

Protein Cohabitation: Long-term Immunoglobulin G Storage at Room Temperature

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Electronic Supporting Information

(ESI[†])

Materials and Methods

Materials.

All the solvents and reagents were used as received. All solvents used in this work were purchased from VWR. gelatin type A porcine skin (80 to 120 g bloom) and Immunoglobulin G > 95 % (GE) from human serum and recombinant Protein G, expressed in *E. coli*, lyophilized powder have been purchased from Sigma Aldrich. Mouse anti-human IgG1, horseradish peroxidase conjugate has been purchased from Invitrogen (Thermo Fischer Scientific). All the proteins were used as received. NaH_2PO_4 , Na_2HPO_4 , glycerol, sodium dodecylsulphate, Tris-HCl were purchased from Sigma Aldrich. Bromoophenol blue and dithiothreitol (DTT) were purchased from Sigma Aldrich.

Methods.

Preparation of the IgG1-gelatin solid formulation.

2.4 mg of IgG1 was dissolved in 1 mL of 0.1 M NaCl solution in Milli-Q water. To this solution added 0.2 g of gelatin Type A, followed by the stirring at 50°C for 10 min., below the melting temperature of IgG1 (55 °C) for complete mixing of the proteins. 250 μL of this solution was then cast on a glass plate, followed by air drying at room temperature for 48 hr. The air-dried IgG1-gelatin solid formulation (transparent film) was then incubated at room temperature until further re-dissolution in phosphate buffer pH. 7.4 for structural and functional analysis. The concentration of IgG1 in the dried gelatin film is 79 $\mu\text{mol.kg}^{-1}$ (Mol. Wt. of IgG1 = 150 kDa). The incubation period is 14 months in this work. A schematic of the preparation is shown in Fig. S1.

Re-dissolution of the IgG1-gelatin film after 14 months for stability analysis

The solid film was dissolved in 400 μL of phosphate buffer pH. 7.4 at 40°C. The viscous stock solution was stored at -20°C until further measurements of CD, SDS-PAGE, and western blot assay. Due to the high concentration of gelatin, it undergoes gelation at a low temperature. Therefore, before measurement, the sample was kept at room temperature at least for 30 min. followed by gently shaking at 40°C for liquefaction. The concentration of the IgG1 in gelatin solution during assay was measured using NanoDrop ONE, Thermo Scientific.

Characterization

Circular dichroism spectroscopy (CD)

CD spectra were recorded in the wavelength range of 190-300 nm using JASCO J-715, spectropolarimeter. Samples were diluted until HT [V] comes down to around 800 to ascertain the accuracy of measurements. The measurement parameters were data pitch = 1nm, scanning mode = continuous, scanning speed = 100 nm, response time = 2 sec. and bandwidth = 1.0 nm, accumulation = 5.

SDS-PAGE of IgG1 and IgG1-gelatin solutions

For SDS-PAGE we used 8–16% Mini-PROTEAN® TGX Stain-Free™ Protein Gels. For this 15 μL each of the protein solutions were mixed with 5 μL of 4x Laemmli Sample Buffer in non-reducing conditions. The concentration of proteins was IgG1 pure = 1mg/mL and IgG1=gelatin solution = 1mg/mL. Electrophoresis was run on polyacrylamide gels with a running buffer (Tris/Glycine/SDS) for 35 min. at 200 V using Bio-RAD Protein Electrophoresis Equipment. After the electrophoresis run, the protein bands were visualized after activating the stain-free gel with UV light. The gel imaging to identify proteins was done using Bio-RAD ChemiDoc™MP imaging system.

HPLC analysis

All experiments were performed on Waters Synapt G2-Si ESI mass spectrometer equipped with a Waters Acquity UPLC system using a C4 RP column (Waters Aquity, UPLC Protein BEH C4, 300 Å, 1.7 μm , 2.1 mm x 50 mm) at 0.4 mL/min. flow rate, 80°C column temperature and 40°C sample manger temperature. Mobile phase A was 0.1% formic acid in MQ-H₂O, mobile phase B was 0.1% formic acid in ACN using 5% eluent B in A for 1 min followed by a gradient of 5% to 95 % eluent B in A over 7.0 min. Samples were analyzed at 280 nm.

IgG1 recovery experiments

A gelatin-IgG1 layer, containing 0.6 mg IgG1 was cut into halves (sample I and sample II, Table S1) and each half was dissolved in 2.5 mL buffer (phosphate, 10 mM, pH 7.2), while gently stirred at 40°C in a glass vial. After the sample was dissolved completely, 500 μL were taken and diluted with 500 μL buffer before subjected to the LC analysis injecting 5 μL into the LC system.

In order to analyze the IgG1 content of the samples, each sample was spiked with two different amounts of a IgG1 reference solution. Since both samples were prepared and measured at different days, fresh IgG1 reference solution (0.51 mg/mL and 0.63 mg/mL, phosphate buffer, 10 mM, pH 7.2) was prepared for each analysis. From this solution 100 μ L (200 μ L) were added to 500 μ L-gelatin-IgG1 sample plus 400 μ L (300 μ L) buffer to give two IgG-spiked IgG-gelatin samples for LC analysis (5 μ L injection volume).

From the average increase of the integral of the IgG1 peak at 3.2 min. (Fig. 4) after addition of the IgG1 reference the IgG1 content of the gelatin-IgG1 sample can be calculated. Since significant carry-over between the samples was observed, two blank runs were run between each sample measurement, which was sufficient to completely remove any carried-over IgG1.

Western Blot Assay

Western blot assay was performed using two methods 1) direct and 2) indirect. In the direct method IgG1 pure or IgG1 in gelatin blotted on a nitrocellulose paper were used as primary antibodies to bind with Mouse-antihuman IgG1-HRP conjugate as a secondary antibody. Due to the poor transfer of high molecular proteins during blotting, we also used an indirect method wherein a low molecular weight protein (protein G) was used as a binding partner between nitrocellulose paper and primary antibody, followed by binding to the secondary antibody.

For western blot assay protein solutions of different concentrations were first run on the SDS-PAGE. For this 15 μ L, each protein solution was mixed with 5 μ L of 4x Laemmli Sample Buffer in non-reducing conditions. The concentrations of proteins used were protein G (0.25 mg/mL, 0.5 mg/mL, and 1mg/mL), IgG1 pure (1mg/mL) and IgG1 in gelatin (0.5 mg/mL and 1 mg/mL). These concentrations were measured using Nano DROP ONE, Thermo Scientific. In addition to the sample assay (IgG1 in gelatin), a control assay (IgG1 pure) was also performed. Electrophoresis was run on polyacrylamide gels with a running buffer for 35 min. at 200 V using Bio-RAD Protein Electrophoresis Equipment. The gel imaging to identify proteins was done using Bio-RAD ChemiDoc™ MP imaging system. For protein blotting, the nitrocellulose paper, ion reservoir stack, and gel were soaked in a 1X-trans blot buffer (200 mL Trans-Blot® Turbo™ 5X transfer buffer + 600 mL MQ water+ 200 mL ethanol) for 10 min. The samples were then stacked in an order (ion reservoir stack, nitrocellulose paper, gel, and ion reservoir stack) followed by a transfer run at 25 V, 1.0 A for 30 min. on a Trans-Blot® Turbo™ transfer system equipment. To check the transfer of proteins to the nitrocellulose paper the gel was imaged using Bio-RAD ChemiDoc™ MP imaging system. Moreover, nitrocellulose paper was stained with ponceau S solution. We observed complete transfer of protein G to the nitrocellulose paper but not of IgG1 pure or IgG1 in gelatin solution. The ponceau S dye was removed from the nitrocellulose paper by rinsing with 1X-TBS buffer (4.84 g Tris, 58.48 g NaCl and 1.5-liter MQ water) at pH 7.5. It was followed by blocking the nitrocellulose membrane by incubating in blocking buffer (TBS buffer containing 10 % BSA) for an hour with gentle shaking. The blocked nitrocellulose membrane was then cut into two pieces. A) sample membrane and B) control membrane containing all proteins with the same concentrations. The control membrane also contains protein markers as well.

Membrane A was soaked in 15 mL of IgG1 in gelatin (0.03 mg/mL) solution in blocking buffer and membrane B was soaked in 15 mL of IgG1 pure (0.01 mg/mL) solution in blocking buffer in 50 mL Eppendorf tubes. Both the sample tube containing membranes A and B were incubated overnight in a cold room with continuous shaking and rolling for binding of IgG1 to protein G. The next day, both the membranes were washed with TBS-T buffer three times (continuous rolling for 5-10 min.) to remove the unbound IgG1. It was followed by the binding of a secondary antibody (mouse anti-human IgG1-HRP conjugate) to IgG1. For this 0.001 mg/mL solution of mouse anti-human IgG1-HRP conjugate was prepared in TBS-T buffer. 10 mL each of this solution was poured into tubes containing membrane A and membrane B respectively. The sample bottles were then allowed to roll for an hour to allow binding of secondary antibody to primary antibody at room temperature. After 1 hr. sample bottles were washed with TBS-T buffer three times (5-10 min. of rolling), followed by 4th washing with TBS buffer. To detect the binding of IgG1 with mouse anti-human IgG1-HRP conjugate in both the sample and control membranes, they were sandwiched between a plastic paper followed by the addition of substrate/activator (Super Signal™ west Pico PLUS containing tetramethylbenzidine). Chemiluminescence signal development was observed in both the membranes due to the oxidation of tetramethylbenzidine-to-tetramethylbenzidine diimine by enzyme horseradish peroxidase (HRP) conjugated to the secondary antibody. The imaging of the Chemiluminescence was done using Bio-RAD ChemiDoc™ MP imaging system. Strong Chemiluminescence bands were observed near the 60 kDa in both sample and control membranes which indicates binding of IgG1 in gelatin or IgG1 pure to the secondary antibody-mediated by protein G. Weak chemiluminescence bands observed at 150 kDa also indicates direct binding of secondary antibody to the IgG1 in gelatin. The low intensity of these bands is accounted for the poor transfer of IgG1 in gelatin to the nitrocellulose membrane owing to its high molecular weight and masking by gelatin. Overall, these results indicate that the functional activity of IgG1 cointegrated with gelatin is retained even after 14 months of storage at room temperature.

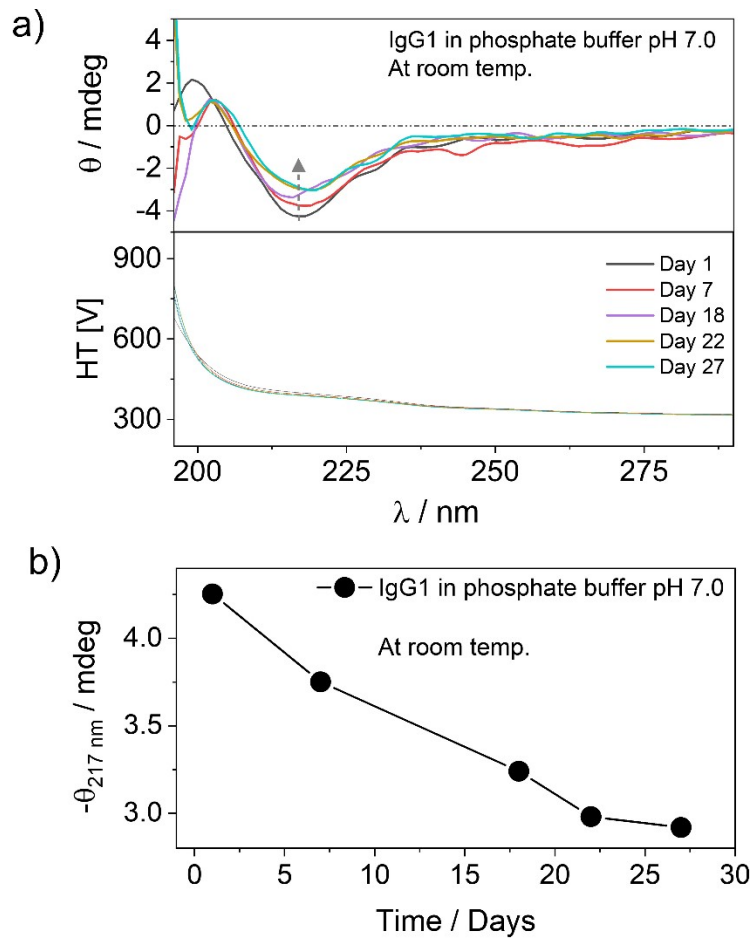


Fig. S1. a) Time dependent CD spectra of IgG1 solution in phosphate buffer pH 7.0 and corresponding HT voltage plot at room temperature. b) Plot showing decrease in ellipticity at 217 nm indicating alterations in the secondary structure of IgG1.

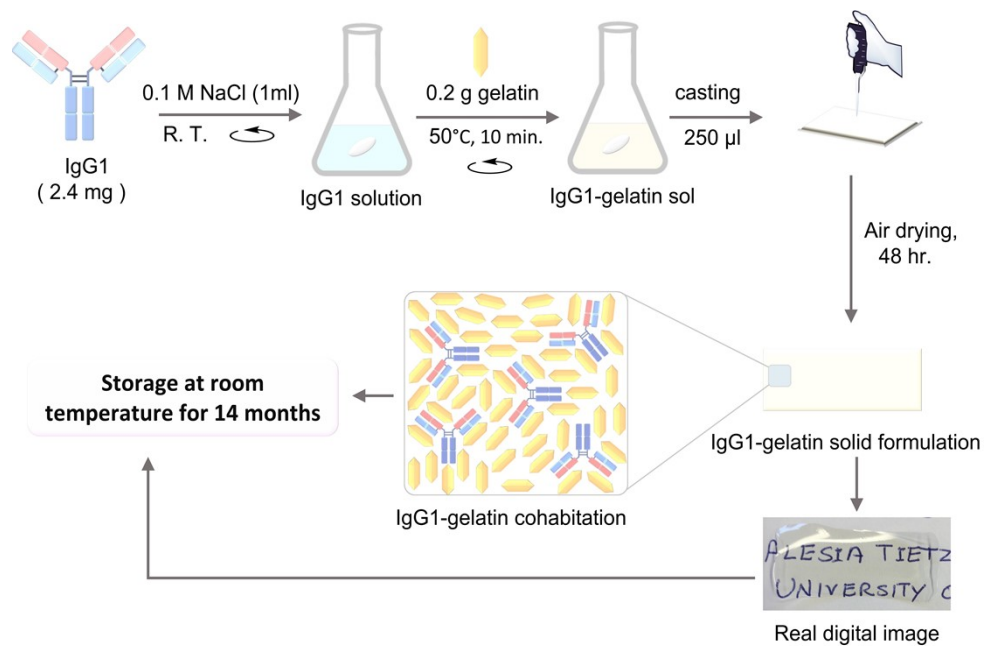


Fig. S2. Schematic of the preparation of IgG1-gelatin solid-formulation and long-term cohabitation.

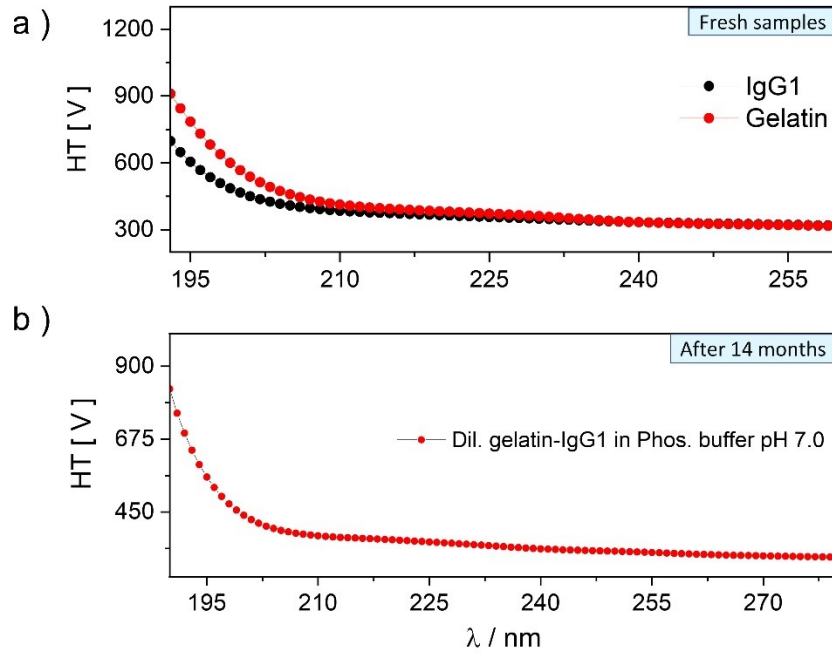


Fig. S3. Circular dichroism HT [V] vs wavelength plots of a) fresh IgG1 and gelatin in the phosphate buffer solution pH 7.0, and b) dil. gelatin-IgG1 solution after 14 months of solid-state cohabitation at room temperature.

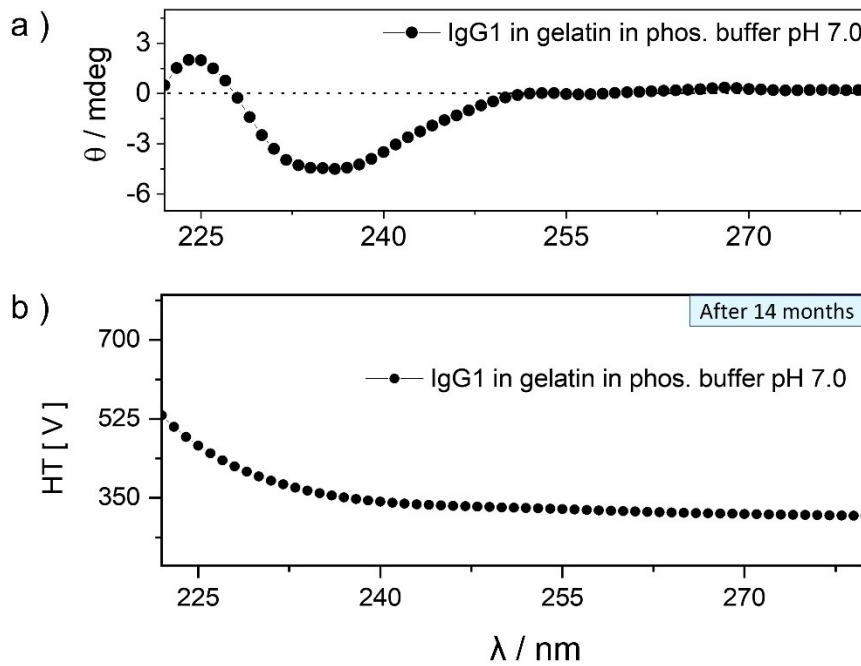


Fig. S4. a) Circular dichroism spectrum, and b) HT [V] vs wavelength plot of IgG1 in the concentrated gelatin solution

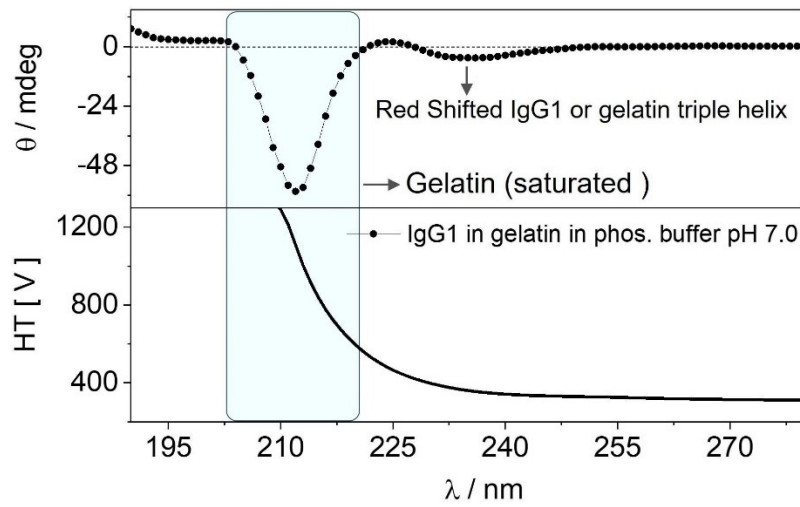


Fig. S5. Circular dichroism spectra of IgG1 showing red-shifted β -sheet conformation or gelatin triple helix peak in the concentrated gelatin solution after 14 months of solid-state cohabitation at room temperature. Due to the high concentration of gelatin, its random coil spectrum is saturated.

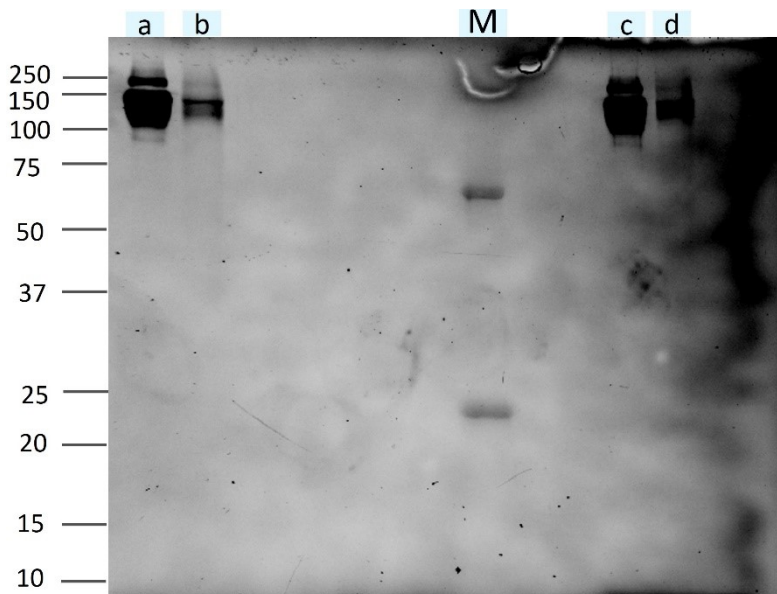


Fig. S6. a) SDS-PAGE gel image of a, c) fresh IgG1 solution and b, d) IgG1 in gelatin solution after 14 months of solid-state cohabitation at room temperature in the solid-state. M is the protein ladder or molecular weight marker

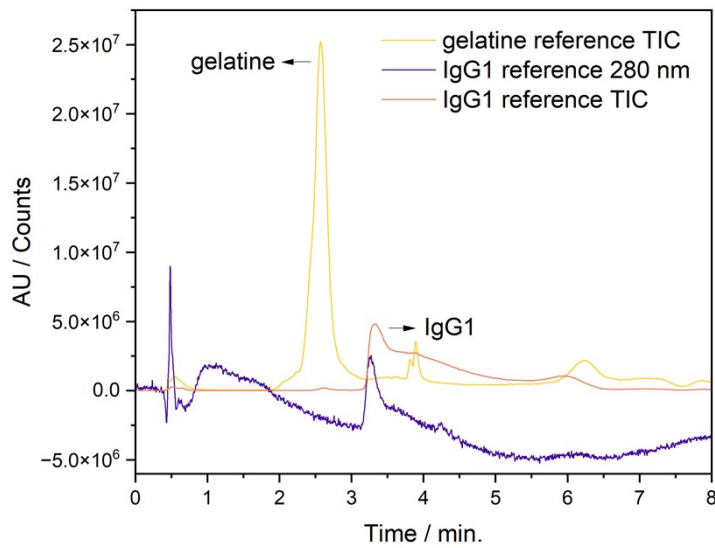


Fig. S7. HPLC reference chromatograms of neat gelatin (yellow line, TIC trace) and IgG1 (orange, violet lines, TIC and 280 nm trace)

Table S1. Summary of HPLC analysis of IgG1 samples

Sample I		Sample + IgG ref.	Sample + 2 x IgG1 ref.
IgG1 integral ($t_{ret}= 3.2$ min)	1684.30	2447.87	3021.50
avg. increase of integral per 0.051 mg/mL ref. spike			668.60
calculated amount of IgG1	0.13 mg/mL \pm 0.03 mg/mL		
calc. total amount of IgG in 14 months old sample	5.2 mg \pm 1.2 mg		
Sample II			
IgG1 integral ($t_{ret}= 3.2$ min)	1754.1	2993.7	4571.9
avg. increase of integral per 0.063 mg/ml ref. spike			1408.9
calculated amount of IgG1	0.078 mg/mL \pm 0.02 mg/mL		
calc. total amount of IgG in 14 months old sample	3.12 mg \pm 0.8 mg		
original IgG1 amount added in gelatin solution on day 1	2.4 mg		

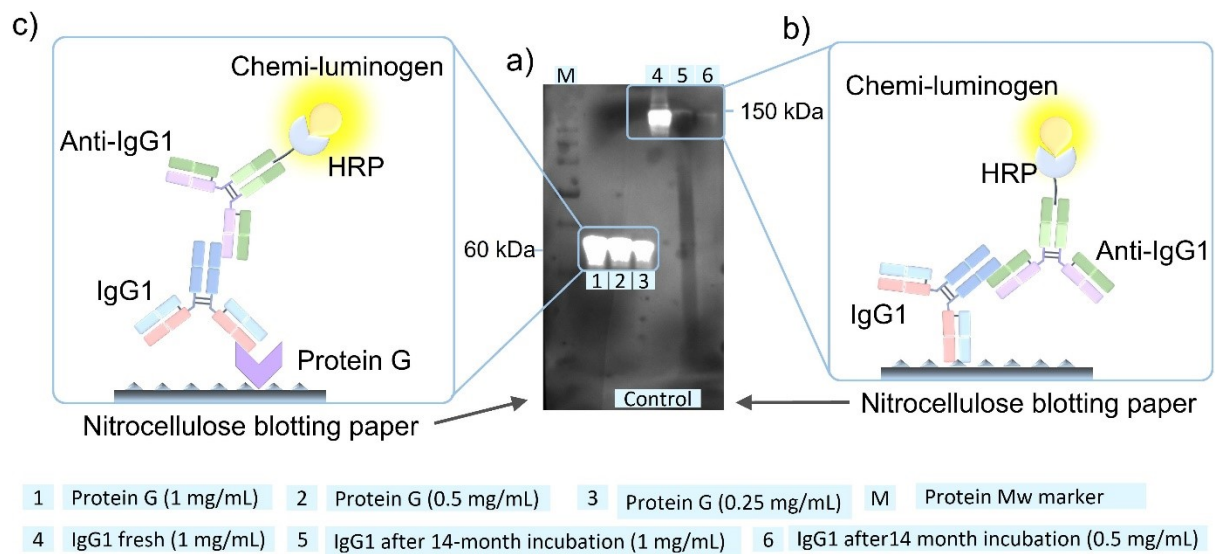


Fig. S8. a) Image of the nitrocellulose blotted paper showing protein G mediated binding of fresh IgG1 to anti-IgG1 (1, 2, 3) and direct binding of anti-IgG1 to fresh IgG1 (4), and 14 months incubated IgG1 (5, 6). b) and c) Schematic presentation of IgG1 binding to anti-IgG1; b) *via* direct binding, and c) *via* protein G mediated binding.

Author Contributions

Pankaj Bharmoria, Alesia Tietze and Kasper Moth-Poulsen conceptualized the idea of this work. Pankaj Bharmoria led the experimental work supported by Saik Ann Ooi, Andreas Cellini, Daniel Tietze, and Hanna Zhdanova. Pankaj Bharmoria and Alesia Tietze wrote the first draft of the manuscript and revised it with the support of other authors including Michal Maj.