrode interface [14]. It is important to notice that, in order to normalize the effect due to possible surface differences among distinct SPGs, in the present paper a percentage variation of R_{ct}, $\Delta R_{ct}\%$, with respect to the immediately preceding functionalization step is used instead of an absolute one, according to the following equation:

$$\Delta R_{ct}\% = \frac{R_{ct,B} - R_{ct,A}}{R_{ct,A}} * 100, \qquad (1)$$

where $R_{ct,A}$ and $R_{ct,B}$ are the charge transfer resistances after the functionalization steps A and B, respectively. An example of a typical electrochemical impedance response of a SPGE is given in Figure 3.

The results of the EIS measurements at different peptide concentrations are reported in Figure 4. The percentage increment $\Delta R_{ct}\%$ is calculated versus the bare gold electrode using equation (1). It is possible to observe that, as expected, the physical adsorption of the peptides onto the surface causes an increase in the R_{ct} value of the electrode. However, the $\Delta R_{ct}\%$ gain after incubation with the peptides falls significantly when their concentration is reduced below 500 μg/ml. For this reason, this value was chosen for validation and calibration of the impedance sensor in order to reduce peptide consumption, while still maintaining optimum surface coverage of the probe molecules.

The feasibility of antigen-antibody binding detection and the specificity of the sensor were also examined by means of EIS measurements. The results are summarized in Figure 5, in which the $\Delta R_{ct}\%$ values after different surface modification steps are reported. As explained before, the percentage variation is always normalized with respect to the previous

functionalization stage using equation (1). Consequently, each column in Figure 5 must be read separately and cannot be used for comparisons. The first column shows that the percentage increment of the R_{ct} value after peptide immobilization with respect to the bare electrode is about 36%, thus proving the effective modification of the surface. A further 37% increase in R_{ct} is achieved versus the peptide-modified electrode when the sensor are incubated with anti-RhD antibodies at 200 μg/ml, as evident from the second column. A relatively high concentration was used to facilitate the investigation of the feasibility of antigen-antibody detection. In the third column the $\Delta R_{ct}\%$ after exposure to the isotope control, that is a non-

Fig. 4. The effect of peptide concentration on $\Delta R_{ct}\%$ values normalized with respect to the R_{ct} values of the bare gold electrodes. This trend is a result of reduced coverage when the peptide concentration is reduced. The error bars refer to the standard deviation among different electrodes.
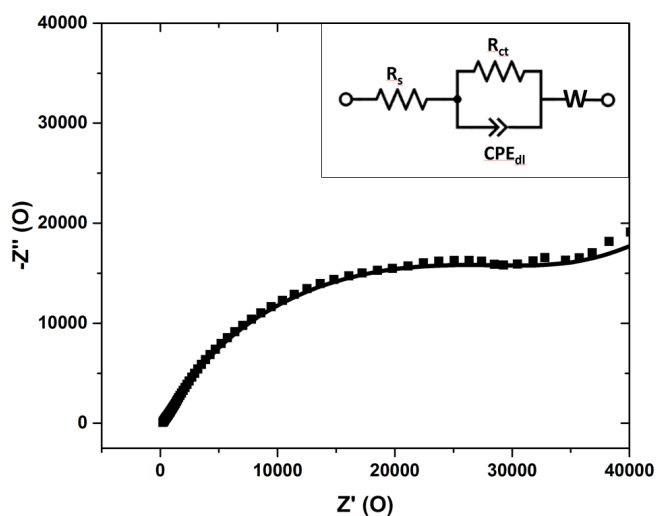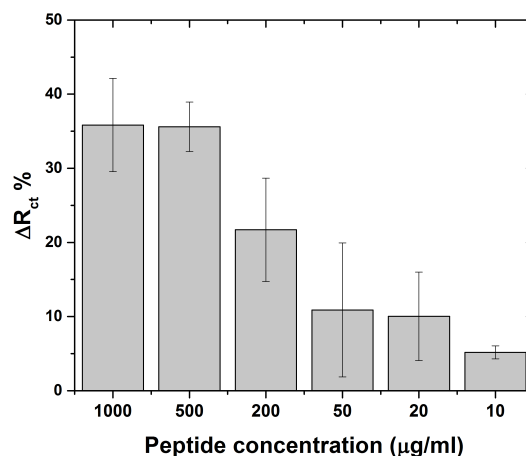

Fig. 3. An example of a typical electrochemical impedance response of a screen printed gold electrode. The fitting was performed by using a modified Randles equivalent circuit.
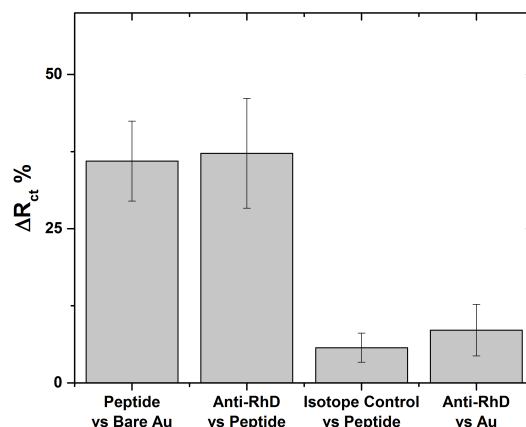

Fig. 5. The $\Delta R_{ct}\%$ values after different surface modification steps. It is important to notice that all percentage increments are normalized with respect to the previous functionalization step. The error bars refer to the standard deviation among different electrodes.
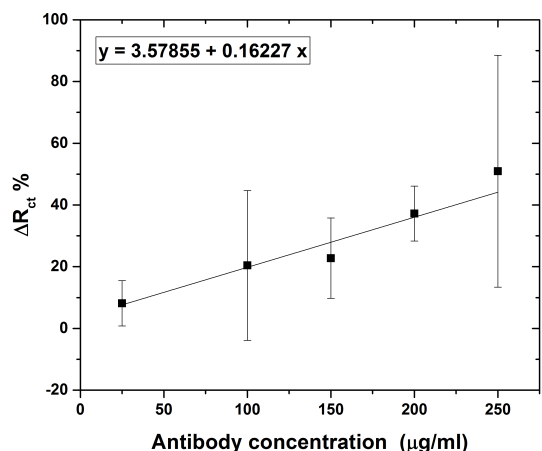
Fig. 6. Calibration curve of the impedimetric sensor. The points reported in the Figure are obtained by means of one-shot measurements. All $\Delta R_{ct}\%$ are calculated with respect to the peptide-modified electrodes.The error bars refer to the standard deviation among different electrodes.

specific antibody, is given versus the peptide-modified surface. A relatively high isotope concentration was used ($250\ \mu g/ml$) as in the case of the specific anti-RhD antibodies. The small percentage increase in R_{ct} (around 5.7%) proves the specificity of the sensor. Finally, the last column shows that the $\Delta R_{ct}\%$ due to the incubation of the bare gold electrodes with anti-RhD antibodies at $250\ \mu g/ml$ is around 8.54%. This modest value proves that the effect of aspecific absorption of anti-RhD antibodies on gold is minimum.

The sensors were exposed to different antibody concentrations in order to obtain a calibration curve (Fig. 6). The points reported in the Figure are obtained by means of one-shot measurements. All $\Delta R_{ct}\%$ are calculated with respect to the peptide-modified electrode according to equation (1).

The antigen-antibody binding is proved by the increase in $\Delta R_{ct}\%$. A higher number of bonds is possible at higher antibody concentrations, leading to larger $\Delta R_{ct}\%$ values. The sensor shows linear behaviour in the range 25-250 $\mu g/ml$ with a lower detected concentration of 25 $\mu g/ml$. This result is comparable to the theoretical Limit Of Detection (LOD) of about 50 $\mu g/ml$, that can be computed as three times the standard deviation of the blank (from the second column of Figure 4) divided by the sensor sensitivity (slope of the calibration curve [15]) [16]. These results demonstrate for the first time the feasibility of the fabrication of electro-chemical immunosensors for quantitative detection of blood group antibodies. This system could be easily integrated in the future with a portable device for decentralized monitoring. Improvement of sensor performance in terms of sensitivity [17] and LOD [18] can be obtained by nanostructuring the electrodes .

In the future other immobilization techniques, like covalent binding using EDC/NHS chemistry can be investigated and compared to physical adsorption. Moreover, the same elec-

trochemical sensing principle can be used for blood typing or for quantitative analysis of other important blood groups antibodies that are relevant for HDFN.

IV. CONCLUSION

A label-free electrochemical sensor for quantitative detection of anti-RhD antibodies was fabricated for the first time through the immobilization of RhD peptides on the surface. This device can represent the first step towards home monitoring of HDFN in pregnant women. This system would significantly improve patients' life. Future work will include nanostructuring of the electrodes and comparison with other immobilization techniques to improve sensor perfomance.

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