

Short communication

A ‘gold cluster-linked immunosorbent assay’: Optical near-field biosensor chip for the detection of allergenic β -lactoglobulin in processed milk matrices

Verena Hohensinner¹, Irene Maier^{*,1}, Fritz Pittner*Max F. Perutz Laboratories, Department of Biochemistry, University of Vienna, Dr. Bohr-Gasse 9, A-1030 Vienna, Austria*

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Abstract

A new optical biosensor based on the resonance enhanced absorption (REA) effect is described. REA effects are observed when noble metal nanoclusters are deposited at a nanometric distance from a highly reflective mirror. The aim of our study was to adopt the REA effect for the rapid testing of proteins in a direct immunoassay format on chip and to adjust a conventional enzyme-linked immunosorbent assay (ELISA) to a cluster-linked immunosorbent assay (CLISA) by labelling the read-out antibody with monodisperse colloidal gold clusters. For generation of a strong REA signal 30 min of coating of the target protein was sufficient. To evaluate our approach we used the milk allergen β -lactoglobulin (β -LG) as analyte, and β -LG-isolations of processed milk products to prove the applicability of our method to the analysis of proteins in complex matrices at even the trace level. For validating the specificity of the CLISA biosensor we used the non-functionalised cluster reagent without antibody and a non-immunoreactive milk matrix as controls. As expected, very weak background signals were obtained with the controls, whereas the purified food samples clearly showed that β -LG was present and detectable. In conclusion, we were able to describe the successful development of a new biosensor chip for assaying proteins using the REA effect.

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Today's bio-analytical questions demand for a ready and easy to use analytical device for time and cost efficient determination of analytes in complex food matrices. These demands can be fulfilled by utilizing noble metal nanoparticles as signal transducers in optical biosensors (Haglmüller et al., 2005a; Bauer et al., 2003; Niemeyer, 2001; Seydack, 2005). Sub-wavelength sized metal nanoclusters exhibit optical resonances (surface plasmons) associated with collective electron oscillations that can be used to monitor changes in the surrounding medium (Hohenau et al., 2006; Eustis and EL-Sayed, 2006) in different ways: The advanced reflectivity-based ‘Surface Plasmon Resonance’ (SPR) method on a very thin gold film, where changes in the angle-dependent intensities of the reflected light are detected in an attenuated total reflection geometry (Karlsson, 2004; Okamoto et al., 2000); and the extinction-based method

that measures the absorptive and scattering properties of excited bio-functionalised nanoparticles (Su, 2006; Li et al., 2006a; Nath and Chilkoti, 2002; Seelenbinder et al., 1999). In the reflectivity-based setup, real time, label-free analysis of all kinds of chemistries – mostly biorecognitive interaction systems – can easily be monitored where traditional optical spectroscopy is limited. Nevertheless, one drawback of this SPR technique is that no visual analysis can be carried out. An alternative approach was developed relying on the measurement of the spectral shift of the plasmon peak's absorbance due to the aggregation of gold nanoclusters by DNA hybridization in bulk solution (Mirkin et al., 1996; Elghanian et al., 1997; Han et al., 2006a,b). This characterization technique was shown to be a very simple colorimetric bioassay, but lacking stability and optical near-field information.

In contrary, we describe immunosensing by (plasmon) resonance enhanced absorption above a reflecting mirror (REA). REA is observed when light-absorbing metal clusters are positioned at a nanodistance from a highly reflective mirror and illuminated with white light of the visible and near-infrared

* Corresponding author. Tel.: +43 1 4277 52808; fax: +43 1 4277 9528.

E-mail address: irene.maier@univie.ac.at (I. Maier).

¹ These authors contributed equally to this work.

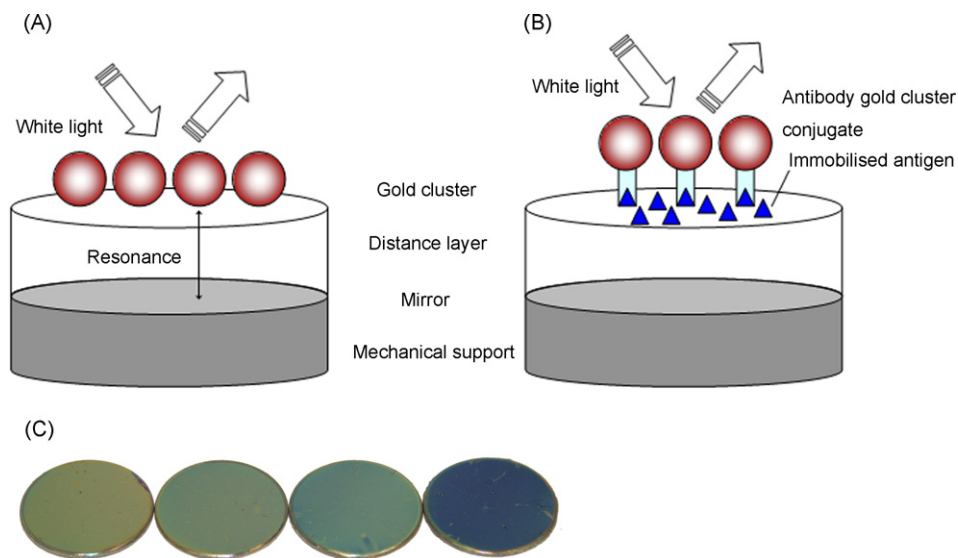


Fig. 1. (A) Schematic illustration of the REA setup. The nanoclusters are positioned above a mirror surface. For a certain distance from the cluster layer to the mirror the electromagnetic field that is reflected by the mirror has the same phase as that of the incident field. This feedback mechanism strongly enhances the extinction coefficients of the clusters. (B) In this capture assay the antigen is immobilised onto the surface of the distance layer. The antibodies which are labelled with gold clusters, bind to the antigen. (C) Variation of the thickness of the distance layer. Decreasing concentrations of the applied polymer solution for spin coating appear as changes in resonance colour (from left to right). The gold cluster layer was applied by sputter coating.

spectrum from the particle side (Fig. 1A). The mirror usually is built up from a flat surface with appropriate chemical, mechanical and thermal stability and metallised with silver or aluminium. Plasmon resonance absorption of mirror-reflected light is phase modulated and, therefore, inherently determined by the distance of the mirror to the excited cluster. This nanointerference system acts similar to a reflection filter and is angle-dependent due to the incident waves. Its feedback mechanism strongly enhances the absorption coefficient of the metal clusters which results in an extremely sensitive colour response that is detectable without instrumentation in the visual range by the ‘naked’ eye. The spectral position of the resonance peak which is associated with the developed colour on the surface is a measure of the distance between the cluster layer and the mirror surface, and can be utilized to determine changes in thickness of a few nanometres (Fig. 1C). As optical interlayer transparent organic polymers are used. The development of colour on chip can be calibrated according to the amount of immobilised antigen, since the intensity of the resonance colour correlates directly to the amount of clusters deposited on the distance layer by affinity binding. Thus, this REA setup allows for monitoring biorecognitive processes showing stable, semi-quantitative, visually detectable signals (Haglmüller et al., 2005b; Mayer et al., 2001).

The aim of the present study was to establish a cluster-linked immunosorbent assay (CLISA) in direct immunoassay format using the REA technology (Fig. 1B). Additionally, we sought to validate the applicability of our test system to the analysis of the milk protein β -lactoglobulin (β -LG) extracted of processed milk matrices. β -LG is the major whey protein and also the major allergen in milk (Besler et al., 2001). A wide study investigating conformational epitopic binding sites of the native protein by different immunochemical and instrumental analysis was reported by Clement et al., 2002.

1. Preparation of biochips

To prepare the biochips, we combined the mechanical support and the mirror using highly polished aluminium foil discs with a diameter of 13 mm (Amag Ranshofen, Austria). A nanoscale optical transparent polymer film was established to serve as a distance layer and binding surface for assay forming biomolecules. The concentration of the polymer solution and the spin coating parameters were adjusted to give a blue resonance colour after applying gold clusters on the chip: 100 μ l of 38 mg ml⁻¹ poly-(styrene/methyl methacrylate) (PS/PMMA 70:30, random copolymer, Polysciences, Eppenheim, Germany) in organic thinner (AZ 1500, Hoechst, Frankfurt, Germany) were spin coated onto the aluminium discs at 4000 rpm for 12 s using a spin coater model P 6700 (Speciality Coating Systems, Inc., Indianapolis, US). To control the thickness of the distance layer, we applied a 3–5 nm layer of gold nanoclusters on control chips by sputter coating with a sputter coater (model 108, series 258, Agar). Fig. 1C shows the effects of varying the thickness of the distance layer by altering the polymer concentration.

2. Preparation of the antibody-gold cluster-conjugate

The gold nanoparticles were prepared according to the literature (Frens, 1973; Li et al., 2006b) by adding 4 ml of 1% (w/v) sodium citrate solution to a boiling solution of 100 ml of 0.01% (w/v) HAuCl₄·3H₂O (Sigma, Steinheim, Germany). The maximum absorption in ultraviolet-visible spectra (plasmon peak) was about 520 nm. According to the literature the particle size was calculated to be 16 nm (Li et al., 2006b). We conjugated purified polyclonal rabbit anti-bovine β -LG IgG (β -LG-AB, 1.0 mg ml⁻¹, Bethyl Laboratories, Montgomery, TX, US) to the gold cluster. The respective amount of β -LG-AB

was added to 10 ml cluster solution under constant stirring and stirred for 20 min at room temperature (RT). To stabilise the conjugate Triton X-100 (Fluka, Buchs, Switzerland) was added to a final concentration of 0.5% (v/v). The solution was then transferred into Eppendorf tubes (10 times à 1 ml) and centrifuged at 13,000 rpm (Biofuge 13, Heraeus Sepatech) for 10 min. The clear and almost particle-free aqueous supernatant was taken off, leaving 50 µl volume left in the tubes for gently resolving the gold cluster pellet. Thus, 10 ml of the conjugated gold cluster solution were reduced to a volume of 500 µl of the final read-out solution. The concentrated β -LG-AB-gold cluster-conjugate was stored at 4 °C.

For optical monitoring of the results, we used a Canon EOS 350D (Canon, Japan) with a zoom lens (18–55 mm, Canon). Photos were taken on a shooting table (Kaiser Fototechnik, Germany) in a fixed angle of 45° and a time value of 1/80 and an aperture value of 5.6.

3. Cluster-linked immunosorbent assay

The antigen was immobilised on the surface of the distance layer (coating). Coating was performed using 1 mg β -LG (Sigma) dissolved in 1 ml 0.01 M phosphate buffered saline (PBS) pH 7.4. To generate a strong signal 30 min of coating were sufficient. After washing and blocking three times with 0.1 M PBS buffer pH 7.4 containing 0.1% (v/v) Tween-20 for 10 min at RT, anti- β -LG-gold cluster-conjugate was applied. To examine the optimal incubation time with the concentrated antibody-cluster-conjugate, it was varied from 30 min to overnight. An

increase in signal intensity with increasing incubation time was observed. As our experiments showed significant signal intensities after 5 h of incubation, this incubation time was applied in further experiments. The upper limit of antibody concentration was reached at 25 µg β -LG-AB per 10 ml gold cluster solution. At higher concentrations partial undesired clotting of the gold particles was observed. The working titre was finally assessed to be 15 µg in 10 ml (before concentrating the detection reagent). The dynamic range of the CLISA method was examined as between 100 µg ml⁻¹ and 100 ng ml⁻¹. To validate the biochip sensor for the specific analysis of allergenic compounds in complex matrices and protein extractions thereof, different controls were carried out. We used a milk hydrolysate (hypoallergenic infant formula, HIPPI, Pfaffenhofen, Germany) as non-immunoreactive control (zero standard). The standard β -LG sample showed high signal intensities, whereas the non-immunoreactive controls exhibited very low signals (Fig. 2A-A). As negative control a concentrated gold cluster solution without antibody was used to estimate unspecific binding of the cluster to the proteins. For the determination of the blank value PBS buffer instead of protein solution was used for the coating step. No signal was obtained with PBS and also when no antibody was used for the zero standard. The chips remained absolutely blank after running the assay as shown in Fig. 2A-A, line 3. Very low signals were obtained when no antibody was applied for detecting the analyte protein. Therefore, our results clearly demonstrate that the response of a REA based chip is evaluated for specifically monitoring immunochemical affinity binding of proteins. To determine if the REA setup is also capable of detecting β -LG in

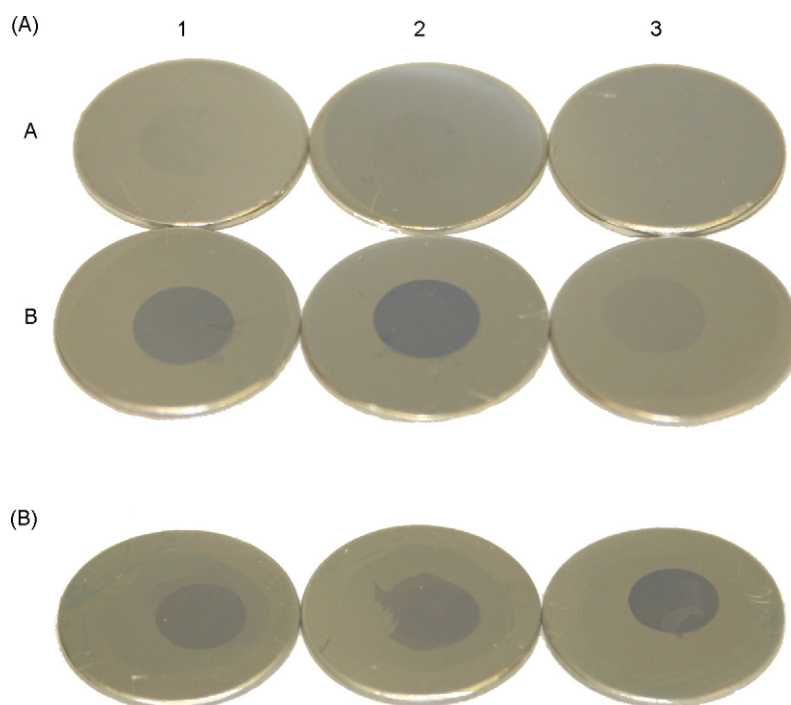


Fig. 2. (A) Validation of CLISA. Line A was coated with the non-immunoreactive control (1 mg ml⁻¹ milk hydrolysate in 0.01 M PBS buffer pH 7.4). Line B was coated with β -LG solution (1 mg ml⁻¹ in 0.01 M PBS buffer pH 7.4). After washing and blocking, the chips were incubated with a concentrated β -LG-AB-gold cluster-conjugate. Lines 1 and 2 show a variation of the antibody titre. In line 1, 10 µg β -LG-AB per 10 ml gold cluster solution were used. In line 2, 20 µg β -LG-AB were applied. Negative controls are depicted in line 3, where the concentrated cluster solution without antibody was applied. (B) Detection of β -LG in processed milk matrices. The purified samples of baby food milk products (from the left to the right: CV, MB and MFJ) showed homogeneously blue coloured signals.

complex matrices, we used differently processed baby food milk products (papescent ‘Vanilla Crème Dessert’ (CV) and ‘Good Night Baby Food’ (MB) – both available in glasses; and a ‘Fruit Yoghurt’ (MFJ) dried powder; HIPPI). β -LG was isolated from those products as described recently (Czerwenka et al., 2006). Allergenic β -LG was not only detectable in the food samples, but the intensities of the signal were also in good agreement with the theoretical and immunochemically detectable β -LG content of these products (Fig. 2B). The response obtained for CV and MB ranged about $500 \mu\text{g ml}^{-1}$, whereas the signal for the dried powder MFJ was significantly higher and ranged about 2 mg ml^{-1} .

We conclude that the presented simple CLISA method is capable of specifically identifying proteins with high sensitivity comparable to so far applied immunoassays. In our report we show clear evidence that a REA based biosensor chip can be used as rapid test for protein detection in routine analysis.

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