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A dot-blot test using gold colloid cluster technology as a miniaturizable alternative to ELISA and hapten inhibition tests

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Abstract A novel miniaturizable method to quantify antigens is described in form of a cluster linked immunosorbent assay (CLISA), using inexpensive nitrocellulose (*NC*) membranes as a support for dot-blotting the antigens. Antibodies for detection are labeled with gold-colloid clusters (*GCC*). After blocking of the membrane with non reacting protein and application of the *GCC*-labeled antibodies the signal is detectable by visual colorimetry and can be compared to a color scale prepared from a dilution series of known sample concentrations. The color reaction product is stable for a very long time and does not fade. The sensitivity of the method is comparable to that of ELISA if not better and furthermore needs only small amounts of antibody for detection or for *GCC*-labeling. This method is an alternative to the use of expensive enzyme-conjugated antibodies for a number of applications, such as tracking of antibodies during purification or hapten inhibition tests.

Keywords Antibody determination; Protein determination; CLISA; Immunoassay.

Introduction

Immunoassays are based on specific antibody-antigen reactions [1] and quantification of the antigen is generally achieved by measuring the amount of a label molecule bound covalently to the antibody, which can then be detected due to its radioactivity, enzyme reaction, fluorescence, chemiluminescence, bioluminescence, or electrical conductivity [1–3]. However, these labels share a common drawback, as they are not suitable for long-term preservation [1]. While some isotopes have half-lives that would allow long-term applications, the use of radioactivity has in general become less desirable, because of issues concerning waste disposal and potential harm to human health. Furthermore, most fluorescence based assays suffer from fading signal intensity due to photo-bleaching [4].

Recently, nanoparticles, in particular gold and silver particles, were used successfully for labeling, as their size can be controlled easily, they are stable over long periods of time, and they are compatible with biological macromolecules, including proteins and nucleic acids [5, 6]. Quantification by visual inspection with the naked eye may help reduce costs and makes the test also applicable for field studies, as most other available techniques require specialized detection equipment, software, and specific sample preparation before the detection step.

The preparation of gold colloid clusters (*GCC*) is well established. Colloidal gold has an orange, red or red-violet color, depending on the size of the particles which can be adjusted by the preparation method. Colloidal gold is prepared by reducing gold salts with different reagents, the most common one being used in this research is trisodium citrate and others

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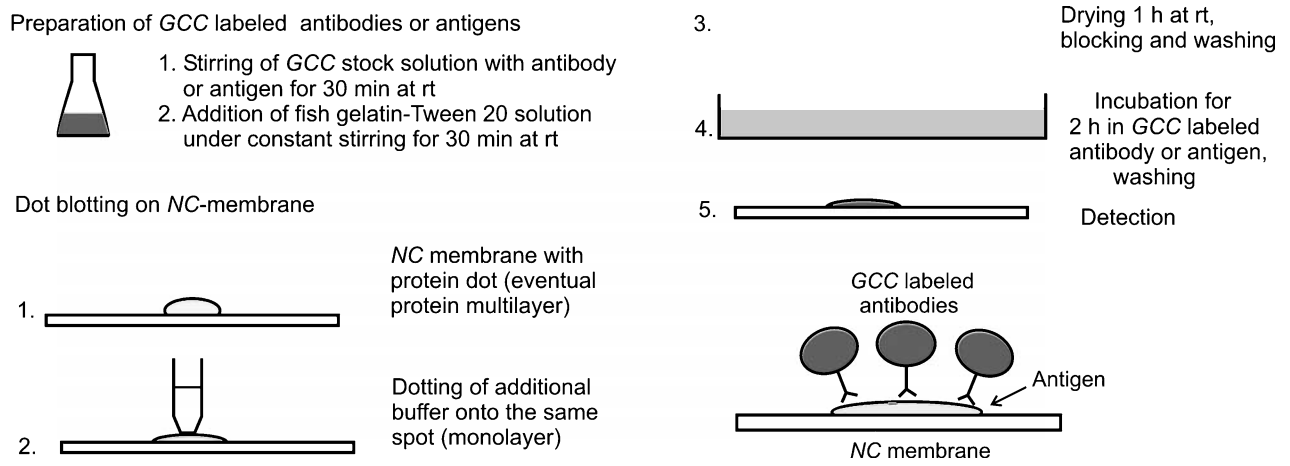

Fig. 1 Setup of the dot blot test compared to experimental results using *BSA* (or *HSA*) as an antigen and *GCC*-labeled polyclonal-*BSA*-antibodies (or monoclonal-*HSA*-antibodies) for detection

are *e.g.* tannic acid, ascorbic acid, and phosphorus. Depending on the method used, *GCC* preparations vary in particle size (expressed as the average particle diameter) and size variability (expressed as the coefficient of variation (CV) of the gold particle diameter in a sol. Such a sol is called monodisperse when the CV is less than 15% [7].

The *GCC* preparation used in this research is carried out according to *Frens* [8]. This method provides monodisperse colloidal gold with a size range from 14 to 50 nm. The size of the clusters can be adjusted by adding different amounts of reducing agent (trisodium citrate). *GCC*s with an average diameter of 17 nm can be prepared by reduction of $HAuCl_4$ with trisodium citrate. Immediately after adding the reducing agent, aggregation of gold atoms forms central icosahedral gold cores of eleven atoms at nucleation sites. The formation of nucleation sites proceeds very quickly. The remaining gold atoms in solution bind to the nucleation sites, until all atoms are removed from the solution.

As a larger number of nucleation sites for a given amount of gold chloride in solution results in smaller final size of each gold particle, the size of the particles is determined by the amount of reducing agent.

To obtain stable gold colloids, they have to be surrounded by a charged layer preferably negative, formed by the residual negative ions in the solution. This negatively charged layer, forming the so called zeta potential, causes the gold particles to repel each other and to stay suspended. Proteins such as antibodies can bind very stably to the surfaces of these gold particles. This phenomenon is utilized to quantify antigens dotted onto the *NC* membrane in form of a cluster linked immunosorbent assay (CLISA, see Fig. 1). Here we demonstrate some successful applications of this principle: firstly, the detection of bovine serum albumin (*BSA*) samples dotted onto *NC* membrane with polyclonal *BSA*-antibodies tagged with *GCC*. Secondly, the interaction between human serum albumin (*HSA*) samples dotted on *NC* membrane and monoclonal *HSA*-antibodies tagged with *GCC*. Thirdly, using an inverse setup, peroxidase-AB dotted onto *NC* membrane and peroxidase tagged to *GCC*. The sensitivity of the method makes it comparable to ELISA and allows for a rapid quantification of antigens at least semi quantitatively. This opens a wide field for applications such as testing for antibody-antigen binding specificity or tracing the purification of antibodies.

Results and discussion

The dot-blot tests presented here can be used for various purposes as *e.g.*, semi quantitative estimation of antigens by binding to *GCC* conjugated antibodies or testing specificity of antibodies. In order to achieve specific binding of the *GCC* conjugates to the respective samples on the *NC* membrane, either the antigen or the antibody can be coupled to the *GCC*. Antibodies bind with their Fc-domain to the clusters and with the Fab-domain to the antigen on the *NC* membrane. In this study 1% aqueous fish gelatin containing 0.1% Tween 20 is used as a stabilizing and blocking reagent for *GCC* as it lacks HS-groups.

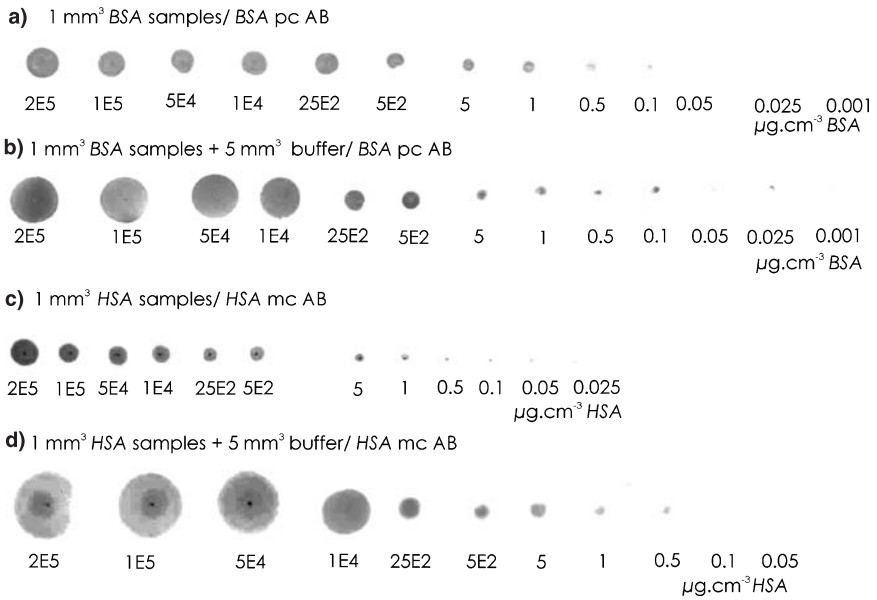

Fig. 2 (a) BSA samples (concentrations in the range between 2E5 and 0.001 $\mu g/cm^3$) dotted onto *NC*-membrane without dotting of additional buffer onto the same spot. (b) *BSA* samples dotted onto *NC*-membrane and dotting additional buffer onto the same spot, to minimize multilayers of protein. (c) *HSA* samples (concentrations in the range between 2E5 and 0.025 $\mu g/cm^3$) dotted onto *NC*-membrane without dotting of additional buffer onto the same spot. (d) HSA samples dotted onto *NC*-membrane and dotting additional buffer onto the same spot, to minimize multilayers of protein

Semi quantitative determination of antigens – BSA and HSA dot blot tests

To test for applicability in a broader field *BSA* as well as *HSA* and their respective *GCC*-labeled antibodies were used as test substances for semi quantitative estimations (see Fig. 2). A signal between *BSA* or *HSA* samples on the membrane and *GCC*-labeled polyclonal *BSA* or monoclonal *HSA*-AB, respectively is observed. The color of the reaction signal depends on sample concentration.

For *BSA* as an antigen the weakest color is observed at 0.025 $\mu g/cm^3$ and almost no signal can be detected at 0.001 $\mu g/cm^3$. The lower limit of detection in this test is considered to be at 0.025 $\mu g/cm^3$ (see Fig. 2a). With increasing concentrations the color gradually shifts towards a dark red. At higher concentrations adding even more sample does not lead to a further increase in color as stacking of protein on the dot will occur. In case of *HSA* decreased color intensity is observed at 0.1 $\mu g/cm^3$ and no signal can be detected at 0.05 $\mu g/cm^3$ (see Fig. 2c).

The test can be improved substantially by dotting a certain amount of buffer onto the spot immediately afterwards, resulting in much bigger spots (see Figs. 2b and d). As the affinity of *NC* membrane and protein is very high only protein not already bound directly to *NC* can be dissolved by the buffer solution migrating to free *NC* surface areas, where it is immediately bound thus resulting in quite even covering of the *NC* on much bigger spots. As is shown in Fig. 3 the amount of bound antigen can then be calculated from the diameter or more precisely from the surface area of the detected spots. In the lower range of sensitivity the diameters of the spots are the same as they are determined by the diameter of the tips used for dotting. Here the amount of antigen may be estimated by color intensity of the spot.

For negative controls *BSA*- and *HSA*-samples in the same concentrations as described above were incubated for 2 h in the same solutions omitting the antibodies and resulting in no detectable signals. As a second negative control antigen samples were incubated in *GCC*-labeled with non binding antibodies (monoclonal *HSA*-AB for *BSA* samples and β-galacosidase antibody for *HSA* samples) also resulting in no color reaction (see Figs. 4b and c).

Testing for specificity of antibodies

Incubation of *HSA* samples with *GCC*-labeled polyclonal anti *BSA*-antibodies showed a certain degree

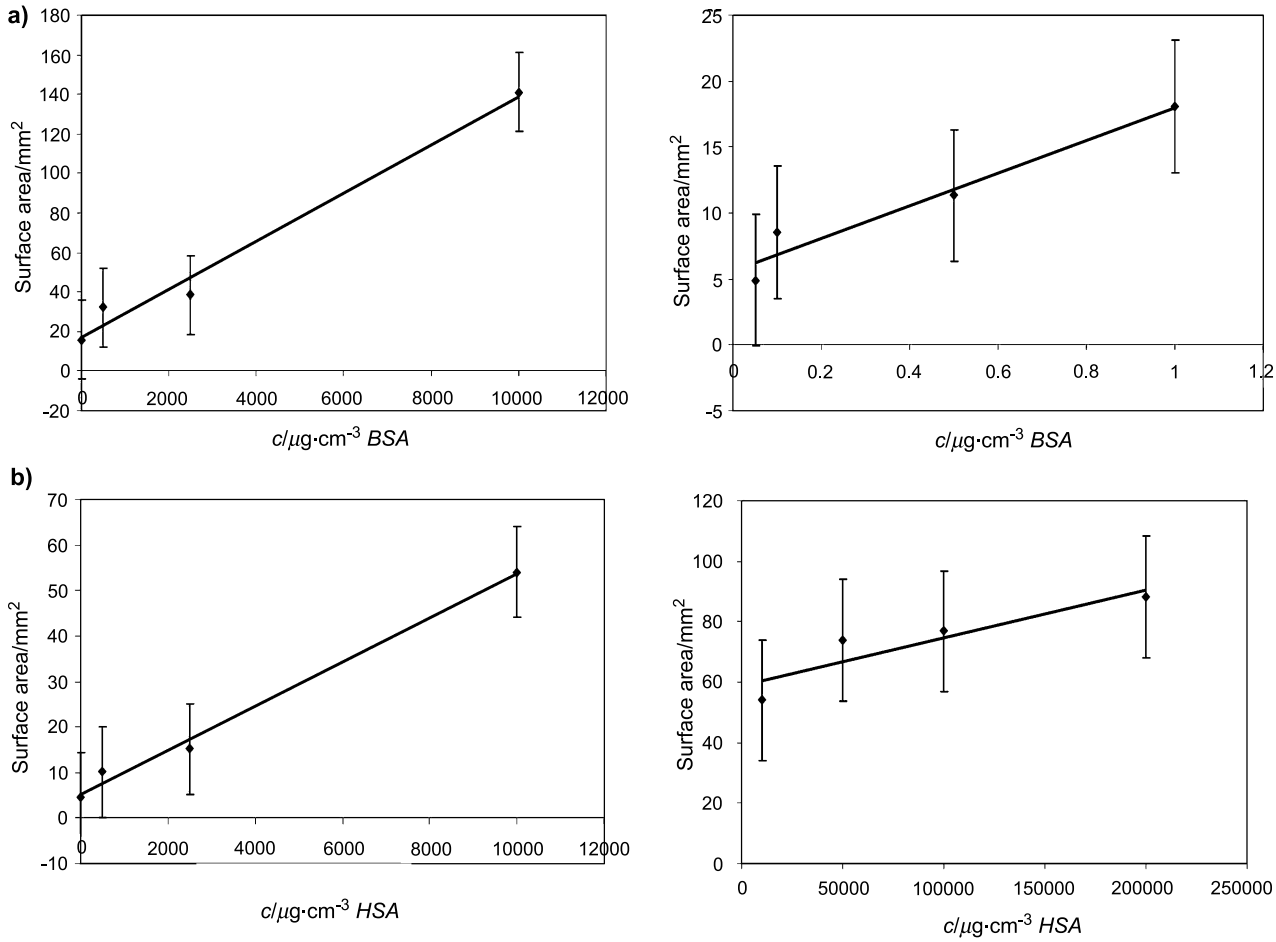

Fig. 3 Calculation of antigen concentration from surface area of spot after addition of 5 mm^3 buffer: The developed dot blot membranes were scanned, the pictures transferred to a graphics program (CorelDRAW 11) and the diameter of the spots then measured (using the zoom tool for small spots). Accuracy of the values on y-axis is dependent on the quality of the scanner. (a) *BSA* samples in various concentrations (left: 5–1E4 $\mu g/cm^3$ and right: 0.05–1 $\mu g/cm^3$). (b) *HSA* samples in various concentrations (left: 5–1E4 $\mu g/cm^3$ and right: 1E4–2E5 $\mu g/cm^3$)

of cross-reactivity due to some degree of homology between human and bovine serum albumins as can be seen from Fig. 4d. This shows that the test presented here is also a helpful means to substitute the more time consuming and inconvenient hapten inhibition tests. Various antigens can be dotted onto the same *NC* membrane and incubated with the *GCC*-labeled antibody to be tested for specificity. This setup can also be used for microarrays and automatisation.

Inverse dot blot setups

In some cases *GCC* tend to aggregate after addition of certain antibodies, positively charged at the working conditions as is the case for *e.g.*, peroxidase antibody. In this case an inverse setup is recommended.

Peroxidase antibody dot blot: Peroxidase antibody (1 mm^3 41 mg/cm^3, Sigma Aldrich) and also monoclonal β-galactosidase antibody (1 mm^3 2–2.5 mg/cm^3, Promega) as a negative control are dotted onto the *NC* membrane. Blocking and washing procedures and also incubation with the antigen *GCC*-labeled peroxidase (40 μg peroxidase used for labeling, Fluka) was done analogous to the previously mentioned protocol and showed only specific binding to the peroxidase antibody on the membrane (see Fig. 4f). As a further negative control *GCC* without peroxidase were reacted with a peroxidase antibody dotted membrane as above, showing no color, thus indicating no unspecific binding.

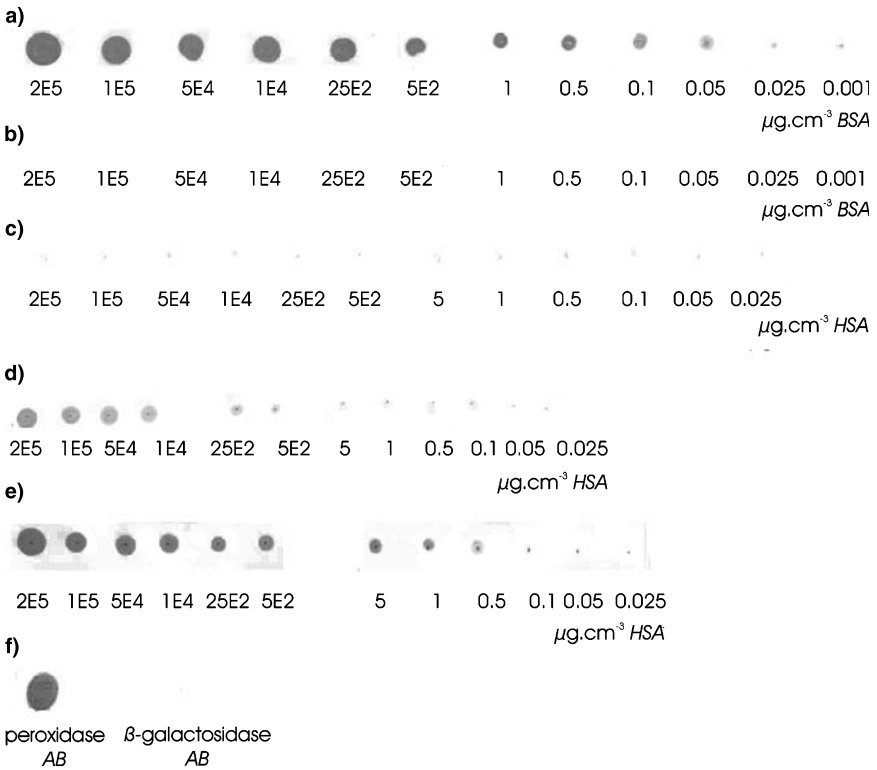

Fig. 4 (a) *BSA* samples reacted with *GCC*-labeled polyclonal *BSA* antibodies. (b) *BSA* samples reacted with *GCC*-labeled monoclonal *HSA* antibodies (negative control). (c) *HSA* samples reacted with *GCC*-labeled β-galactosidase antibody (negative control). (d) *HSA* samples reacted with *GCC*-labeled polyclonal *BSA* antibodies (cross-reactivity). (e) *HSA* samples reacted with *GCC*-labeled monoclonal *HSA* antibodies. (f) Peroxidase antibody reacted with labeled Peroxidase but not with negative control (β-galactosidase antibody)

Concluding remarks

The aim of this paper is to introduce a simple dot blot test using cherry-red colored *GCC*s as markers for protein binding reagents. This assay may be used in many cases as an alternative to ELISA. It has a number of advantages such as employing cheap *NC* membranes as used for western blotting. The detection of an antigen or an antibody in a sample is feasible in the presented technology using *GCC* even if no enzyme conjugated secondary antibodies are available. The signal detection does not require any instruments and the detection of cherry-red color of *GCC* by the naked eye may be preferable. This makes it useful also for field studies, especially if unstable probes might deteriorate during the way to a lab. As the signal color is stable and does not bleach, the examination in detail can then be done in the lab and documentation for a long time is also facilitated. The diversity of the different combinations using *GCC*-labeled antibodies or antigens in the developed technique makes it a useful tool for a general, rapid, sensitive, simple and cheap test for determining serum antibody or for detecting the presence of an antigen.

Experimental

Synthesis of 17 nm gold colloid clusters (GCC) according to Frens

According to the method of *Frens* [8] it is possible to produce mono-disperse colloidal gold with a size range from 14 to 50 nm. Preparation of 17 nm gold clusters: in a very clean *Erlenmeyer* flask rinsed additionally several times with doubly distilled water (dd H_2O) and covered with aluminum foil 10 mg $HAuCl_4 \cdot 3H_2O$ (Sigma Aldrich) is added to 100 cm^3 dd H_2O. The solution is heated to boiling under vigorous stirring. Then 4 cm^3 of sterile filtered 1% (w/v) trisodium citrate (Riedel-de Haen) is added very quickly (the solution is blown out of a 5-cm^3 glass pipette). Starting of nucleation can be seen after about 25 sec when the slightly yellowish solution will turn into a faint blue color. After approximately 4 min the blue color changes into cherry-red, indicating the formation of monodisperse spherical particles. The solution is boiled for another 10 min to complete the reduction of the gold chloride under constant stirring.

Spectrophotometric analysis at excitation wavelengths from 300 to 800 nm shows the plasmonic absorption as a single peak around 520 nm. The *GCC* solution can be stored at 4°C for prolonged period and used until a color change is observable.

Preparation of GCC-labeled antibodies

In order to achieve specific binding of the labeled *GCC* to the samples dotted on *NC* membrane, either the antigen or the antibody has to be bound or adsorbed to the *GCC*. Stock solution (10 cm^3) of *GCC* (17 nm) is mixed with 1 mm^3 antibody under gentle stirring for 30 min at room temperature (rt) to generate the labeled *GCC*-antibody conjugate. Polyclonal anti-rabbit bovine serum albumin (*BSA*) antibody (Sigma Aldrich, 3.6 mg/cm^3), and monoclonal anti-human serum albumin (*HAS*) antibody (Sigma Aldrich, 7.8 mg/cm^3) were used in this study. Then 1 cm^3 aqueous 1% fish gelatin (Sigma Aldrich) including 0.1% Tween 20 (Sigma Aldrich) is added under stirring for 30 min. Fish gelatin stabilizes *GCC*s during the following incubation steps and blocks free binding sites of *GCC*s avoiding their direct binding to detected antigen on the *NC* membrane. The concentration of fish gelatin was assessed using a salt flocculation test for *GCC*; 10 mm^3 of 1% fish gelatin dissolved in dd H_2O stabilizes 100 mm^3 17 nm *GCC*.

Preparation of GCC-labeled antigen

The protocol for labeling antigens used in this study is done analogous to the protocol for the preparation of *GCC*-labeled antibodies Peroxidase-*GCC* conjugate 10 cm^3 of 17 nm synthesized *GCC* stock solution is mixed with 40 μg peroxidase (Fluka, 502 U/mg) to generate the conjugate. The following incubation step of peroxidase-*GCC* conjugate in fish gelatin and Tween 20 is carried out as described above.

Dot blotting of test-samples

A test sample (1 mm^3, antigen or antibody) is dotted onto the surface of a *NC* membrane (pore size 0.45 μm, Protran BA 85, Schleicher & Schuell). For semi quantitative tests 5 mm^3 *Tris*-HCl buffer (50 m*M Tris*-HCl, 0.15 NaCl *pH* 7.3) is then dotted onto the same sample spot to increase the diameter of the protein monolayer avoiding multilayers of protein on the sample spot (Fig. 1a). This step can be omitted if only testing for antigen-specificity is desired. The spots are dried for 1 h at rt. Then the membrane is immersed in blocking solution containing 2% fish gelatin (Sigma Aldrich), 0.1% Tween 20 (polyethylene glycol sorbitan monolaurate, Sigma Aldrich) and *Tris*-HCl buffer for 20 min at rt under gentle shaking. Fish gelatin is used for eliminating unspecific background staining as it does not contain free reactive HS-groups that would form a tight chemical bond with the *GCC*. Therefore, blocking with HS-group containing reagents, such as *BSA* or milk, is not recommended.

Blocking is further enhanced by the addition of the non-ionic detergent Tween 20. After blocking, the membrane is treated 2×5 min in excess washing solution (*Tris*-HCl buffer containing 0.5% Tween 20) and 3×1 min in dd H_2O to remove residual NaCl, which otherwise causes flocculation of *GCC*s. Flocculation occurs immediately if the gold sol is unstable and the red color of *GCC* turns to violet and blue. The washed membrane is then incubated for 2 h in the *GCC*-labeled antibody or antigen solution (inverse setup) at rt under gentle shaking, followed by washing for 3×10 min under gentle shaking at rt in the above mentioned solution.

Dilution series of antigen samples

BSA dilution series in *Tris*-HCl buffer *pH* 7.3 were made in a concentration range between 2E5 $\mu g/cm^3$ and 0.001 $\mu g/cm^3$, *HSA* dilution series in *Tris*/HCl buffer *pH* 7.3 in the range from 2E5 $\mu g/cm^3$ and 0.025 $\mu g/cm^3$.

Salt flocculation test

Gold particles in a gold colloid are surrounded by a layer of negative charges, deriving from negatively charged ions present in the reaction mixture (trisodium citrate) during synthesis. Due to this charged layer the gold particles repel each other and stay dispersed. During labeling with *GCC* the colloidal gold sols become unstable in the presence of electrolytes. Adding of electrolytes turns the red color of *GCC* into violet or blue, and clusters flocculate. However, when poly-electrolytes are added under appropriate conditions, these adsorb spontaneously to the gold particles, and sols are rendered hydrophilic and stable in the presence of added salt. The binding of poly-electrolytes to gold is irreversible in most cases [9]. In this study the *GCC* is stabilized using fish gelatin. The salt flocculation test is carried out according to *Walter* and *Bauer* [10].

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