Supporting Information

Porous Antibody-Containing Protein Microparticles as Novel Carriers for ELISA

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Experimental Procedures

Materials:

Horseradish peroxidase (EC 1.11.1.7) (HRP; CAS: 9003-99-0); anti-H.IgG-goat antibodies conjugated with FITC (GAH; F5512); bovine serum albumin (BSA; CAS: 9048-46-8); human Immunoglobulin G (H.IgG; 56834); fluorescein isothiocyanate (FITC; F3651); glutaraldehyde (CAS: 111-30-8); ethylenediaminetetraacetic acid (EDTA; CAS: 60-00-4); luminol (CAS: 521-31-3), Tris–HCl (CAS: 77-86-1), $CaCl_2$ (CAS: 10035-04-8); NaCl (CAS: 7647-14-5); 4-morpholinopyridine (CAS: 2767-91-1); Na_2CO_3 (CAS: 497-19-8) were purchased from Sigma (USA).

Goat anti-FITC-polyclonal antibodies (A150-112A) was from Bethyl (USA).

PBS (A0964.9100) and Tween-20 (CAS: 9005-64-5) were from AppliChem (Germany). $NaHCO_3$ (Art.-Nr. 0965.3) and H_2O_2 (Art.-Nr. 9681.3) – from Carl Roth GmbH (Germany).

Conjugate of H.IgG with HRP was synthesized by a periodate method as described in. [1]

Sodium 3-(10'-phenothiazinyl)propane-1-sulfonate (SPTZ) was synthesized as described in. [2]

All salts were of analytical or chemical purity grade.

The HRP concentration was measured using ε_{402} = 102 000 M^{-1} cm^{-1}.[3] The H_2O_2 concentration was determined by monitoring A_{240}, using ε = 43.6 M^{-1} cm^{-1}.[4] The required H_2O_2 dilutions were prepared daily.

All solutions were prepared using a water getting by three-stage Millipore Milli-Q Plus 185 purification system and having a resistivity higher than 18.2 MΩcm.

Methods:

Optimization of the conventional ELISA.

ELISA was carried out using 96-well black polystyrene microplates (High Binding, 3925, Costar, USA). The microplate wells were coated by adding into each well 50 µL of 2, 0.7, 0.2 or 0.07 µg mL^{-1} of GAH in 100 mM carbonate buffer, pH=9.5 and incubated at 4°C overnight. The microplate wells were then washed using PBST (PBS with 0.05% Tween-20) four times. Then, 25 µL of H.IgG (0 or 60 µg mL^{-1}) in PBS, pH 7.4 and 25 µL of H.IgG-HRP in PBS, pH 7.4 (dilutions are 1:15000, 1:45000, 1:135000 or 1:405000 of the stock H.IgG-HRP solution) were added to each well and incubated for 1 hour at 37°C. The microplate was washed again as described above. Then, 50 µL of freshly prepared substrate solution (80 mM Tris buffer, pH 8.3, containing 0.17 mM luminol, 2.1 mM SPTZ, 8.75 mM 4-morpholinopyridine and 1.75 mM H_2O_2) [5] was added to each well. Chemiluminescence was monitored for 6 minutes after initiating the luminol oxidation on a luminometer OMEGA (BMG LABTECH GmbH, Germany).

Determination of H.IgG by conventional ELISA with chemiluminescent detection.

The scheme of the conventional ELISA for the determination of H.IgG is presented at Figure S1. The microplate wells were coated by adding into each well 50 µL of 2 µg mL^{-1} GAH dissolved in 100 mM carbonate buffer, pH=9.5 and incubated at 4°C overnight. The microplate wells were then washed using PBST four times. Then, 25 µL of H.IgG (0-60 µg mL^{-1}) in PBS, pH 7.4 and 25 µL of a 1:135000 dilution of the stock H.IgG-HRP solution in PBS, pH=7.4 were added to each well and incubated for 1 hour at 37°C. The microplate was washed again as described above. Then, 50 µL of freshly prepared substrate solution (80 mM Tris buffer, pH 8.3, containing 0.17 mM luminol, 2.1 mM SPTZ, 8.75 mM 4-morpholinopyridine and 1.75 mM H_2O_2) [5] was added to each well. Chemiluminescence was monitored for 6 minutes after initiating the luminol oxidation on a luminometer OMEGA (BMG LABTECH GmbH, Germany).

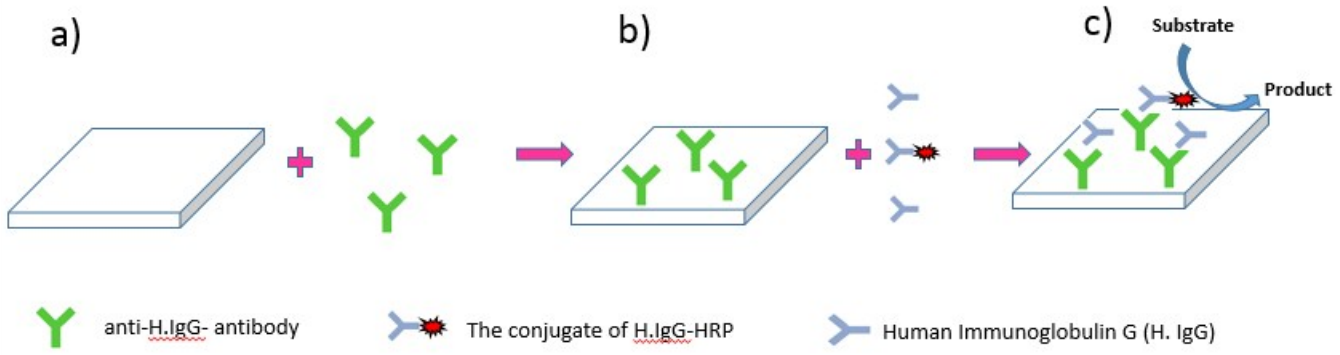

Figure S1. The scheme of the convencional ELISA for the determination of the H.IgG: a) the immobilization of the anti-H.IgG-antibodies onto solid surface of the ELISA plate; b) the adding of the analyte H.IgG and the H.IgG-HRP conjugate; c) the adding of the substrate mixture and registration of the analytical signal.

Fabrication of porous antibody-containing protein microparticles (PACPMs).

Calcium carbonate vaterite crystals containing proteins were fabricated on the basis of the previously established co-precipitation approach[6] as described below. All the solutions and procedures described were performed at room temperature.

Briefly, equal volumes (80 µL) of 1M $CaCl_2$ and 1M Na_2CO_3 solutions were added to 240 µL of protein solution (314.5 µg mL^{-1} GAH or anti-FITC-antibodies and 18.5 µg mL^{-1} BSA), which was in water in a cylindrical glass. The resulting suspension was stirred for 10 sec with speed equal 50 rpm, followed by incubation for another 4 minutes (no stirring) to complete the crystal growth. After centrifugation for 20 sec at 13000 rpm of samples and removal of the supernatant, the solution of glutaraldehyde in water (till the molar ratio to GAH (or anti-FITC-antibodies) and BSA equal 14000:1 and 1167:1, respectively) was added. After 20 minutes of incubation with shaking, samples were washed 3 times in Millipore water by centrifugation.

To get PACPMs 1 mL of 100 mM of EDTA was added to the 50 µL of obtained suspension of the $CaCO_3$-microcrystals. After template dissolving the suspension of PACPMs was dialyzed (Dialysis tubing cellulose membrane, D9277-100 FT, cut-off = 14 kDa, Sigma, USA) overnight with stirring (4°C) against Millipore water and then used in assay. The final concentration of antibodies in PACPMs was calculated taking into account the dilution factor during the synthesis and the percentage of loaded antibodies.

Determination of the loaded amount of GAH in fabricated PACPMs.

The microplate wells were coated by adding into each well 50 µL of 2 µg mL^{-1} GAH dissolved in 100 mM carbonate buffer, pH=9.5 and incubated at 4°C overnight. The microplate wells were then washed using PBST four times. Then, 25 µL of GAH (0-60 µg mL^{-1}) or supernatant after the fabrication of PACPMs in different dilutions in PBS, pH 7.4 and 25 µL of a 1:135000 dilution of the stock H.IgG-HRP solution in PBS, pH=7.4 were added to each well and incubated for 1 hour at 37°C. The microplate was washed again as described above. Then, 50 µL of freshly prepared substrate solution (80 mM Tris buffer, pH 8.3, containing 0.17 mM luminol, 2.1 mM SPTZ, 8.75 mM 4-morpholinopyridine and 1.75 mM H_2O_2)[5] was added to each well. Chemiluminescence was monitored for 6 minutes after initiating the luminol oxidation on a luminometer OMEGA (BMG LABTECH GmbH, Germany). The concentration of loaded GAH in PACPMs was estimated as a difference between the GAH concentrations before synthesis and determined in the supernatant.

Determination of residual active GAH in PACPM.

The microplate wells were coated by adding into each well 50 µL of 2 µg mL^{-1} GAH dissolved in 100 mM carbonate buffer, pH=9.5 and incubated at 4°C overnight. The microplate wells were then washed using PBST four times. Then, 25 µL of GAH (0-60 µg mL^{-1}) or samples of synthesized PACPMs in different dilutions in PBS, pH 7.4 and 25 µL of a 1:135000 dilution of the stock H.IgG-HRP solution in PBS, pH=7.4 were added to each well and incubated for 1 hour at 37°C. The microplate was washed again as described above. Then, 50 µL of freshly prepared substrate solution (80 mM Tris buffer, pH 8.3, containing 0.17 mM luminol, 2.1 mM SPTZ, 8.75 mM 4-morpholinopyridine and 1.75 mM H_2O_2)[5] was added to each well. Chemiluminescence was monitored for 6 minutes after initiating the luminol oxidation on a luminometer OMEGA (BMG LABTECH GmbH, Germany). The amount of active GAH in PACPMs was estimated by using the calibration curve for the determination of GAH. The percent of residual active GAH in PACPMs was calculated from the ratio between the estimated concentration of GAH in PACPMs and the loaded amount of GAH during the procedure of co-precipitation.

Synthesis of FITC-HRP

The conjugate of HRP labeled by FITC was synthesized by the standard method of protein labeling with FITC. In brief, to 1 mg of HRP dissolved in 912 µL of 100 mM carbonate buffer, pH=9.5, the 88 µL of 0.1 mg mL^{-1} FITC in ethanol were added drop by drop with stirring. After the incubation for 4 hours at room temperature in the dark place with stirring the dialysis against PBS, pH 7.4, (Dialysis tubing cellulose membrane, D9277-100 FT, cut-off = 14 kDa, Sigma, USA) at 4°C with stirring was repeated twice. The labeled HRP was stored at -20°C.

Determination of the loaded amount of anti-FITC-antibodies in fabricated PACPMs.

The microplate wells were coated by adding into each well 50 µL of 0.3 µg mL^{-1} anti-FITC-antibodies dissolved in 100 mM carbonate buffer, pH=9.5 and incubated at 4°C overnight. The microplate wells were then washed using PBST four times. Then, 25 µL of anti-FITC-antibodies (0-20 µg mL^{-1}) or supernatant after the fabrication of the PACPMs in different dilutions in PBS, pH 7.4 and 25 µL of a 1:500 dilution of the stock FITC-HRP solution in PBS, pH=7.4 were added to each well and incubated for 1 hour at 37°C. The microplate was washed again as described above. Then, 50 µL of freshly prepared substrate solution (80 mM Tris buffer, pH 8.3, containing 0.17 mM luminol, 2.1 mM SPTZ, 8.75 mM 4-morpholinopyridine and 1.75 mM H_2O_2)[5] was added to each well. Chemiluminescence was monitored for 6 minutes after initiating the luminol oxidation on a luminometer OMEGA (BMG LABTECH GmbH, Germany). The concentration of loaded anti-FITC-antibodies in

PACPMs was estimated as a difference between the anti-FITC-antibodies concentrations before synthesis and determined in the supernatant.

Determination of residual active anti-FITC-antibodies in PACPMs.

The microplate wells were coated by adding into each well 50 µL of 0.3 µg mL^{-1} anti-FITC-antibodies dissolved in 100 mM carbonate buffer, pH=9.5 and incubated at 4°C overnight. The microplate wells were then washed using PBST four times. Then, 25 µL of anti-FITC-antibodies (0-20 µg mL^{-1}) or samples of synthesized PACPMs in different dilutions in PBS, pH 7.4 and 25 µL of a 1:500 dilution of the stock FITC-HRP solution in PBS, pH=7.4 were added to each well and incubated for 1 hour at 37°C. The microplate was washed again as described above. Then, 50 µL of freshly prepared substrate solution (80 mM Tris buffer, pH 8.3, containing 0.17 mM luminol, 2.1 mM SPTZ, 8.75 mM 4-morpholinopyridine and 1.75 mM H_2O_2)[5] was added to each well. Chemiluminescence was monitored for 6 minutes after initiating the luminol oxidation on a luminometer OMEGA (BMG LABTECH GmbH, Germany). The amount of active anti-FITC-antibodies in PACPMs was estimate by using the calibration curve for the determination of anti-FITC-antibodies. The percent of the residual active anti-FITC-antibodies in PACPMs was calculated from the ratio between the estimated concentration of anti-FITC-antibodies in PACPMs and the loaded amount of anti-FITC-antibodies during the procedure of co-precipitation.

Optimization of the PACPM-based ELISA.

The microplate wells were coated by adding into each well 50 µL of 0.07; 0.2 or 0.7 µg mL^{-1} of GAH in PACPMs suspended in 100 mM carbonate buffer, pH=9.5 and incubated at 4°C overnight. The microplate wells were then washed using PBST four times. Then, 25 µL of H.IgG (0 or 60 µg mL^{-1}) in PBS, pH 7.4 and 25 µL of a 1:5000 or 1:15000 dilution of the stock H.IgG-HRP solution in PBS, pH=7.4 were added to each well and incubated for 1 hour at 37°C. The microplate was washed again as described above. Then, 50 µL of freshly prepared substrate solution (80 mM Tris buffer, pH 8.3, containing 0.17 mM luminol, 2.1 mM SPTZ, 8.75 mM 4-morpholinopyridine and 1.75 mM H_2O_2)[5] was added to each well. Chemiluminescence was monitored for 6 minutes after initiating the luminol oxidation on a luminometer OMEGA (BMG LABTECH GmbH, Germany).

Determination of H.IgG by PACPM-based ELISA with chemiluminescent detection.

The scheme of the PACPM-based ELISA for the determination of H.IgG is presented at Figure S2. The microplate wells were coated by adding into each well 50 µL of 0.2 µg mL^{-1} of GAH in PACPMs dissolved in 100 mM carbonate buffer, pH=9.5 and incubated at 4°C overnight. The microplate wells were then washed using PBST four times. Then, 25 µL of H.IgG (0-60 µg mL^{-1}) in PBS, pH 7.4 and 25 µL of a 1:5000 dilution of the stock H.IgG-HRP solution in PBS, pH=7.4 were added to each well and incubated for 1 hour at 37°C. The microplate was washed again as described above. Then, 50 µL of freshly prepared substrate solution (80 mM Tris buffer, pH 8.3, containing 0.17 mM luminol, 2.1 mM SPTZ, 8.75 mM 4-morpholinopyridine and 1.75 mM H_2O_2)[5] was added to each well. Chemiluminescence was monitored for 6 minutes after initiating the luminol oxidation on a luminometer OMEGA (BMG LABTECH GmbH, Germany).

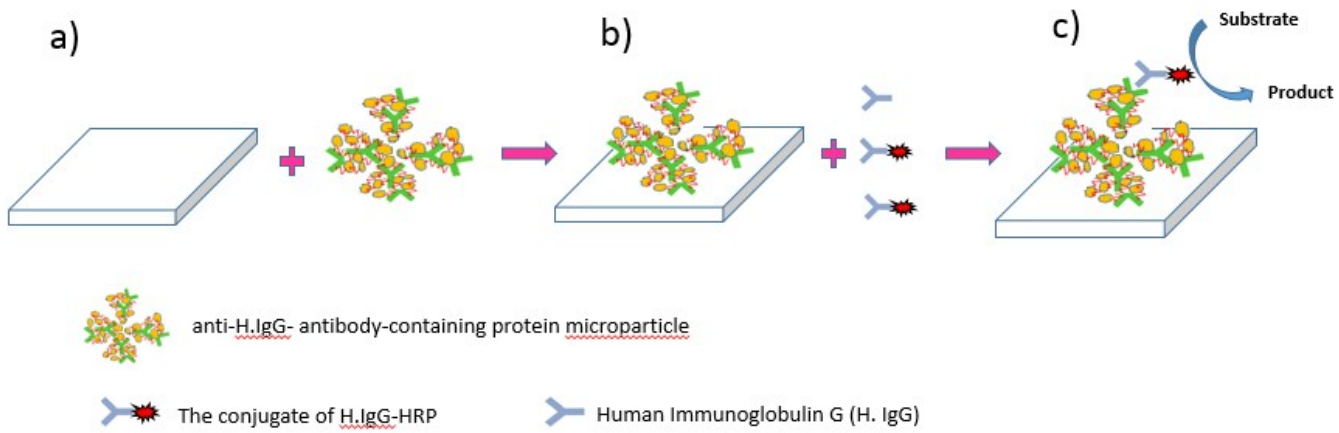

Figure S2. The scheme of the PACPM-based ELISA for the determination of the H.IgG: a) the immobilization of the PACPMs onto solid surface of the ELISA plate; b) the adding of the analyte H.IgG and the H.IgG-HRP conjugate; c) the adding of the substrate mixture and registration of the analytical signal.

Confocal Laser Scanning Microscopy (CLSM).

CLSM was used to estimate the distribution of the GAH in protein mixture encapsulated in $CaCO_3$ microcrystals before and after template dissolving. CLSM images were obtained using a Zeiss LSM 510 Meta installation (Zeiss, Germany). An oil-immersion objective with 40× magnification and numerical aperture of 1.3 was used. Standard filter settings for excitation and emission of FITC were used for the laser sources with wavelengths of 488 and 633 nm. All of the CLSM experiments were performed at room temperature. Analysis of the CLSM images has been done by means of ImageJ[7] and LSM Image Browser.[8]

Scanning Electron Microscope (SEM).

For SEM analysis of PACPMs samples were prepared by applying a drop of the PACPMs suspension after template dissolving to a glass slide and then drying overnight at room temperature. Samples of $CaCO_3$ microcrystals before template dissolving were first dried overnight at 45°C and then placed to a glass slide. Samples were sputtered with gold and measurements were conducted using a Gemini Leo 1550 instrument at an operation voltage of 3 keV. Porosity of the PACPMs before and after template removal was analyzed by profiles taken along to the PACPM's surface. We used Image J[7] to analyze the intensity of the grey value after taking the profiles.

References

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7 Public software from National Institutes of Health; http:// rsbweb.nih.gov/ij/.

8 https://www.embl.de/eamnet/html/body_image_browser.html