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AN IMMUNOSENSOR FOR THE DETECTION OF HUMAN B-LYMPHOCYTES

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Abstract. The number of purified human B-lymphocytes in blood samples was determined with a piezoelectric immunosensor. Immobilizing an anti-B-cell antibody layer onto a 10 MHz-AT cut crystal resulted for 5 x 10^3 to 5.6 x 10^5 cells in a linear frequency change and a long term stability of six weeks. The coated crystal can be used eight times without detectable loss of activity.

Keywords. Piezoelectric Immunosensor. Human B-lymphocytes. Anti-B-lymphocyte Antibody. Whole Blood.

B-lymphocytes belong to the familiy of leucocytes and are found in primary and secondary lymphoid organs as well as in body fluids[1]. Because B-cells play a central role in the immune system of mammalia, it is of great interest to determine the number of these cells in whole blood samples. This is generally done with (i) the haemocytometer, or (ii) electronic particle counting[1]. A possible alternative is the use of piezoelectric antibody-based biosensors[2].

Antibody-based biosensors detect the antigen concentration either by indirect competitive and displacement reactions similar to immunoassays, or by direct changes in transducer output[2]. An example for the latter type of system is the piezoelectric (PZ) crystal detector. Piezoelectric devices consist of an oscillating quartz containing an adsorbent (antibody) on its surface that selectively interacts with the analyte. Adsorption of the analyte (antigen) increases the mass of the crystal and decreases proportionally the resonance frequency of oscillation.

Since recently antibodies are available in large amounts, biosensors are being developed for a wide range of applications such as food industry, environmental monitoring and processing, biotechnology, and now mainly clinical diagnostics[3-7]. The piezoimmunosensor system offers many of the properties desired for ideal immunosensors such as sensitivity, specificity, stability and simplicity of use.

Table I

Frequency changes (ΔF) due to binding of purified human B-lymphocytes (1.5×10^5 cells) and B-lymphocytes in whole human blood to the anti-B-cell antibody modified crystal surface with three different immobilization procedures. Average of five experiments ± SEM (with one electrode).

Immobilization method	ΔF purified B-lymphocytes	ΔF whole human blood
polyethyleneimine	8318 ± 482	8764 ± 838
γ-amino propyltriethoxy silane	8007 ± 476	8645 ± 895
protein A	8138 ± 481	12068 ± 1478

To develop such a system, we have tested three different immobilization procedures to fix a B-cell specific monoclonal antibody to the gold electrodes. The antibody (anti-CD 19 gp 95kD antibody) binds a 95 kD glycoprotein displayed on the surface of the lymphocytes[8].The best results in sensitivity, reusability and stability were observed with the polyethyleneimine (PEI) method (Table I and figure 1). The crystal precoated with PEI is stable for six weeks and provided the most reproducible results of all of the immobilization procedures tested (Table I). These results are in agreement with earlier results by Prusak-Sochaczewski et al.[9]. Figure 2 shows the plot of the frequency change versus number of pure human B-lymphocytes. The signal is linear from 5×10^3 to 5.6×10^5 cells (correlation coefficient: 0.993). No enhanced frequency change is observed when detecting B-lymphocytes in whole human blood instead of purified B-lymphocytes (Figure 2). With an increased number of cells however ($> 4.5 \times 10^5$) a 5% enhanced signal for whole human blood is seen. Additional control experiments were done to show the specificity of our system. With increasing amounts of non-lymphocyte whole human blood samples a linear increase of the frequency shift is observed. No saturation is seen and the signal height is about 7% of the height of the B-lymphocyte signal (Figure 2). Similar results have been observed with other samples like human serum albumin, immunoglobulin fractions and purified erythrocytes (data not shown). However, the 5% increased signal for whole human blood and cell numbers of more than 4.5×10^5 indicated that a slight amount of unspecific material contained in the blood sample adheres to the modified electrode, as can also be seen from the control experiments with other blood cells or proteins. We believe that this is due to the increased cell density on the electrode surface, resulting in an increased probability for nonspecific cell aggregation.

Immobilization via γ-amino propyltriethoxy silane (APTES) has similar results with a slightly (4%) reduced sensitivity. Also linear from 5 x 10^3 to 5.6 x 10^5 cells (correlation coefficient: 0.999), the APTES modified electrode showed a 9% increased frequency change when detecting B-lymphocytes in whole blood samples compared to the results of purified B-lymphocytes (Table I). We also tested the long-term stability of this system. When stored desiccated at room temperature the modified crystal surface is stable for only five weeks and also the number of regenerations (six) is reduced (data not shown).

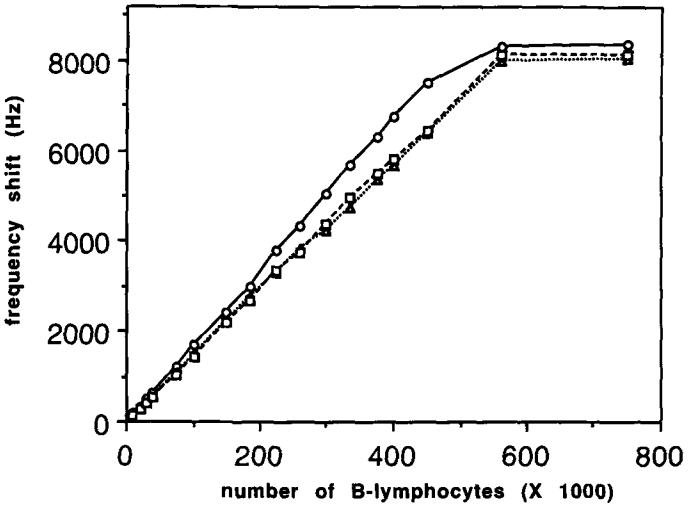

Fig. 1. Comparison of three different immobilization procedures and its relationship between the number of human B-cells on the crystal and the frequency change of the anti-CD 19 gp 95kD antibody coated crystal. ○ immobilization via PEI; Δ immobilization via APTES; ❑ immobilization via protein A. The plot shows that the frequency change is linear from 5 x 10^3 to 5.6 x 10^5 cells. Each point represents the average of ten experiments (different crystals used for each set of experiment testing the response from 5 x 10^3 to 7.5 x 10^5 cells). The number of purified human B-cells were determined with a standard haemocytometer (improved Neubauer). Incubation time: 60 minutes. The experiments were done as described earlier[6] and B-lymphocytes have been purified according to standard procedures[1].

Sensors prepared by immobilization via protein A gave the most unfavorable results (Table I and figure 1). In our hands this method seems unsuitable for directly detecting B-cells in whole blood, because of the high interference with other blood

cells. Although the observed frequency change is comparable to the PEI method when using purified B-lymphocytes (2% difference, which is within experimental error), the highest interference from unspecifically bound material (33%) with whole human blood samples is observed (Table I). Additionally, the crystal shows a slightly reduced life-time (five-weeks; data not shown), because it could not withstand the regeneration procedure. When the crystal was immersed in a solution containing 8 M urea, partial desorption of the immobilized antibody occured. Other dissociating agents such as 0.2 M glycine-HCl (pH 2.8) and 0.2 M ethanolamine (pH 8)

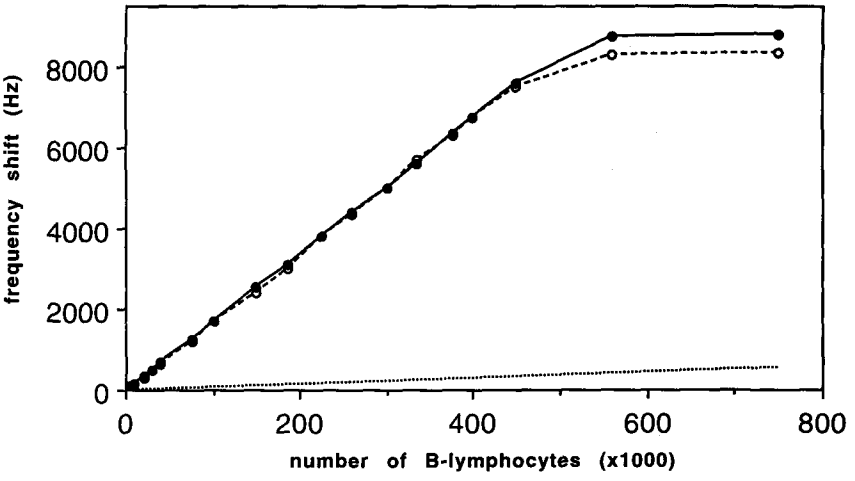

Fig. 2. Comparison of frequency changes with increasing numbers of purified human B-lymphocytes (○) and B-lymphocytes in whole human blood (●) (PEI immobilization procedure). The frequency change is linear in the range of 5 x 10^3 to 5.6 x 10^5 cells. Up to 4.5 x 10^5 cells the purified B-lymphocytes and B-lymphocytes in whole human blood gave nearly identical results. A 5% increased frequency change with whole human blood is observed when larger amounts (> 4.5 x 10^5) of B-cells are used. Control experiments with non-lymphocyte human blood showed a linear increase of the signal height with increasing amounts of sample material added to the crystal surface (doted line). Crystals were regenerated with 8 M urea for 10 minutes at room temperature. The number of purified human B-cells was determined with a standard haemocytometer (improved Neubauer). For experimental conditions see figure 1.

did, however, not totally remove the adsorbed cells. This is in contrast to the results published by Plomer and coworkers[10], who obtained the best results with this immobilization procedure. A possible explanation is that due to the non-

homogeneous electrode surface that exposes hydrophilic as well as hydrophobic parts, the protein A derivatized electrodes exhibit enhanced nonspecific interaction with non B-lymphocytic cells contained in the blood.

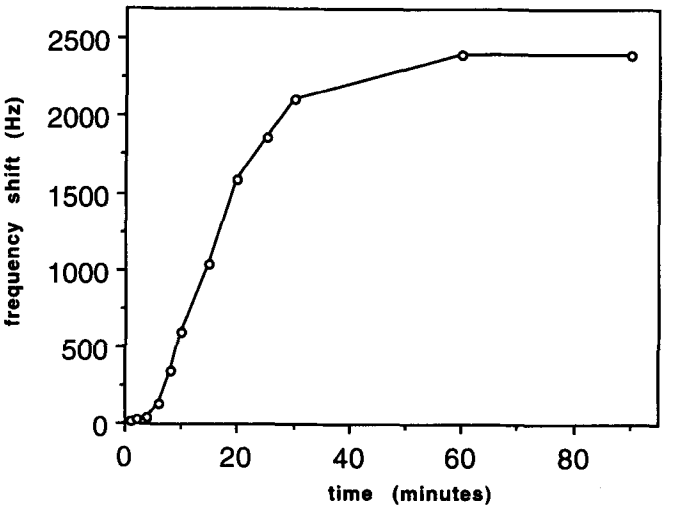

Fig. 3. Correlation between human B-lymphocytes and anti-B-cell antibody reaction time resonance frequency shift (ΔF) for cell concentrations of 1.5×10^5 cells ml^{-1}. After 60 minutes the antigen-antibody reaction is nearly complete. Each point represents the average of five experiments (done with five different cristals). The experiments were done as described earlier[6] and B-lymphocytes has been purified according to standard procedures[1].

An important factor affecting the sensitivity of the piezoelectric detecting system is the time of incubation of the antibody-coated crystal with antigen. In order to optimize this parameter the crystal was exposed to a solution of 1.5×10^5 B-lymphocytes ml^{-1} for different periods. The results show that an incubation time of 60 minutes gives optimal sensitivity (Figure 3). Prolonged exposure up to 90 minutes, did not improve the observed frequency change essentially. Thus, in all subsequent experiments a 60 minute incubation time was used.

Using an ELISA for the determination of B-lymphocytes in blood samples showed that the detection limit with this technique is about 2×10^3 cells (data not shown) and is therefore about three-times more sensitve than our piezoelectric immuno-

sensor. However, the linear response of the ELISA is limited to one decade (data not shown), compared with two decades for the piezoelectric cristal detector (Fig. 2).

The results reported above demonstrate that a piezoelectric crystal coated with PEI and the anti-B-lymphocyte antibody can be applied for determination of purified human B-lymphocytes as well as B-lymphocytes in whole human blood. This very promissing technique offers the possibility for the rapid determination of the number of B-cells in whole human blood. The piezoelectric system seems to be optimal for such tests, because the coating process with the polymer (polyethyleneimine) is simple, cheap and reproducible and compared to the ELISA the piezoelectric immunosensor is, with nearly similar sensitivity and specificity, faster and has an increased linear response. Additionally from the plots of frequency change versus number of B-lymphocytes one can estimate 5.6×10^5 B-lymphocytes as the maximum number of cells bound to the electrode area. Comparing this number with the theoretical maximum possible number of B-lymphocytes (6×10^5 for an area of 4.4×10^7 μm^2 and a diameter of 8.5 μm for the B-lymphocytes) showed that more than 90% of the electrode surfaces are occupied with cells. Therefore, we believe that in the near future this technique may replace the methods now in use.

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