Chapter

A New Alternative Approach for RhD Incompatibility; Determination Fetal RhD Status via Biosensor Technology

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Abstract

Prenatal detection of the fetal RHD status in early stage of pregnancy is observed to be useful in the management of RhD incompatibility to identify fetuses at risk of hemolytic disease. The routine use of antenatal and postnatal anti-D prophylaxis reduces the incidence of hemolytic disease of the fetus and newborn. Cell-free fetal DNA in maternal plasma is in use today for routine genotyping fetal RHD status. Fetal RhD antigens can be detected in the blood of RhD-negative pregnant women using a nanopolymer-coated biosensor and could be an alternative method for medical diagnosis. We detected RhD-positive fetal antibodies with biosensor in maternal blood of RhD-negative mothers. The electrochemical measurements were performed on a PalmSens potentiostat and corundum ceramic-based screen-printed gold electrode. The demonstrated method has a different view for the detection of fetal RhD status in early pregnancy. The biosensor technology is useful and can be carried out rapidly in clinical diagnosis. Biosensors are also reproducible methods which give results quickly compared to noninvasive fetal RHD genotyping with real-time PCR-based techniques. We suggest that this method could become an alternative part of fetal RHD genotyping from maternal plasma as a prenatal screening in the management of RhD incompatibility.

Keywords: RhD incompatibility, fetal RhD, biosensor, hemolytic disease, RhD antigen

1. Introduction

The discovery of circulating fetal DNA by Lo et al. [1] has opened new possibilities for noninvasive prenatal diagnosis for investigators. It has been shown that this new source of fetal DNA also could be used for noninvasive prenatal determination of fetal RhD genotyping using the plasma of RhD-negative pregnant women [2]. RhD genotyping from maternal plasma is a valuable method to identify pregnancies that has a risk of hemolytic disease of the fetus and newborn (HDFN) [3].

The HDFN is caused by IgG antibodies of the mother that cross the placenta to red cell surface antigens and facilitate destruction to the immune defense of fetal red cells or erythroid progenitors. This causes a significant rate of morbidity and mortality for the fetus. RhD antigen of the rhesus system is the most commonly

implicated antigen [4, 5]. Prophylaxis after delivery with anti-D immunoglobulin reduces the alloimmunization of RhD-negative women [4]. RhD alloimmunization has to be monitored for fetal anemia in complicated pregnancies for effective pre-/postnatal transfusion treatment to prevent the baby from hydrops fetalis [3, 6].

Postnatal prophylaxis was used since the 1960s, with serology test used to identify the baby's RhD status [7]. The routine antenatal prophylaxis in the third trimester of pregnancy is now a standard implementation in many countries [4, 8, 9]. This application reduces the maternal sensitization and the HDFN in babies [7].

Invasive procedures should be avoided in alloimmunized pregnant women because of the risk of transplacental hemorrhage (amniocentesis has the risk up to 17%), and the risk of pregnancy loss was found to be up to 2% after amniocentesis and chorionic villous sampling (CVS), respectively [10].

In this chapter we aimed to share our experiences about the determination of fetal RhD genotyping with cffDNA and detection of fetal RhD antigens from maternal blood using a new biosensor as a candidate method for management of RhD incompatibility.

2. RhD incompatibility and management

The knowledge about the fetal RhD type supports the management of alloimmunized pregnancies in RhD-negative women [11, 12].

Prophylaxis after delivery is offered only to RhD-negative women who have given birth to an RhD-positive baby [9, 13]. This prevents babies from rhesus disease and reduces maternal sensitization. Routine antenatal anti-D prophylaxis use was first introduced in the mid-1990s. The sensitization rates were then reported to reduce from 1.2% for the earlier policy to 0.28% [7]. Commonly in white population, however, about 38% of these women would be carrying an RhD-negative fetus and thus receive anti-RhD immunoglobulin, a pooled human plasma product, unnecessarily [14, 15]. Fetal RHD genotyping with cell-free fetal DNA (cffDNA) is accepted as a useful method by obstetricians in early pregnancy for the management of RhD incompatibility. Since 2001, several European countries use cffDNA in maternal blood for noninvasive prenatal diagnosis of fetal RhD status [3]. There is also change in the measurement method in the hemolysis detection. This invasive method which detects the optical density at a wavelength of 450 nm in amnion fluid replaced by detect the fetal anemia by the Doppler measurement of the peak velocity of systolic blood flow in the middle cerebral artery [16].

3. Fetal RhD genotyping with cell-free fetal DNA

Prenatal care strategies for the fetus with RhD have been changed significantly during the last few decades. Discovered cffDNA from plasma of pregnant women by Lo et al., in 1997, has been used for the noninvasive detection of fetal RhD status which avoids RhD-negative women from antenatal anti-RhD prophylaxis [17–20].

3.1 Methods and sample preparation

3.1.1 Sample preparation

Maternal blood (10 cc) was collected from each pregnant woman and placed into an EDTA tube. Centrifugation step was applied within 1 h (at 1600 × *g*, 10 min) after separating maternal plasma. After centrifugation the plasma was removed

carefully from the collection tubes and transferred into polypropylene tubes. Another centrifugation step was done at 16,000 × *g* (10 min). The plasma supernatants removed to new polypropylene tubes and stored at −20°C until other processes. Collected plasmas were thawed, and the DNA was automatically extracted from 1 mL of plasma as reported previously [9, 21, 22].

3.1.2 Fetal RhD genotyping

RHD genes (exons 5 and 7) were analyzed from isolated cffDNA samples. The oligonucleotide primers used to perform real-time quantitative PCR are reported in **Table 1** [9]. The gene of DYS14 was tested to confirm the presence of male fetal DNA, and the beta globin (β-globin) gene was used as a reference to confirm the presence of cffDNA [10]. Real-time PCR performed in a LightCycler 480 (Roche Applied Science, Basel, Switzerland) using 96-well plates. The PCR mixture was 50 μL in total volume that contains 300 nM of each primer, 50 nM probe, 2 × TaqMan Universal PCR master mix (Roche Diagnostics, Basel, Switzerland), and 15 μL of template DNA of plasma samples. The PCR cycling conditions were as follows. Incubation step was 50°C for 2 min and 95°C for 10 min. Amplification step was 95°C 15 s and 60°C 60 s (50 cycles). The β-globin gene protocol was the initialization step at 95°C for 10 min, followed by 95°C 15 s, 57°C 10 s, and 72°C 10 s (40 cycles). Samples were analyzed in triplicate. Calibration curves were run also for each analysis [21].

The clinical features of the subjects studied (mean age and week of pregnancy) are shown in **Table 2**. Fifteen fetuses were found to be RhD-negative females. The RhD status of the fetus was predicted in 70 pregnancies in our study. The gender determination of the fetuses was shown in **Table 3**.

We have shown that fetal RHD genotyping by multiplex real-time PCR is applicable and readily performed, with a high accuracy rate, as a routine clinical test in prenatal diagnostic laboratories in Turkey. This method avoids unnecessary

Primer	**Sequence (5′–3′)**	**Label**
RHD exon 7		
Forward	GGGTGTTGTAACCGAGTGCTG	None
Reverse	CCGGCTCCGACGGTATC	None
TaqMan probe	CCCACAGCTCCATCATGGGCTACAA	FAM-TAMRA
RHD exon 5		
Forward	CGCCCTCTTCTTGTGGATG	None
Reverse	GAACACGGCATTCTTCCTTTC	None
TaqMan probe	TCTGGCCAAGTTTCAACTCTGCTCTGCT	VIC-TAMRA
DYS14		
Forward	CATCCAGAGCGTCCC TGG	None
Reverse	TTCCCCTTTGTTCCCCAAA	None
TaqMan probe	CGAAGCCGAGCTGCCCATCA	FAM-TAMRA
Beta globin		
Forward	ACACAACTGTGT TCACTAGC	None
Reverse	CAACTTCATCCACGTTCACC	None
TaqMan probe	GAAGTCTGCCGTTACTGCCCTG	LC-Red

Table 1.
Primer and probe sequences used in RHD genotyping [9].

RhD status	Age (years)	Gestational age	Immunization	
	X ± SD (min–max)	X ± SD (min–max)	Yes (n, %)	No (n, %)
RhD-negative (n = 26)	29.1 ± 6.0 (20–39)	20.2 ± 8.6 (10–38)	12 (46.2)	14 (53.8)
RhD-positive (n = 44)	28.6 ± 6.1 (18–39)	16.9 ± 6.9 (9–38)	11 (25.0)	33 (75.0)

Table 2.
Clinical features of the study population [9].

Gestation weeks	N	RhD positive, n (%)		RhD negative, n (%)		Accuracy of *RHD* and sex genotyping, %
		Male	Female	Male	Female	
6–12	38	10	18	3	7	100
13–28	20	3	5	7	5	100
29–40	12	6	2	1	3	100
Subtotal		19	25	11	15	
Total	70	44 (62.8%)		26 (37.2%)		100

Table 3.
Fetal RhD and sex status of maternal plasma samples [9].

immunoprophylaxis in RhD-negative women bearing RhD-negative fetuses. We suggest that RHD genotyping should become an essential part of prenatal screening in the management of RhD incompatibility [9].

4. Biosensor in use to detect fetal RhD in maternal blood

Nowadays biosensors are universal devices which is used in biomedical diagnosis such as point-of-care monitoring of treatment and disease progression, drug discovery, forensics, and biomedical research [23]. They are widely used in different areas of healthcare [24]. The two main examples of biosensors are pregnancy tests and glucometers which are very successful devices. Biosensors have different transducing mechanisms based on signal generation (such as an electrochemical or optical signal) following the formation of antigen-antibody complexes [25]. Antibodies, enzymes, and synthetic biomolecules that are high-affinity reagents can be coupled to the transducer in order to provide specificity of the biosensors [23, 26].

We designed a new nanopolymer-coated electrochemical biosensor which is specific for the detection of fetal RhD antigens in the blood of pregnant women and results compared with cffDNA RHD genotyping with real-time PCR [26]. Biosensor technology is reproducible which can be used many times. The results can be generated quickly within a few minutes when compared to noninvasive fetal RHD genotyping with real-time PCR-based techniques. We suggest that biosensor technology could become a candidate method in early pregnancy in the management of RhD incompatibility.

4.1 Materials and methods

The bioelectrochemical measurements were performed with PalmSens potentiostat systems and gold working electrode combined with the auxiliary Au/Pd (98/2%) electrode and the reference Ag/AgCl electrode. Thermostatic working cell, magnetic stirrer, automatic pipets, and Milli-Q ultrapure water were used in the experiments.

4.1.1 Preparation procedure of the Au electrode surface

Cleaning electrode. First off all, the base of the working electrode surface was polished with alumina. And then the polished working electrode was sonicated in pure ethanol and Milli-Q ultrapure water for 10 min for removing undesired absorbable particles, respectively. For the last step of the electrode cleaning, five successive cyclic voltammogram sweeps were taken with bare working electrode between −1.0 and +1.0 V in 0.1 M HNO3 solution.

RhD antibody immobilization onto Au electrode surface. Poly(Hema-Mac) nanopolymer was immobilized on the clean electrode's surface at room temperature via anilin (20 μL anilin and 20 μL RhD antibody). For trapping the antibody, a cross-linking agent (2.5% glutaraldehyde) was used. The modified working electrode was cleaned with Milli-Q ultrapure water for removing unbinding materials.

Principle of the electrobiochemical measurement. The measurement is based on the oxidation-reduction reactions of the RhD antibodies. All the measurements performed with thermostatic reaction cell included phosphate buffer (50 mM, pH 7.0) and potassium ferrocyanide [$K_4Fe(CN)_6$] as mediator complex, at 35°C. The charge transfer capacitance (electrochemical potential difference) of antigen-antibody interaction difference was measured by biosensor system (**Figure 1**).

Preparation of the samples. The working group has 26 RhD-negative primigravidas. All of them were admitted to the Department of Gynecology and Obstetrics and to the Department of Medical Biochemistry for prenatal diagnosis in different gestational ages (8th–36th weeks) that were analyzed in biosensor study for RhD status (**Table 4**). Written informed consent that was approved by the Ethics Committee of the Faculty of Medicine of Cukurova University was obtained from each subject. Blood samples were collected at ethylenediaminetetraacetic acid (EDTA) tube (Becton Dickinson, Bangkok, Thailand). Blood group test was identified by the Blood Bank Centre using slide/tube agglutination test, which includes antibodies against red blood cell antigens.

4.2 RhD antibody immobilization

UV polymerization of anilin was used for RhD antibody immobilization. Anilin's reduction potential is reducing at the UV light. A reversible manner was showed on the uncovered working electrode of the cyclic voltammogram of redox probe $Fe(CN)_6^{4-/3-}$ (**Figure 2**). To inhibit the charge transfer among redox probe in solution on the Au electrode, a bioactive layer was applied on the surface of the electrode. The reversible behavior of the cyclic voltammograms turned into a capacitive shape (**Figure 2**).

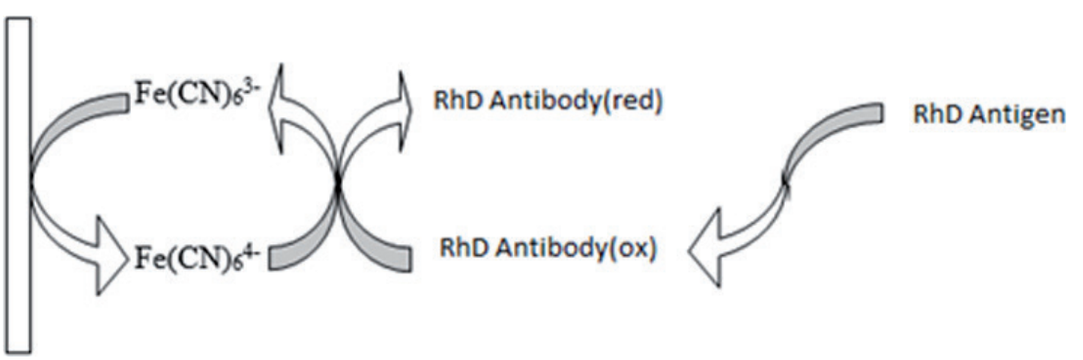

Figure 1.
The principle of the biosensor [26].

Fetal RhD Status|	Age (years)		Gestational Age		Immunization
	X ± SD (min-max)	Range	X ± SD (min-max)	Range	
RhD positive (n = 21)	29.5 ± 5.9 (19–37)	18	15.1 ± 6.7 (8–36)	28	No
RhD negative (n = 5)	25.6 ± 3.2 (21–30)	9	11 ± 2.0 (8–13)	5	No

Table 4.
Clinical features of the samples in biosensor study for RhD status [26].

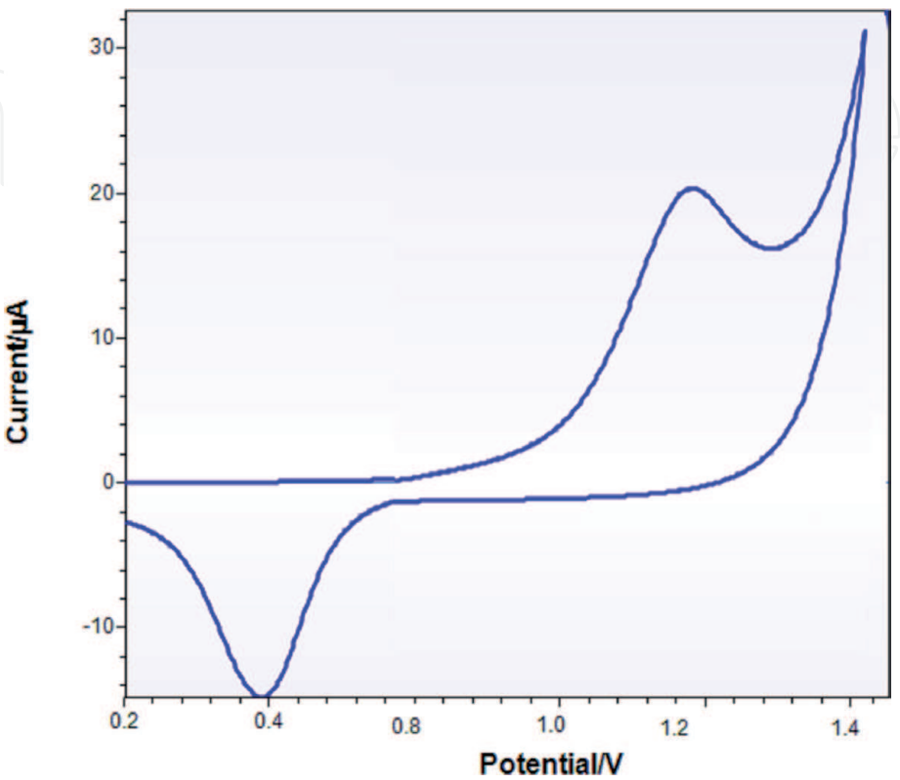

Figure 2.
RhD biosensors cyclic voltammogram for the immobilization steps. Red line: uncovered gold electrode; blue line: UV polymerized. (Working conditions: incubation time 1 h for RhD antibody; 50 mM electrochemical redox probe solution; and mediator complex pH 7.0 potassium ferrocyanide [$K_4Fe(CN)_6$]). For detection of RhD antigen in maternal sample, the optimal curve of the biosensors potential range was 0.2–1.4 V [26].

4.3 Biosensors optimization trials

Working condition optimization studies were performed to determine the most suitable working conditions for using the biosensor. For this aim, the mediator concentration, cross-linker concentration, RhD antibody concentration, temperature effect, pH, and repeatability were studied.

Concentration of RhD antibody. Determination of the antibody concentration effect on the biosensor response, different RhD antibody concentrations (0.05, 0.10, 0.15, 0.20 ng/mL) were applied on the surface of biosensor. The RhD antibodies optimum concentration was determined at 0.10 ng/mL.

Mediator and cross-linker concentration. In order to investigate the effect of the mediator concentration on the biosensor response, potassium ferrocyanide of 1.25 and 2.5 mg/dL was used in the preparation of the biosensor. To determine the effect of cross-linker concentration on the biosensor, the concentrations of glutaraldehyde of 12.5 and 2.5% were used. The optimum was value obtained at 2.5%. According to the results obtained from the experiments, the mediator complex of 1.25 mg/dL was assigned as the most effective result for the biosensor.

The pH effect. For the pH values' effect on the biosensor response, different buffer systems were investigated. For this aim, acetate (50 mM, pH 5.0 ± 5.5), phosphate (50 mM, pH 6.0 ± 6.5 ± 7.0 ± 7.5), and Tris-HCl (50 mM, 8.0 ± 8.5) buffers were used. The optimum pH value was found at 7.0 due to 100% activity rate. Above and below pH 7.0 can cause a decrease in the biosensor response.

Temperature effect. To examine the temperature effect on the biosensor response, the assay was performed by different temperatures (10 ± 55°C). The optimum working temperature of the biosensor system was detected as 35°C. The biosensor response is directly increased with temperature until 35°C, but further increase in temperature caused a decrease on the biosensor response.

Repeatability. Determination of the repeatability of the biosensor experiments were also studied for 1 μM RhD concentration (n = 10). From the assays the mean value ($\bar{x}$), standard deviation (SD), and coefficient of variation (CV %) were found to be 2.68 ± 0.06 μM and 2.23%, respectively. From the results, the repeatability of the biosensor response can be accepted as well as within the given concentration of RhD according to the 95% confidence interval.

4.4 Characterization of RhD antibody biosensor

The graphic shown as **Figure 3** is the concentrations of RhD in different gestational age of pregnant women. The slope of the curves increased with the increasing fetal RhD antigen concentration which depends on gestational ages of the samples (**Figure 3**).

Linearity. The linearity study for the RhD biosensor was obtained in concentration range between 1 and 250 ng/mL. At higher concentrations, standard curve showed a deviation from linearity.

Fetal RHD genotyping. The cffDNA used for fetal RhD status of the fetus is studied in 26 pregnancies with multiplex real-time PCR for RHD gene exons 5 and 7. Twenty-one of 26 cffDNA were detected as RhD positive, and 5 of 26 were detected as RhD negative (the same results as detection with RhD biosensor). The results of the fetuses were confirmed after the delivery by serological and molecular tests.

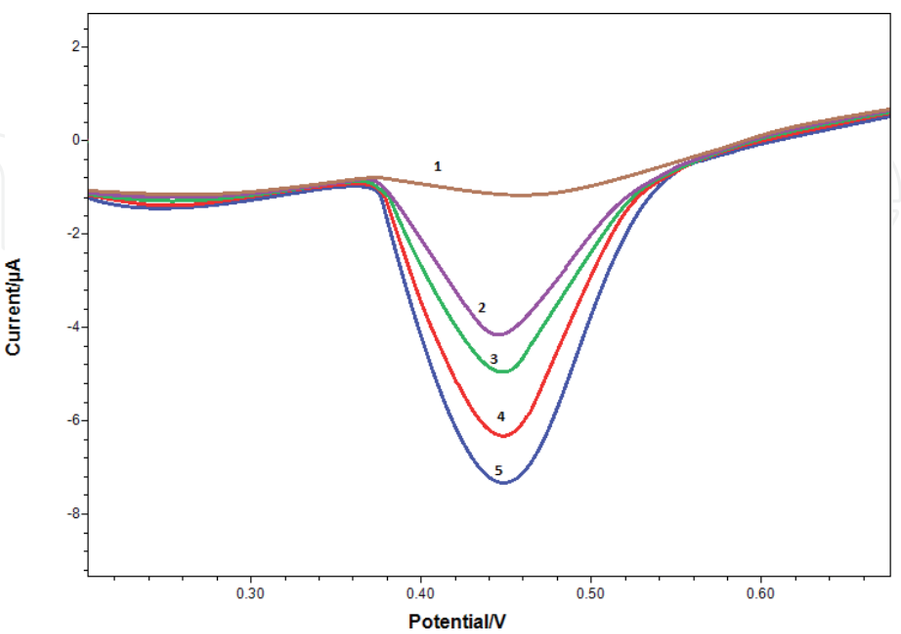

Figure 3.
Detection of increasing fetal RhD antigen with biosensor in different gestational age and mother's blood. Sloped line 1: RhD-negative sample; sloped line 2: sample 8th week of gestation; sloped line 3: sample 13th week of gestation; sloped line 4: sample 21th week of gestation; sloped line 5: sample 36th week of gestation [26].

5. Conclusions

The new biosensor design, which detects RhD status of the fetus in the early stage of pregnancy in RhD-negative pregnant women blood, is suggested as a candidate method in fetal RhD management. RhD antibody is immobilized using UV polymerization of anilin. To characterize the electrochemical properties of the biosensor surface, impedance measurements were applied. For binding the formed stable bioactive layer showed binding of RhD antigen of fetus. The significant impedance biosensor response concentration to detect RhD antigen-antibody binding was 1 ng/mL RhD. The fetus RhD status was approved with real-time PCR fetal RHD genotyping. The detection of the RhD status of the fetus with antigen-antibody biosensor system has more advantage as being fast compared to the noninvasive fetal *RHD* genotyping using fetal DNA. Up to now, common serological-based techniques were used for the detection fetal RhD status on delivery. There is a requirement for fast, sensitive, and low-cost techniques on clinical and molecular diagnostic. Using NIPD for the fetal blood group, detection studies were accelerated after the discovery of fetal DNA in maternal plasma. The noninvasive technique of fetal RhD status of cffDNA with qPCR has been recently introduced and now is a strong alternative for traditional tests in early pregnancy. The early detection of RhD status with NIPD is advantageous and also avoids the mother from anti-RhD prophylaxis [5, 27]. For the detection of fetal *RHD* from maternal plasma, the fetal DNA extraction is a better way. In the last decade, there were significant improvements in the accurate management of pregnancies in RhD-negative pregnant women (not immunized and/or alloimmunized) by noninvasive fetal *RHD* genotyping [12, 28].

The fetal nucleated red blood cells (RBCs) are well known in maternal blood [29]. Bianchi et al. disclosed that in the first three-month period of the gestation, the fetus blood contains plenty of RBCs [30]. The RBC membrane has the RhD antigen, and when the fetus genotype is RhD positive, the alloimmunization arise when the fetal RBCs enter maternal blood. The cause is the anti-D antibodies developed by RhD-negative mother. The fetal RhD antigens can be detected on the 30–40th day of pregnancy. During the measurement with biosensor, the fetal RhD antigens cause signals (the signals increased in proportion to the gestational week). This chemical signals mean that the fetal RhD antigens on fetal RBCs bind on the surface of the biosensor that is coated with RhD antibodies (antigen-antibody complex). In RhD-positive fetuses, this chemical signal is converted into an electrical signal by a transducer. In our RhD-negative samples (five of the fetus were RhD negative), there was no signal change detected on the biosensor. The biosensor detects the fetal RhD-positive antigens in the blood of RhD-negative mothers. This study demonstrates an original, quick, reliable, and easy detection method with biosensor technologies. The design of an immunospecific biosensor offers a candidate noninvasive prenatal detection for fetal RhD status to manage the RhD incompatibility between the fetus and mother. This method is able to capture fetal RhD antigens in maternal blood in the early stage of pregnancy (8th week of pregnancy). The biosensor-based detection of fetal RhD status takes several minutes using a gold electrode covered by RhD antibody. The determined biosensor method is more suitable, simple to construct, sensitive, and specific and does not require any expensive apparatus compared with the routine fetal RhD determination in early pregnancy. The biosensor instrument exhibits low cost with regard to real-time PCR devices. The biosensors can be used several times (up to 400-fold) and so decreases the cost. The most commonly used technique for NIPD is the qRT-PCR. Studies that based on biosensor technologies for NIPD application with cffDNA for monogenic diseases reported previously [31]. Some studies

demonstrated PCR-free applications by SPR-imaging [32]. We prepared a study which detects fetal RHD genotypes from cffDNA using SPR-based biosensor.

In conclusion, the biosensor-based technologies which have used less amount of sample and low cost and determine the RhD status of the fetus in a very short time make the biosensors more advantageous than NIPD of RhD based on real-time quantitative PCR technologies.

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Conflict of interest

There is no conflict of interest between authors.

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