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# Surface plasmon resonance shows a gender difference in circulating annexin A5 in human

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ABSTRACT

The level of circulating anxA5 is correlated to various diseases such as acute myocardial infarction, trauma, thrombosis, inflammation and in some cancers. Our aim was to assess whether a direct approach using surface plasmon resonance (SPR) could be easily used to provide a rapid and cheap alternative to detect anxA5 in blood samples in human. Our results indicate that SPR permits to detect and quantify circulating anxA5 in serum with a minimum time of manipulation. Furthermore, we report here, for the first time, that the level of circulating anxA5 is significantly higher in male than in female (5.43 (±0.02) and 4.41 (±0.2) ng/ml, respectively). In conclusion, we found that SPR can be used to rapidly quantify anxA5 in patients and that a gender difference has to be taken into account to explain gender differences in the prevalence of some diseases.



## 1. Introduction

Annexins form a group of 12 highly conserved proteins sharing similarity in structure and function. All annexins share a common core structure with four repeats of the conserved 70-amino-acid domain, except eight repeats of annexin VI. These regions have the ability to bind calcium-dependently to phospholipids. They exhibit anti-inflammatory and anticoagulant properties [1–4]. They are involved in exocytosis and in cell proliferation [5,6]. Annexin A5 (anxA5, 35 kDa) is widely distributed in human tissues [7–9]. It is characterized by an ability to bind phospholipids in a reversible and $Ca^{2+}$-dependent manner and it is involved in many physiological processes, such as $Ca^{2+}$ regulated exocytosis and endocytosis, signal transduction, cell differentiation, membrane repair and coagulation. AnxA5 mostly binds to the negatively charged phospholipid phosphatidylserine (PS) [10–14]. Its interaction with phosphatidylcholine or sphingomyelin is also reported. PS resides constitutively in the inner plasma membrane leaflet but is rapidly externalized in response to an increased intracellular $Ca^{2+}$ concentration, cell injury or apoptosis. AnxA5 has also been shown to play an important role in blood coagulation and efficient recognition and removal of apoptotic cells [15,16]. As an anticoagulant factor, anxA5 prevents the interaction of exposed PS on platelets with coagulation factors such as prothrombin. Disruption of anxA5 binding to PS results in an increase in PS exposure and deregulation of blood coagulation as in the anti-phospholipid syndrome. It is also involved in the pathophysiology of many diseases such as heart disease [17–21], cystic fibrosis [22,23] and thrombosis. Reduction of circulating anxA5 levels and resistance to anxA5 anticoagulant activity was found in women with recurrent spontaneous pregnancy losses [16]. Furthermore, anxA5 is elevated 1–5.5 h in blood samples of patients with acute myocardial infarction, in old myocardial infarction, in valvular heart disease, in liver disease, in trauma, in the early stage of uterine cervical cancer and in cancer of the endometrium [15–21]. Finally, circulating anxA5 level has been inversely related to the severity of coronary stenosis [19]. These results show the increasing interest in testing the circulating anxA5 in patients, mostly in acute phase of heart disease. Actually, plasma anxA5 can be measured using commercial two-site enzyme immuno-assay. Its concentration in the plasma of healthy individuals is given to be 1.9 (±0.7) ng/ml [19] or 0.6–28 ng/ml [20].

Due to the importance to correlate the amount of circulating anxA5 with various diseases, our aim was to assess whether a direct approach using surface plasmon resonance (SPR) could be easily used to provide a more rapid and cheaper alternative to detect it in blood samples. SPR is a biosensor-based method to measure the

*Abbreviations:* SPR, surface plasmon resonance; RU, resonance units; anxA5, annexin A5.

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association of a free molecule (analyte) with an immobilized ligand. The response is observed as a curve (sensorgram), in real time, representing a SPR signal (resonance units, RU) over time [24,25]. The interest of such a methodology is that the results are obtained in real time with no labeling of the interacting molecules, providing an answer within minutes.

Our results indicate that SPR permits to detect and quantify circulating anxA5 in human serum with a minimum time of manipulation using SPR. We observed for the first time a gender difference in the amount of circulating anxA5. We found that the level of circulating anxA5 is significantly higher in male than in female (5.43 (±0.02) and 4.41 (±0.2) ng/ml, respectively).

The elevated concentration of plasma anxA5 in the early stage after the onset of acute myocardial infarction [20], in familial Mediterranean fever [26], its involvement in thrombosis, in inflammation and in many other diseases, show the importance to measure the circulating anxA5 in patients. Our results indicate that measurement of plasma anxA5 concentration in serum of patients could be performed in the emergency room using SPR. Indeed, anxA5 measurements by SPR are time consuming than ELISA and are also less expensive. Furthermore, the higher amount of circulating anxA5 in male could in part explain the gender difference observed in the prevalence of cardiovascular disease [27]. Indeed, decreased anxA5 binding to endothelial cells is now seen as a novel mechanism of atherothrombosis [28].

## 2. Experimental

### 2.1. Antibody and chemicals

AnxA5 antibody (sc-1929) and pure anxA5 (A9460) were from Santa Cruz Biotechnology and Sigma, respectively. Sensor chip CM5, Amine coupling kit (*N*-hydroxysuccinimide (NHS)), *N*-ethyl-*N*-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC), ethanolamine (1 M, pH 8.5) and HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 at pH 7.4) were obtained from GE Healthcare Bio-Sciences AB. Human serum from healthy blood donors (10 females and 10 males) was obtained from the Etablissement Français du Sang (Brest, France). Full agreement of the donors was obtained. The median age of the donors was 51 and 33 years for male and female, respectively.

All the experiments were performed at the *PurIProb* core facility (Inserm, Unité 613, Brest).

### 2.2. Antibody immobilization

Real-time detection and quantification of human anxA5 in serum analysis were performed using the Biacore system (Biacore 3000; GE Healthcare) and its Control Software version 3.2. All injections were performed at 25 °C in HBS-EP 1× running buffer (GE Healthcare). Biacore 3000 was set at 25 °C for all steps during the analytical process, and experimental data were collected at a medium rate. Biacore sensorgrams were analyzed using the BIAevaluation software. For each sample the indicated RU value is the value on the active flow cell (FC) minus the value of the reference FC, 20 s after the beginning of the dissociation phase.

Binding of anxA5 antibody was performed according to Biacore recommendations on a CM5 sensor chips which is a carboxymethylated dextran matrix covalently attached to a gold surface. Monoclonal antibody was immobilized on a CM5 biosensor chip using the Amine Coupling Kit (GE Healthcare). Before final immobilization, the immobilization pH scouting test was performed in order to determine the buffer to use for anti-anxA5 dilution. The surfaces (FC2 and FC4) were activated with equal volumes of NHS and EDC for 7 min with a 5 μl/min flow. The binding events at a sensor surface induce changes in SPR signal which are expressed in resonance units (RU). One RU is given to be equivalent to one picogram of protein per square millimeter on the sensor surface. The anxA5 antibody was injected to achieve about 2000 and 4000 RU on FC2 and FC4, respectively. The surfaces were then blocked with 1 M ethanolamine hydrochloride (pH 8.5) injection for 7 min with a 5 μl/min flow. The reference channels (FC1 and FC3) were activated with equal volumes of NHS and EDC and immediately saturated with ethanolamine.

### 2.3. Calibration and Annexin A5 detection in serum

Calibration curves consisting of relative response versus concentration of pure anxA5 in HBS-EP ranging from 0 to 5 ng/ml, were fitted with a 4 parameters logistic equation (using BIAeval 3.1 software for Biacore measurements): $f(c) = \mathrm{Rhi} - ((\mathrm{Rhi} - \mathrm{Rlo})/1 + (c/A1)A2)$, where $\mathrm{Rhi} = f(c)$ when $c \to \infty$, $\mathrm{Rlo} = f(c)$ when $c = 0$, $A1$ is fitting constant 1 and $A2$ is fitting constant 2.

Binding experiments with serum were carried out at a flow rate of 10 μl/min using HBS-EP buffer pH 7.4 as running buffer, in triplicate, at 25 °C. Venous blood of donors was immediately transported to the laboratory. Because anxA5 may bind on platelets microparticules which are present in plasma, serums were used. Bloods were collected in dry tubes without any coagulation activator. The tubes were stored at 4 °C until a blood clot was formed. The serums were then centrifuged at 2200 × *g* for 10 min at room temperature and separated. Serums were then frozen (−20 °C) in aliquots until used. In order to evaluate the best serum dilution to be used, injections of 20 μl of diluted serums in HBS-EP (0–100%) were performed over the activated surfaces and the corresponding sensorgrams were recorded. In between injections, the surface was regenerated by a 2 μl pulse of 50 mM NaOH, which was found to be a suitable condition for removing the bound analyte on the immobilized antibody with a very low impairment of the ligands. Indeed, regeneration scouting was performed using 10 mM glycine with increasing pH (1.5, 2, 2.5, 3) and 50 nm NaOH. The regeneration with acidic solutions did not permit to reach the base line whereas NaOH gave good results with very low removal of the linked antibody. Measurements were performed on FC2 and FC4. Because no difference was observed between FCs, the results presented here are from FC2 to FC1, FC1 being the reference cell.

In order to evaluate the reproducibility of this new assay, 3 among 20 serums were injected 5 times. The same procedure was repeated 3 different days to evaluate the assay reproducibility by measuring the differences between injections and from day to day.

### 2.4. Controls

To assess the specificity of the observed interactions an irrelevant protein (BSA) was used as negative control. Another control experiment was performed. A serum sample was injected on the immobilized anti-anxA5 antibody and the signal was measured. The same sample was pre-incubated with 100 ng anti-anxA5 antibody for 1 h at 4 °C and then injected.

### 2.5. Statistical analysis

Student's *t*-test was used and differences were considered significant when $p < 0.05$ (*).

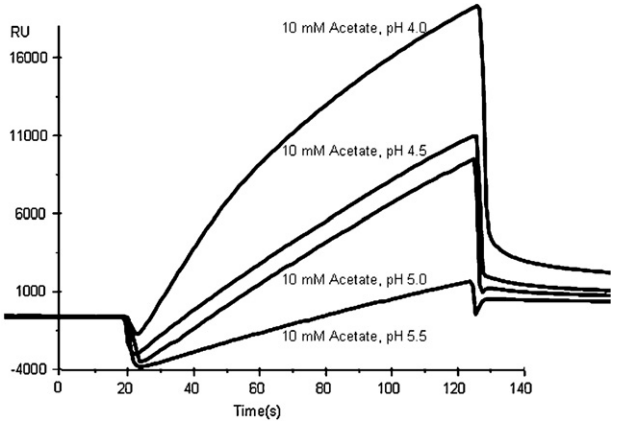


**Fig. 1.** (A) Curves of the pH scouting experiments showing that 10 mM acetate pH = 4.0 gave the best immobilization of the anti-anxA5 antibody on the sensorchip. (B) Example of sensorgram obtained during the immobilization procedure.

## 3. Results

### 3.1. Immobilization, calibration curve and first control experiment

Immobilization pH scouting test was performed in order to determine the buffer to use for anti-anxA5 dilution. 10 mM acetate pH 4, 4.5, 5, 5.5 were tested to dilute the antibody. As it can be observed in Fig. 1A, 10 mM acetate pH 4 gave the highest binding result and was compatible with the isoelectric point of anxA5 which is 4.8. Therefore, the anxA5 antibody was diluted in this buffer at a 30 μg/ml concentration. Immobilization of the anti-anxA5 antibody with a conserved activity was necessary for the biosensor analysis. Sensor chip CM5 with a dextran matrix was used because proteins covalently attached to such matrix retain their biological activity over time. Under these conditions the immobilization response varied from 2000 to 3000 RU, corresponding to a binding of 2–3 ng/mm$^2$, respectively. An example of immobilization curve is given in Fig. 1B.

Increasing quantities of pure anxA5 diluted in the running buffer HBS-EP were injected over the immobilized anti-anxA5 antibody in order to obtain a calibration curve. Examples of sensorgrams are shown in Fig. 2. An increased response was obtained accordingly to the quantity of pure anxA5 and a curve was fitted for further quantification of anxA5 in serum samples (Fig. 2, inset). The obtained RU number followed an exponential curve and linearity was observed between 3.3 and 3.8 ng/ml, with a good correlation ($R^2$ = 0.9511).

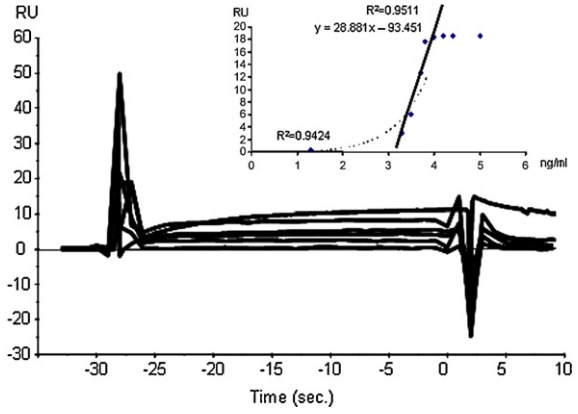


**Fig. 2.** Example of sensorgrams obtained during the injection of pure anxA5 (1.3; 3.3; 3.5; 3.7; 3.8 ng/ml) leading to a linear increase of the RU values. The inset shows the calibration curves.

Above 3.8 ng/ml, the obtained response reached a plateau phase with no further increase in the RU values.

A first control experiment was performed to assess the specificity of the observed response. BSA was diluted in HBS-EP buffer (100 μg/ml) and was injected over the anti-anxA5 antibody. No detectable binding to the antibody was observed as it can be seen in Fig. 2, lowest curve, indicating that an irrelevant protein did not bind to the immobilized antibody.

### 3.2. Injection of human serum, regeneration and second control experiment

Before injecting the serum samples, the question was to know whether serum had to be diluted in the running buffer. To answer this question a serum sample was randomly chosen, diluted in HBS-EP (0–100% serum) and injected on the activated sensorchip (Fig. 3A). It was observed that for all the used dilution a sensorgram could be obtained. After each injection, the RU values were noted 20 s after the end of injection, reflecting the amount of bound anxA5 on the linked antibody. A curve (log(RU) = % of dilution) was then drawn (Fig. 3B) and it was observed that above the 10% dilution of the serum in HBS-EP the RU values did not increase. Therefore, all further experiments were performed with the 10% dilution.

The regeneration is aimed to remove the bound anxA5 between each injection. Unsuitable proteins present in serum can affect the immunoassay by binding to the sensor surface, producing a non-specific response that hides the real interaction. These proteins can additionally interfere in the binding of the antigen to the immobilized antibody, leading to a reduced specific signal. Antigen–antibody interactions can be disrupted by different strategies and it is often obtained by the injection of extreme pH solutions. Therefore, we tested the recommended buffers to disrupt the interaction on the sensorchip (Fig. 4). 10 mM glycine with pH values ranging from 1.5 to 3 and 50 mM NaOH were tested. We observed that the injection of 2–5 μl NaOH permitted to reach the base line after each injection with a minimal alteration of the signals between samples. The stability of the immunosensor upon

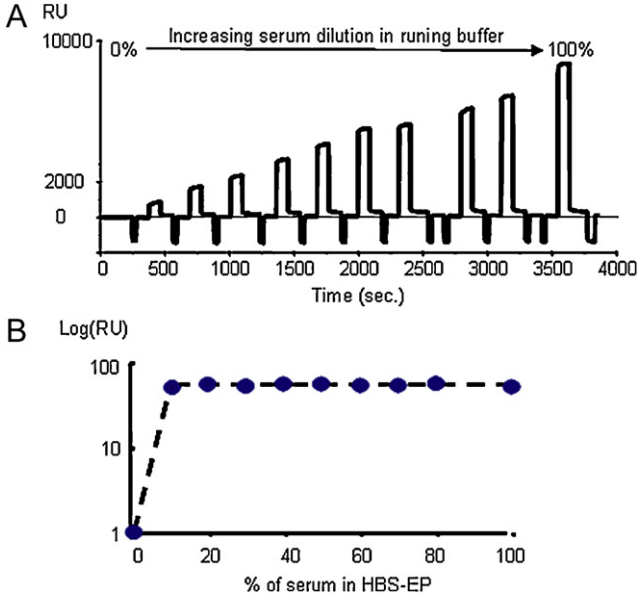


**Fig. 3.** (A) Sensorgram obtained when increasing dilution of serum in HBS-EP was injected on the immobilized anti-anxA5 antibody. (B) Curve showing the RU values in function of the dilution of the injected serum. This curve shows that above 10% dilution, the RU values did not increase, indicating that this dilution could be further used in the study. Pure serums correspond to 91.9 mg/ml (±9.2).

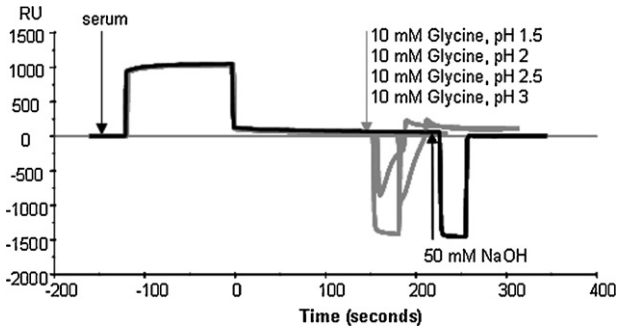


**Fig. 4.** Sensorgrams obtained during the determination of the regeneration buffer. Grey curves obtained with glycine show that the base line is not reach after injection. The black curve obtained with 50 mM NaOH shows that the base line was reached, showing that this buffer was suitable for the regeneration procedure.

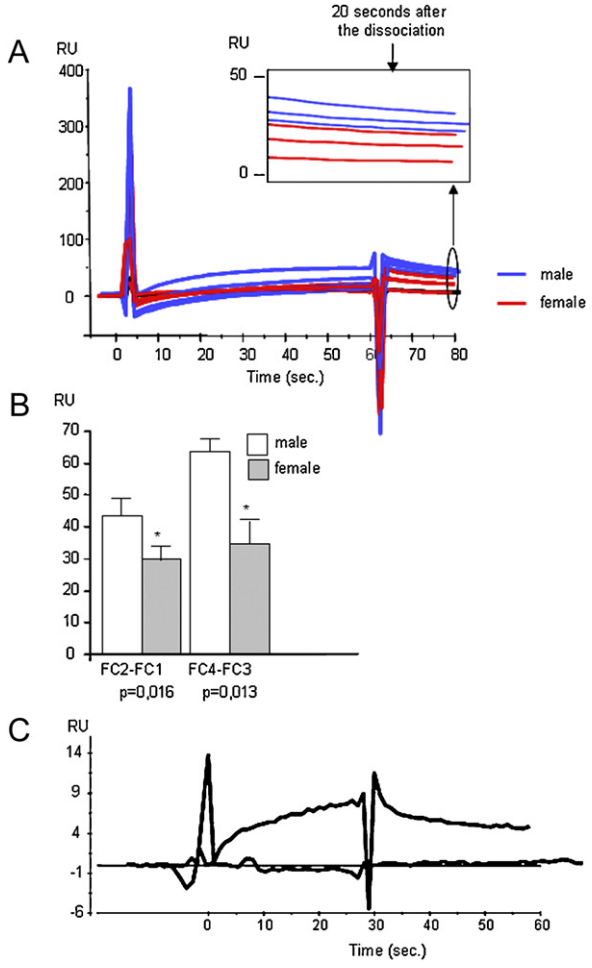


**Fig. 5.** (A) Example of sensorgrams obtained when serum were injected. It can be observed that the curves are higher with serum from males (blue) than with serum from female (red). Inset represents an enlarged view of the sensorgrams 20 s after the end of injection showing the difference between male and female. (B) Statistical analysis of the RU obtained with serum. The RU values were taken 20 s after the end of injection. Histograms show that the RU values were significantly higher in male than in female on FC2 as well as on FC4. (C) Example of sensorgram obtained during the control experiment in which serum were pre-incubated with the anti-anxA5 antibody prior the injection. This pre-incubation abolished the response. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

regeneration was tested by measuring the variations of the base line (not shown).

Serum samples were then injected on the immobilized anti-anxA5 antibody and sensorgrams were recorded and plotted (Fig. 5A). It was observed that the curves obtained with male's serum were higher than that of female. The RU value for each sample was noted 20 s after the beginning of the dissociation phase and a statistical analysis was performed in order to quantify the observed difference among gender. The results clearly showed that the amount of circulating anxA5 was significantly higher in male than in female, on the FC2 as well as on the FC4 flow cells (Fig. 5B). In male, circulating anxA5 was 5.43 (±0.02) ng/ml in serum whereas in female it was 4.41 (±0.2) ng/ml ($p = 0.002$).

Another control experiment was performed, using serum. A serum sample was separated in two parts. The first part was directly injected and the second part was pre-incubated in the presence of an excess of anxA5 antibody before its injection over the active surface (Fig. 5C). The pre-incubated serum did not show any binding resulting in a flat sensorgram showing that the observed results with serum were really due to the presence of anxA5 in the samples, excluding a non specific binding.

### 3.3. Reproducibility

The immunoassay reproducibility was studied by the assessment of the intra- and inter-day variability. The same serum was passed over the sensorchip 5 times on 3 different days to assess within-day and day-to-day variation. The coefficients of variation (CV%) were calculated. All the individual coefficients of variations were below 5% and thus by far within the accepted variability values for analytical methods (Fig. 6A). The day-to-day variations were assessed using Student-*t* test. There was a significant variation between day 1 and day 2 or 3, indicating that a standardization has to be performed for each set of experiments (Fig. 6B). In the present work, all the experiments were thus performed the same day.

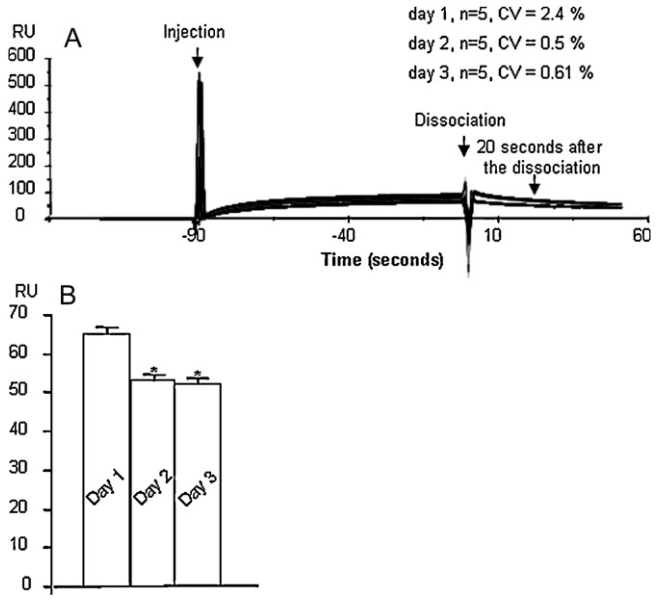


**Fig. 6.** (A) Example of sensorgrams obtained for a single sample injected three different days. All the curves obtained the same day are superimposed showing that experiments were reproducible within given day. (B) Histograms represent the variations observed in the RU values from day to day for a given sample. Lower signals were obtained after day 1 explaining why all experiments were performed the same day.

## 4. Discussion

The SPR-sensor approach was used to detect and quantify anxA5 in human serum. Our results indicate that this technology permits the quantification and that the obtained results are in the range of the previous results obtained by ELISA. Circulating anxA5 is given to be 1.9 (±0.7) ng/ml [19] or 0.6–28 ng/ml [20], depending on the authors. We found that it is 4–5 ng/ml. Therefore, our results suggest that the label-free SPR technique can serve as a very good alternative to the label-based conventional ELISA immunoassay. SPR was found to be quite comparable to ELISA with some advantages such as sensitivity, reproducibility, absence of labeling and low time consuming. The need for only one antibody for the direct detection of circulating anxA5 protein in human serum makes SPR a very attractive strategy for disease biomarker studies. Nevertheless, the development of SPR for clinical applications is hindered by matrix effects due to high molecular weight species present in any complex biological samples such as serum. The applications of SPR using serum generally require pre-treatment of the samples. In the present work we achieved anxA5 determination in serum directly with a 1/10 dilution in HBS-EP and without any pre-treatment. The regeneration between injections also reduces the sensor life time and alters the signal due to the removing of some linked antibodies. The biosensor reusability depends on the stability of the surface among measurements and we found that the reutilization of the sensorchip is accomplished very simply with a complete dissociation of the antigen–antibody complex without affecting the affinity of the immobilized molecule.

SPR distinguished, for the first time, different anxA5 levels in male and female without amplification or labeling steps. This latest point is of importance. Indeed, circulating anxA5 is increased in patients with acute myocardial infarction and other heart disease which are more prevalent in men. Therefore, the higher level of circulating anxA5 that we observed could in part explain this gender difference.

In conclusion, the present work shows that circulating anxA5 can be rapidly measured in human using SPR and that its levels vary with gender. These findings are of importance for clinical diagnosis.

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## References

[1] B.P. Wallner, R.J. Mattaliano, C. Hession, et al., Nature 320 (1986) 77–81.
[2] J.S. Brugge, Cell 46 (1986) 149–150.
[3] T. Funakoshi, R.L. Heimark, L.E. Hendrickson, B.A. McMullen, K. Fujikawa, Biochemistry 2 (1987) 5572–5578.
[4] C. Ravanat, G. Archipoff, A. Beretz, G. Freund, J.P. Cazenave, J.M. Freyssinet, Biochem. J. 282 (1992) 7–13.
[5] T.J. Yeatman, T.V. Updyke, M.A. Kaetzel, J.R. Dedman, G.L. Nicolson, Clin. Exp. Metastasis 11 (1993) 37–44.
[6] T. Nakata, K. Sobue, N. Hirokawa, J. Cell Biol. 110 (1990) 13–25.
[7] C.P. Reutelingsperger, Thromb. Haemost. 86 (1) (2001) 413–419.
[8] P. Trouvé, S. Legot, I. Belikova, F. Marotte, D. Benevolensky, F. Russo-Marie, J.L. Samuel, D. Charlemagne, Am. J. Physiol. 276 (1999) H1159–H1166.
[9] D. Benevolensky, Y. Belikova, R. Mohammadzadeh, P. Trouvé, F. Marotte, F. Russo-Marie, J.L. Samuel, D. Charlemagne, Lab. Invest. 80 (2000) 123–133.
[10] P. Comfurius, E.F. Smeets, G.M. Willems, E.M. Bevers, R.F. Zwaal, Biochemistry 33 (1994) 10319–10324.
[11] C.P. Chang, J. Zhao, T. Wiedmer, P.J. Sims, J. Biol. Chem. 268 (1993) 7171–7178.
[12] J. Zhao, Q. Zhou, T. Wiedmer, P.J. Sims, J. Biol. Chem. 273 (1998) 6603–6606.
[13] V. Gerke, C.E. Creutz, S.E. Moss, Nat. Rev. Mol. Cell Biol. 6 (6) (2005) 449–461.
[14] A. Bouter, C. Gounou, R. Bérat, S. Tan, B. Gallois, T. Granier, B.L. d'Estaintot, E. Pöschl, B. Brachvogel, A.R. Brisson, Nat. Commun. 2 (2011) 270.
[15] F.A. Kuypers, S.K. Larkin, J.J. Emeis, A.C. Allison, Thromb. Haemost. 97 (2007) 478–486.
[16] J.H. Rand, A.A. Arslan, X.X. Wu, R. Wein, J. Mulholland, M. Shah, W.L. van Heerde, C.P. Reutelingsperger, C.J. Lockwood, E. Kuczynski, Am. J. Obstet. Gynecol. 194 (2006) 182–188.
[17] N. Kaneko, R. Matsuda, S. Hosoda, T. Kajita, Y. Ohta, Clin. Chim. Acta 25 (1996) 65–80.
[18] S. Shirotake, N. Inaba, Y. Ohta, I. Fukazawa, H. Takamizawa, H. Bohn, Chem. Pharm. Bull. 34 (1986) 2191–2195.
[19] L.J.H. van Tits, W.L. van Heerde, G.M. van der Vleuten, J. de Graaf, D.E. Grobbee, L.P.L. van de Vijver, A.F. Stalenhoef, H.M. Princen, Biochem. Biophys. Res. Commun. 356 (2007) 674–680.
[20] R. Matsuda, N. Kaneko, M. Kikuchi, F. Chiwaki, M. Toda, T. Ieiri, Y. Horikawa, M. Shimizu, K. Shimamoto, Resuscitation 57 (2003) 171–177.
[21] R. Gonzalez-Conejero, J. Corral, V. Roldan, C. Martinez, F. Marin, J. Rivera, J.A. Iniesta, M.L. Lozano, P. Marco, V. Vicente, Blood 100 (2002) 2081–2086.
[22] P. Trouvé, M.A. Le Drévo, M. Kerbiriou, Y. Fichou, D. Gillet, C. Férec, Biochim. Biophys. Acta 1772 (10) (2007) 1121–1133.
[23] M.A. Le Drévo, N. Benz, M. Kerbiriou, M.A. Giroux-Metges, J.P. Pennec, P. Trouvé, C. Férec, Biochim. Biophys. Acta 1782 (10) (2008) 605–614.
[24] G.R. Marchesini, K. Koopal, E. Meulenberg, W. Haasnoot, H. Irth, Biosens. Bioelectron. 22 (2007) 1908–1915.
[25] R. Gutiérrez-Gallego, J. Bosch, G. Such-Sanmartín, J. Segura, Growth Horm. IGF Res. 19 (2009) 388–398.
[26] A.S. Boyajyan, G.M. Mkrtchyan, L.P. Hovhannisyan, T.J. Hovsepyan, J. Inflamm. 7 (2010) 55.
[27] T. Gijón-Conde, J.R. Banegas, Rev. Clin. Esp. 212 (2) (2012) 55–62.
[28] A.G. Frostegard, J. Su, P. Von Landenberg, J. Frostegard, Scand. J. Rheumatol. 39 (1) (2010) 77–83.