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Development of a reusable protein G based SPR immunosensor for direct human growth hormone detection in real samples

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Although it has been shown that protein G based site-directed antibody capture is an advantageous immobilization technique in immunosensor development, the application of such immunosensors for direct repeated real sample measurements has not been addressed. In this surface plasmon resonance analysis based study random covalent antibody immobilization was employed as a reference technique in contrast to site-directed protein G mediated affinity capture and protein G mediated antibody capture stabilized by chemical cross-linking aimed at direct repeated human growth hormone detection in real samples. Site-directed affinity capture increased the analytical signal 2.6 times compared to random antibody immobilization. However, this approach resulted in significant antibody dissociation from the sensor surface during the measurements, which was detrimental for signal evaluation, immunosensor reusability and formed a potential platform for non-specific binding of molecules present in real samples. In contrast, chemical cross-linking of the protein G and antibody complexes under optimal conditions enabled repeated measurements and improved signal evaluation. Moreover, the analytical signal was increased 3.5 times in comparison to random antibody immobilization. The obtained results put emphasis on sensing molecule orientation, stability surface mass density and remaining activity after the immobilization. Based on these findings an immunosensor model for human growth hormone detection was developed. The limit of direct detection of the developed immunosensor was 0.99 nM, the limit of quantification was 3.31 nM and the linear detection range was from 3 to 9 nM. It has been demonstrated that the immunosensor model was prone to non-specific binding of serum proteins that could be minimized following a one-step sample pre-treatment procedure.

Introduction

An immunosensor is an analytical device comprising analyte specific immune system molecules coupled to a signal transducer. Upon analyte binding the physicochemical changes of the recognition molecules are converted to a measureable signal proportional to the analyte concentration. A lot of excellent effort has been put in developing this technique, as it is applicable for the detection of numerous analytes, especially considering the broad choice of monoclonal antibodies that might be employed as biorecognition elements.[1] The most important advantage of immunosensors is quick real-time analysis not requiring labelling or complicated sample pre-treatment procedures. Moreover, a range of available signal transducers can yield output signals with valuable additional information, such as kinetic parameters of the interaction[2] or physicochemical properties of the biomolecular layers.[3]

The first application of surface plasmon resonance (SPR) spectroscopy for immunosensor development was reported in 1983.[4,5] Since then antibodies have been extensively used in SPR immunosensing. When developing these sensors it is particularly important to take into account antibody immobilization since SPR analysis is based on the changes of the dielectric constant at the interface of the gold-coated sensor and the sample.[6] For that reason the formed immunorecognition surfaces should be stable and not contribute to signal interferences.

Antibody adsorption is the simplest surface modification technique which is very often used in conventional immunoassays[7] and immunosensors.[8] However, it has several disadvantages in terms of SPR analysis. First, adsorption is difficult to control and the resulting sensor surface inconsistencies might cause dispersion of analytical signals. Second, conformational changes that proteins undergo upon adsorption might result in reduced antigen-binding activity and lead to non-specific

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binding especially in real sample analysis. Finally, if the system is to be used for multiple measurements, proteins from real samples might displace the adsorbed antibodies.[9,10] As a consequence, adsorption has not been widely used in SPR immunosensor development.

One of the most popular approaches to antibody immobilization is covalent coupling to carboxyl terminated supports by 1-ethyl-3-(3-diaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) chemistry.[11,12] Although this immobilization method solves the surface instability problem, it results in random orientation of the antibody molecules. Subsequently, some antigen-binding sites become unavailable to analyte molecules and partial antibody inactivation occurs due to covalent bonds formed in the effector region. This leads to decreased analytical signals and problems in kinetic parameter evaluation.[3,13]

Consequently, site-directed antibody immobilization has been used to increase sensitivity and experimental reproducibility.[14] Antibodies can be immobilized in a site-directed manner by either direct covalent binding or affinity capture. While methods based on covalent binding yield stable surfaces with well-oriented effector sites, they require chemical modification that often results in damage of antibody molecules and decreased analytical signals. In contrast, since affinity capture is based on non-covalent interactions, antibody structure and activity can be retained. Bacterial proteins A and G specific to the Fc region of antibody molecules are the most commonly used affinity capture reagents.[15,16] A study comparing different antibody immobilization methods in the development of an SPR immunosensor revealed that the highest analytical signal was registered using protein G mediated antibody immobilization.[13] However, only a general principle of applying this technique in SPR immunosensing has been shown. The applicability of protein G based SPR immunosensors aimed at real sample analysis has not been addressed. The main challenge posed in successful application of this immobilization technique is surface instability due to affinity interactions. It leads to two major limitations. First, the main benefit of SPR immunosensing lies in the opportunity to perform multiple automated measurements, so the biorecognition surfaces of SPR immunosensors have to be stable enough to withstand analyte binding and regeneration cycles. Second, antibodies coupled to the surface by affinity interactions might get displaced by naturally occurring plasma or serum proteins.[17] Chemical cross-linking has been employed to circumvent the instability problem. However, additional chemical modification of the antibody molecules might result in the same drawbacks as in the case of direct covalent coupling.[18,19] The effect of such surface modification on the characteristics of an SPR immunosensor aimed at real sample measurements has not been investigated.

For these reasons in the present study we explored protein G-mediated antibody immobilization focusing on its successful application in the development of a reusable SPR based immunosensor for potential analysis in real samples. A model system of mouse monoclonal anti-human growth hormone antibodies (anti-hGH) and their antigen, human growth hormone (hGH), has been chosen. Analytical characteristics of the immunosensor exhibiting the best analytical signal have been determined. Finally, we demonstrate herein problems rising in serum sample analysis and present an uncomplicated sample pre-treatment procedure enabling analyte detection by the developed immunosensor in real serum samples.

Experimental

Reagents

Mouse monoclonal anti-human growth hormone IgG2B antibodies (anti-hGH) and recombinant human growth hormone from *E. coli* (hGH) were obtained from R&D Systems (United Kingdom). Recombinant protein G from *Streptococcus* sp., 11-mercaptoundecanoic acid (11-MUA), 1-ethyl-3-(3-diaminopropyl)carbodiimide hydrochloride (EDC), sodium acetate trihydrate, acetic acid, dimethylpimelimidate hydrochloride (DMP), Tween 80, methanol, sodium borate decahydrate and hexane were obtained from Sigma-Aldrich (Germany). *N*-Hydroxysuccinimide (NHS) and ethanolamine were purchased from Merck (Germany). Sodium dodecylsulfate (SDS) was obtained from AppliChem (Germany). Hydrochloric acid 37% was purchased from Scharlau Chemie S.A. (Spain). PBS tablets (0.14 M NaCl, 0.0027 M KCl, and 0.01 M phosphate buffer pH 7.4) were acquired from AB Medicago (Sweden). All chemicals were of analytical grade or better. All aqueous solutions were prepared in UHQ water (conductivity less than 1 μS cm^{-1}) purified using a DEMIWA rosa 5 (WATEK, Czech Republic). Human serum was provided by the National Blood Center (Lithuania).

SPR sensor disk surface preparation

The surfaces of the gold-coated SPR sensor disks (Metrohm Autolab B.V., The Netherlands) were cleaned according to a protocol provided by the manufacturer. Briefly, the disks were incubated in methanol for 30 min and hexane for 2 min. Afterwards the sensor disks were transferred to 1 mM 11-MUA in methanol and stored overnight to form a self-assembled monolayer (SAM). The disks were then rinsed with methanol and deionised water, dried with argon and assembled on a hemi-cylinder *via* a refractive index matching fluid (n_D = 1.460–1.570) (Cargille Laboratories, USA). The hemi-cylinder was installed in a double channel SPR-analyser Autolab Esprit (Metrohm Autolab B.V., The Netherlands) with a fixed sample volume (50 μl) cuvette and mixing. The SPR measurement surface area in one channel was 7.9 mm^2. The measurements were performed at 23 °C. All incubation, immobilization and interaction steps discussed in the following chapters were performed in the SPR cuvette under mixing.

Anti-hGH immobilization

Random covalent anti-hGH immobilization. The carboxyl groups of 11-MUA SAM were activated using a mixture of 400 mM EDC and 100 mM NHS for 300 s. The resulting surface was exposed to 330 nM anti-hGH in 10 mM sodium acetate buffer, pH 4.5 for 1000 s. The deactivation of the activated carboxyl

groups on the surface was carried out using 1 M ethanolamine, pH 8.5 for 900 s.

Site-directed anti-hGH immobilization. Covalent protein G immobilization. The carboxyl groups of 11-MUA SAM were activated by incubating with a mixture of 400 mM EDC and 100 mM NHS for 300 s. The activated surface was then exposed to 9.1 μM protein G in 10 mM sodium acetate buffer, pH 4.5 for 1000 s. The deactivation of the activated carboxyl groups on the surface was carried out using 1 M ethanolamine, pH 8.5 for 900 s.

Protein G mediated anti-hGH affinity capture. Protein G mediated anti-hGH capture was performed by exposing the protein G surface to 330 nM anti-hGH in 10 mM PBS with 0.005% Tween 80, pH 7.4. After obtaining a stable baseline, an association phase was carried out for 500 s and was followed by a dissociation phase of 500 s.

Protein G mediated anti-hGH capture with covalent cross-linking. After anti-hGH protein G affinity capture the surface was incubated with DMP solutions of varied concentrations in 0.1 M sodium borate buffer, pH 9.2 for different incubation times. Then the unreacted groups were blocked using 1 M ethanolamine, pH 8.5 for 1500 s. Finally, the surfaces were stabilized with 10 mM PBS with 0.005% Tween 80, pH 7.4 and 10 mM NaOH/0.5% SDS aqueous solution for 1300 s.

Anti-hGH/hGH interaction

Anti-hGH/hGH interaction was carried out for 100 s when comparing different anti-hGH immobilization approaches and for 200 s when evaluating the analytical characteristics of the sensor. The association phase of anti-hGH/hGH interaction was followed by dissociation with 10 mM PBS with 0.005% Tween 80, pH 7.4, and surface regeneration with a gentle regeneration solution of 10 mM NaOH/0.5% SDS. One channel of the SPR analyser was used for anti-hGH/hGH interaction measurements and the other channel was used as the reference. hGH in buffer or serum was added to the measurement channel. Buffer or serum lacking hGH was added to the reference channel for the negative control. The difference between these channels was used for the evaluation of anti-hGH/hGH interaction.

Anti-hGH/hGH interaction in buffer. The developed recognition surfaces were allowed to react with 600 nM hGH dissolved in 10 mM PBS with 0.005% Tween 80, pH 7.4. The immunosensor based on the best immobilization method was exposed to a range of different hGH concentrations (3–200 nM).

Anti-hGH/hGH interaction in human serum. The sensing surface exhibiting the best performance was allowed to react with 10% human serum spiked with a range of hGH concentrations (5–600 nM). Serum was spiked after serum pre-treatment procedures immediately prior to measurements.

Serum pre-treatment procedures

Serum was obtained frozen and was thawed immediately before the experiments. Then it was filtered using 0.22 μm Whatman® Puradisc PES sterile filters Sigma (GE Healthcare, UK).

Ammonium sulfate IgG precipitation from serum samples. Ammonium sulfate IgG precipitation was performed according to a previously published protocol.[20] Briefly, saturated ammonium sulfate solution was prepared 24 h prior to the precipitation procedure and was stored at 4 °C. Then serum was cooled to 4 °C and a saturated ammonium sulfate solution was added under stirring to yield a final saturation of 45%. The mixture was then stored at 4 °C under stirring. Afterwards the mixture was divided into 1 ml aliquots and centrifuged at $4000g_{av}$ for 2 min. The pellet was discarded and the supernatant aliquots were combined and used for further analysis.

IgG and BSA affinity extraction from serum samples. ProteoExtract Albumin/IgG removal kit (Calbiochem, Germany) was used for IgG and BSA extraction from serum samples. First, the BSA removal column was equilibrated with 2 ml binding buffer. Then 1 ml serum was applied to the column and the flow-through was collected. Bound proteins were eluted from the column with 2 ml elution buffer. Finally, the column was re-equilibrated with 2 ml binding buffer. BSA and IgG extraction was repeated from one to three times and serum fractions collected after each BSA and IgG extraction step were used for further analysis.

Evaluation of anti-hGH immobilization and interaction with hGH

To evaluate anti-hGH immobilization or anti-hGH/hGH interaction, two report points were selected. One was placed 10 s prior to anti-hGH or anti-hGH/hGH association phase and the other was placed 15 s after the start of the dissociation phase. The surface mass densities were calculated from a linear relationship between the SPR angle shift and the amount of bound material. A 122 m° change in the SPR angle corresponds to 1 ng mm^{-2} change in protein surface mass density.

Results and discussion

Anti-hGH immobilization

The schematic representation of the investigated antibody immobilization techniques is shown in Fig. 1. The registered SPR responses and calculated molecule surface mass densities are provided in Table 1.

Random covalent immobilization from a 330 nM anti-hGH solution resulted in an SPR response of 314 m° and a surface mass density of 2.57 ng mm^{-2}. This value compared well with values suggested by other authors for antibody molecule monolayer coverage. Theoretical antibody amounts adsorbed in monolayers of extreme orientations are in the range of 2.0–5.5 ng mm^{-2} with 2.0 ng mm^{-2} corresponding to a monolayer with antibodies in 'side-on' orientation and 5.5 ng mm^{-2} to a layer of antibodies oriented in an 'end-on' manner.[9,21] In addition, the results were in good agreement with a surface mass density value for a monolayer of polyclonal antibodies (2.1 ng mm^{-2}) reported in a study based on total internal reflection ellipsometry.[3] The discrepancies were most likely influenced by varying immobilization conditions between the experiments, such as proteins used, concentrations, temperatures, ionic strength of the solutions and incubation times.

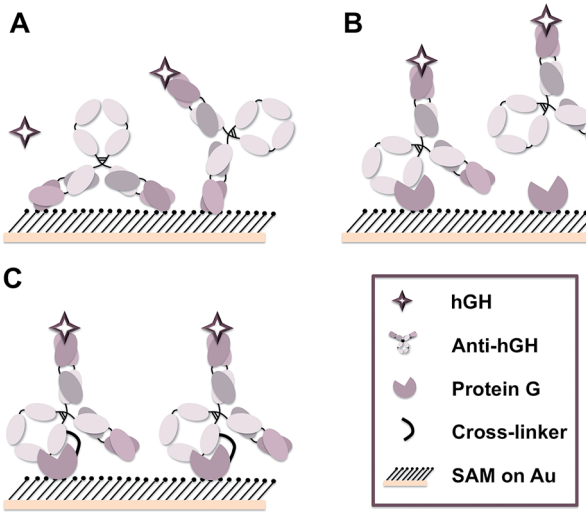

Fig. 1 Schematic representation of different anti-hGH immobilization techniques. (A) – Random covalent immobilization. (B) – Site-directed affinity protein G-mediated immobilization; (C) – site-directed protein G-mediated immobilization stabilized with chemical cross-linking. hGH – human growth hormone, anti-hGH – antibodies against human growth hormone, and SAM – self-assembled monolayer.

In order to immobilize anti-hGH in a site-directed manner using an intermediary layer of a capture molecule, protein G was first coupled to an amine reactive 11-MUA SAM surface. An SPR response of 171.10 m° was registered following protein G immobilization. This value was equal to a surface mass density value of 1.41 ng mm^{-2}. Next the protein G-modified surface was exposed to 330 nM anti-hGH. After antibody immobilization a 591.26 m° SPR response was registered. The surface mass density value was equal to 4.85 ng mm^{-2} (32.33 fM mm^{-2}). A very similar surface mass density of 33 fM mm^{-2} was reported for monoclonal antibodies against progesterone immobilized *via* protein G.[22]

Site-directed anti-hGH immobilization resulted in a 1.9 times increase in anti-hGH surface mass if compared to that obtained by random immobilization. These results were most likely influenced by different anti-hGH interfacial positions. Randomly immobilized anti-hGH should adopt various steric positions, *e.g.*, 'flat-on', 'side-on' or 'end-on' resulting in less antibody molecules on the surface. In contrast, anti-hGH immobilized in a site-directed manner should be deposited more uniformly with anti-hGH in either 'side-on' or 'end-on' orientation leading to a more compressed antibody layer. Therefore, the results confirmed that anti-hGH was immobilized in a site-directed manner and formed a more densely packed layer as opposed to randomly immobilized anti-hGH.

However, in the case of affinity anti-hGH immobilization an overall downward shift was observed in the SPR sensogram. It could be attributed to gradual antibody dissociation from the protein G surface. As it can be seen from Table 1, although a report point placed at the beginning of the dissociation phase indicated an SPR response value of 591.26 m°, the SPR response kept diminishing throughout anti-hGH immobilization and was equal to 391.97 m° before interaction with hGH.

Anti-hGH dissociation most likely occurred due to protein G/anti-hGH binding kinetics, more specifically, fast anti-hGH dissociation influenced by the saturation of protein G antibody binding sites and the subsequent loss of rebinding. One option of circumventing such anti-hGH leakage is using smaller anti-hGH concentrations. However, in an attempt to develop a stable surface suitable for multiple measurements, an option of chemical cross-linking was considered. To achieve this, protein G/anti-hGH complexes were incubated with a bifunctional cross-linker DMP. At alkaline pH values (pH 8.0–9.0) DMP reacts with primary α and ε amines of protein molecules and forms amidine bonds retaining the net charge character of protein primary amines with which it reacts. This is important for preserving protein conformation.

First, the protein G/anti-hGH complex was cross-linked with 0.4 mM DMP for 60 min. The decrease of anti-hGH dissociation rate was registered during the cross-linking procedure until anti-hGH leaking was gradually suspended. As can be seen from Table 1, a 729.89 m° SPR angle change corresponding to 5.98 ng mm^{-2} anti-hGH surface mass density was registered following anti-hGH affinity coupling. These values were reduced to 259.46 m° and 2.13 ng mm^{-2} after cross-linking, which meant that only 35.61% of the captured anti-hGH remained on the surface. Although a significant decrease in anti-hGH surface mass density as opposed to affinity coupling was observed, unlike the

Table 1 Comparison of anti-hGH immobilization techniques after anti-hGH immobilization and after interaction with hGH in terms of SPR responses (R) and calculated surface mass densities (Γ). CV < 0.05

	Anti-hGH after immobilization		Anti-hGH before interaction		Anti-hGH/hGH interaction	
Anti-hGH immobilization technique	R (m°)	Γ (ng mm^{-2})	R (m°)	Γ (ng mm^{-2})	R (m°)	Γ (ng mm^{-2})
Random	314.00 ± 11.60	2.57 ± 0.10	314.00 ± 11.90	2.57 ± 0.10	16.66 ± 0.66	0.14 ± 0.01
Affinity	591.26 ± 26.10	4.85 ± 0.21	391.97 ± 16.26	3.21 ± 0.13	43.77 ± 1.93	0.36 ± 0.02
Cross-linking						
0.4 mM DMP, 60 min	729.89 ± 28.00	5.98 ± 0.23	259.46 ± 10.60	2.13 ± 0.09	58.21 ± 1.75	0.48 ± 0.01
2 mM DMP, 60 min	720.14 ± 29.96	5.90 ± 0.25	383.82 ± 16.23	3.15 ± 0.13	44.00 ± 1.84	0.36 ± 0.02
20 mM DMP, 60 min	718.96 ± 29.01	5.89 ± 0.24	638.07 ± 24.93	5.23 ± 0.20	42.43 ± 1.59	0.35 ± 0.01
20 mM DMP, 180 min	763.57 ± 23.29	6.26 ± 0.19	792.98 ± 32.99	6.50 ± 0.27	17.78 ± 0.64	0.15 ± 0.01
50 mM DMP, 180 min	791.95 ± 25.97	6.49 ± 0.21	809.53 ± 29.36	6.64 ± 0.24	13.77 ± 0.67	0.11 ± 0.01

latter case, the cross-linked surface was stable and could be used for repeated measurements. In an effort to further increase the anti-hGH surface mass-density, higher DMP concentrations of 2 mM and 20 mM for 60 min were used. The SPR responses following anti-hGH affinity immobilization were 720.14 m° (5.90 ng mm^{-2}) and 718.96 m° (5.89 ng mm^{-2}) respectively. After cross-linking SPR responses decreased to 383.82 m° (3.15 ng mm^{-2}) in the case of 2 mM DMP and to 638.07 m° (5.23 ng mm^{-2}) when using 20 mM DMP. It enabled the increase of anti-hGH surface mass density up to 2.5 times (88.8% anti-hGH remained as opposed to the initial value). To yield even higher anti-hGH surface loading and get a completely stable anti-hGH surface, the DMP cross-linking time was prolonged. After cross-linking with 20 mM DMP for 180 min no anti-hGH dissociation from the surface was registered and even a small increase in the SPR response after the cross-linking was observed. Cross-linking with a higher DMP concentration of 50 mM for 180 min revealed the same tendency. In this case the observed SPR response was slightly increased as well. These increases could be most likely attributed to the refractive index of DMP that was probably not completely rinsed from the sensing surface.

Anti-hGH and hGH interaction

The sensing surfaces prepared using different anti-hGH immobilization techniques were compared by exposing them to 600 nM hGH. An SPR response of 16.66 m° corresponding to 0.14 ng mm^{-2} surface mass density was registered by the surface modified with randomly oriented anti-hGH (Table 1 and Fig. 2A).

In contrast, protein G mediated anti-hGH affinity capture resulted in a 43.77 m° SPR response to hGH. It was equal to 0.36 ng mm^{-2} hGH surface mass density. The difference between the responses to hGH of random and site-directed affinity capture anti-hGH surfaces was 2.6 times. However, a continuous downward shift of the SPR signal upon hGH binding could be observed (Fig. 2A). The downward shift was observed from as early as the baseline phase and by the end of the hGH dissociation phase the SPR signal returned to the protein G level. Although the most obvious limitation of this technique is that sensor surface is single use, it is also important to note that antibody–antigen interaction kinetics is very difficult to evaluate, especially if data fitting is required which is often the case in SPR analysis. Additionally, if the immunosensors were to be used for analyte detection in real samples, the unoccupied antibody binding sites would be an excellent platform for antibodies naturally occurring in the samples to bind.

Cross-linking the protein G/anti-hGH complexes with 0.4 mM DMP for 60 min improved the surface stability. The SPR signal could be repeatedly returned to a stable anti-hGH baseline value after the regeneration. This surface preparation technique yielded an SPR response of 58.21 m° accounting for 0.49 ng mm^{-2} hGH surface mass density (Table 1, Fig. 2B). This response was improved 3.5 times as opposed to that obtained using the random antibody immobilization based immunosensor. Similar results were presented in a study examining protein G mediated antibody immobilization on matrix-assisted laser desorption/ionization targets.[23] In general, antigen binding capacity can be improved 2–8 times using site-directed antibody immobilization methods.[24]

After cross-linking protein G/anti-hGH complexes with 2 mM DMP for 60 min, a 44.00 m° SPR response equal to 0.36 ng mm^{-2} hGH surface mass density was registered. This value was smaller than that obtained previously but still was a 2.6-fold improvement over the results obtained by random antibody immobilization. Incubating the sensor surface with 20 mM DMP for 60 min and exposing it to 600 nM hGH resulted in an SPR signal change of 42.43 m° corresponding to 0.35 ng mm^{-2} hGH surface mass density. Higher DMP concentrations and longer incubation times correlated with lower SPR responses. Cross-linking with 20 mM and 50 mM DMP for 180 min yielded 17.78 m° and 13.77 m° SPR responses to 600 nM hGH, which corresponded to 0.15 ng mm^{-2} and 0.11 ng mm^{-2} hGH, respectively. These values were the lowest out of all the tested cross-linked systems and even lower than that obtained using

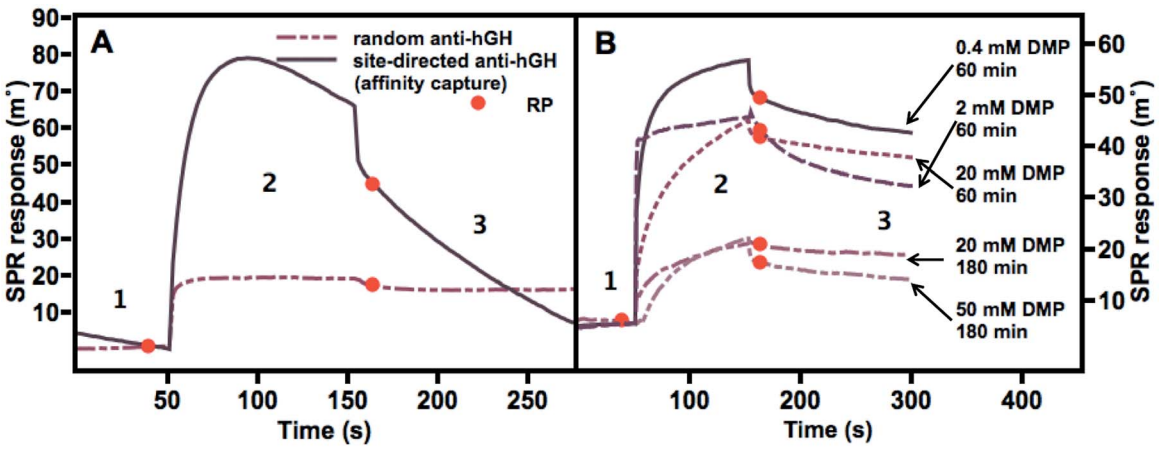

Fig. 2 SPR responses to 600 nM hGH by sensor surfaces prepared by different anti-hGH immobilization techniques. (A) – Anti-hGH immobilized in a random (- -) and site-directed (–) manner. (B) – Protein G mediated anti-hGH immobilization after different DMP cross-linking procedures. (1) – Baseline phase, (2) – association phase, (3) – dissociation phase, and RP – report points.

the affinity coupled protein G/anti-hGH system. In fact, these results were very close to those obtained by randomly immobilized anti-hGH (16.66 m°). Such a decrease in anti-hGH binding capacity could be explained by partial inactivation of anti-hGH molecules due to DMP attachment in antigen binding sites.

It is interesting to note that the highest response to hGH correlated with the lowest anti-hGH surface mass density. From the results summarized in Table 1 and Fig. 2 it can be seen that although antibody orientation has a weighty influence on the immunosensor response, it is also important to consider steric limitations. Despite similar surface mass densities of the randomly oriented and site-directed anti-hGH surfaces after cross-linking with 0.4 mM DMP for 60 min, an increase in the SPR response in the site-directed system was observed. On the other hand, when comparing site-directed systems, the decrease in the anti-hGH surface densities yielded better responses to hGH. In addition, it is important to consider the effect of chemical cross-linking conditions on the immobilized antibodies. On the one hand, high cross-linker concentrations and long incubation times might damage antibody structure by interfering with the epitopes. Conversely, low cross-linker concentrations might affect the surface mass density of the immobilized molecules. Apparently, all these aspects play an important role in hGH immunosensor development. Taking into account these aspects can significantly improve the characteristics of the developed immunosensor.

Analytical characteristics of the cross-linked protein G/anti-hGH based immunosensor

A reusable SPR immunosensor using hGH as the model antigen system was developed based on the most efficient anti-hGH immobilization method. When a stable and sensitive protein mediated anti-hGH sensing surface was obtained after cross-linking with 0.4 nM DMP for 60 min, it was exposed to a range of hGH concentrations (3–200 nM). The SPR response dependence on different concentrations of hGH is depicted in Fig. 3A. All phases of anti-hGH/hGH interaction are presented including the report points that were used for further analysis of the SPR data. The stability of the anti-hGH surface was confirmed since the baseline phase of the repeated measurements was consistent.

It is interesting to note that at the beginning and in the end of the association phase, some sensograms exhibit the so-called 'bulk shifts', particularly the sensogram of the SPR response to 200 nM hGH, which displays a significant negative shift. These shifts are caused by mismatched refractive indices of the sample and running buffer and could not be completely eliminated despite the identical compositions of sample and running buffers and subtracting the response in the reference channel from the response in the measurement channel. Since our aim was concentration measurements, bulk shifts could be circumvented by placing report points prior to the association phase and at the beginning of the dissociation phase. However, if other types of measurements are to be considered (*e.g.*, kinetics), bulk shifts have to be corrected by precisely matching the refractive indices of the sample and running buffers or by employing special methods, such as solvent correction.

The regeneration step (Fig. 3A, phase 4) was carried out with a gentle regeneration solution of 10 mM NaOH/0.5% SDS. Although 10 mM glycine pH 1.5–2.5 is often used for this purpose, we chose an alkaline solution since the amidine bonds between the DMP and proteins are reversible at low pH values. Regeneration was very efficient and resulted in SPR response decreasing to anti-hGH baseline levels. The sensing surface could be used for 25 analyses before a gradual decrease in the baseline value and a decrease in the SPR response was observed (Fig. 4).

After SPR responses to a range of hGH concentrations were recorded, a calibration curve and linear detection range were prepared. As can be seen from Fig. 3B, analytical signals were of good precision with relative standard deviation (RSD) values less than 5% and measurement repeatability approx. 95.8%. The linear detection range was defined in the range of 3 to 9 nM

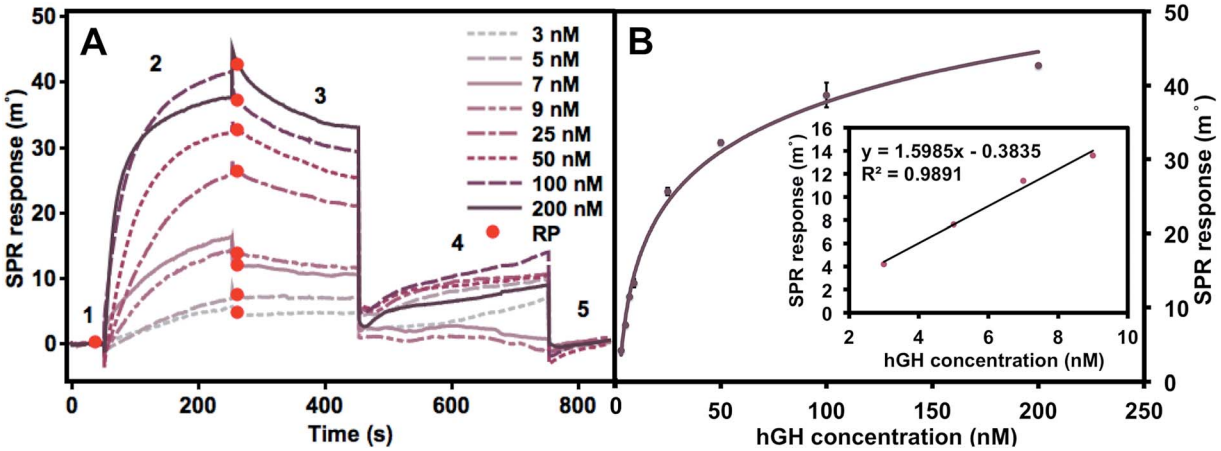

Fig. 3 The responses of the developed SPR immunosensor to a range of hGH concentrations in PBS (A) and the corresponding calibration curve (B). Inset – linear range interval. (1) – Baseline phase, (2) – association phase, (3) – dissociation phase, (4) – regeneration phase (10 mM NaOH/0.5% SDS), (5) – back to baseline phase, and RP – report points.

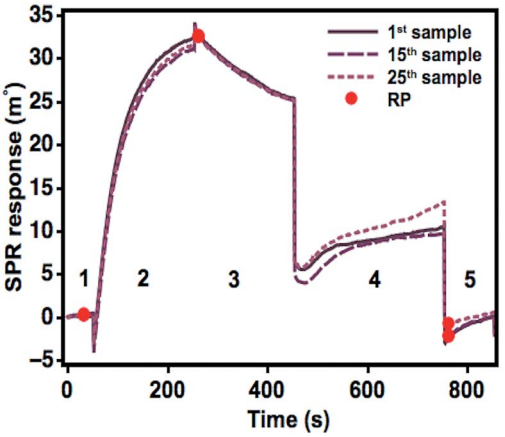

Fig. 4 The responses of the developed SPR immunosensor to 50 nM hGH samples upon repeated measurements.

hGH ($R^2 = 0.9891$ and $n = 3$). The limit of detection (LOD) was calculated as the lowest concentration of the analyte giving an analytical signal greater than the background value plus three times the standard deviation and was equal to 0.99 nM. In addition, the determined LOD was 4.6 times lower than that obtained in a direct SPR immunosensor format using randomly oriented polyclonal antibodies against a recombinant hGH isoform.[25] The limit of quantification (LOQ) was determined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy and was equal to 3.31 nM. The concentration limits for the detection using the Autolab SPR device are 10^{-11} to 10^{-3} M, so the analytical characteristics of the developed immunosensor were well within the sensitivity limits of the device. In order to test the specificity of the developed assay, the sensing surface has been exposed to 50 nM porcine human growth hormone (pGH). Upon sample injection no SPR response was observed and subsequently no analytical signal was registered at the report point, which accounted for no cross-reactivity with pGH.

Application of the developed immunosensor in serum samples

Finally, the performance of the developed immunosensor in real samples was determined in order to evaluate the influence of serum protein matrix on the analytical signal. When the immunosensor was exposed to serum samples spiked with 600 nM hGH, the SPR angle shift of 895.61 m° was observed in the measurement channel (Fig. 5A). However, a high SPR angle shift was registered in the reference channel as well where serum without hGH was added. Despite the increased signals in both channels, an SPR response difference of 18.83 m° was registered between them.

High SPR angle shifts indicated large amounts of molecules binding to the sensor surface. Most likely it was serum immunoglobulins binding to the unoccupied binding sites of protein G. However, it was not clear whether the SPR signal difference between the channels was caused by hGH binding or by

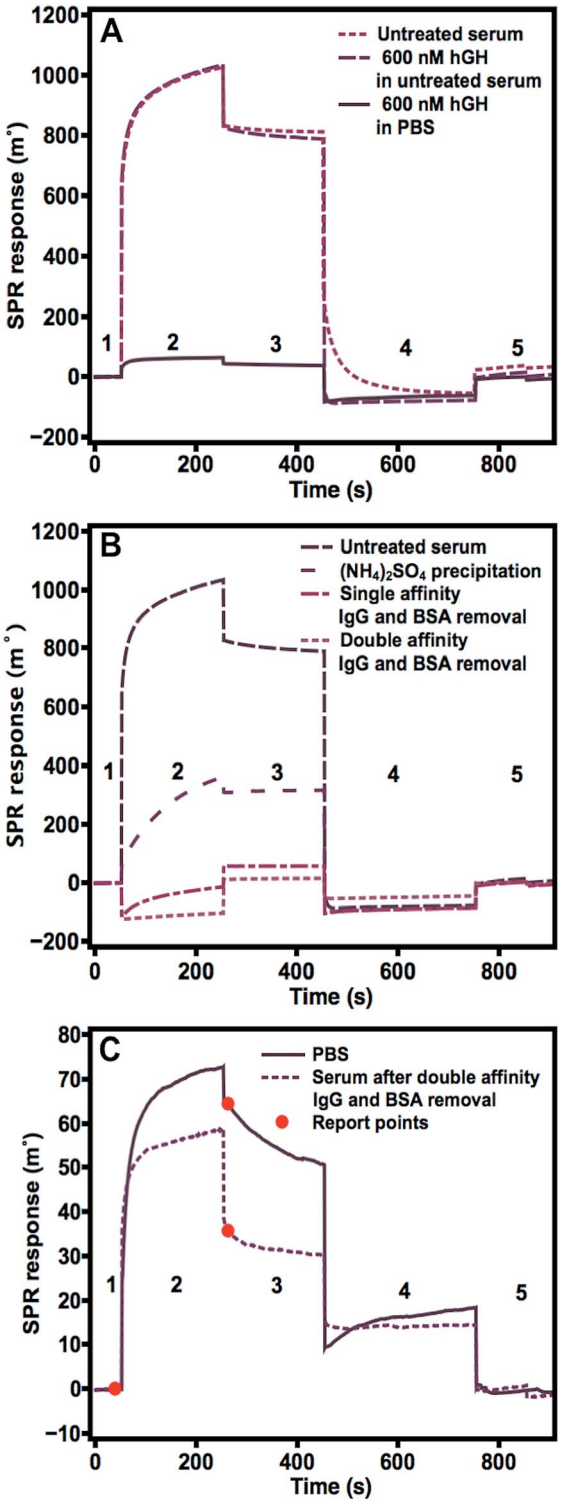

Fig. 5 The responses of the developed SPR immunosensor to human serum samples. (A) – Comparison of 600 nM hGH spiked PBS and untreated serum samples as opposed to a serum sample not spiked with hGH. (B) – Comparison of differently pre-treated serum samples and an untreated serum sample. (C) – Comparison of 600 nM hGH spiked PBS and a human serum sample after double albumin/IgG extraction. hGH – human growth hormone.

variations in the non-specific binding. To see what part of the response could be attributed to hGH, not spiked serum samples were added to both channels. SPR angle shifts of 821.87 and 817.55 m° were registered in the measurement and reference channels, respectively. These results confirmed that the signal difference between the two channels was mainly caused by hGH binding. Principally it would be possible to evaluate hGH concentration from the difference between the channels if the responses registered in serum samples not spiked with hGH were considered as the negative control. However, in this study aliquots of the same serum sample were used for all measurements. Conversely, serum samples obtained from different individuals might strongly differ, especially in immunoglobulin composition[26–28] which makes such analysis impossible. Therefore, in an effort to investigate whether the reason of high non-specific responses was immunoglobulin binding and in order to adapt the immunosensor for human sample analysis, it was decided to remove immunoglobulins from serum prior to the measurements.

Two immunoglobulin extraction methods were tested. The first one was immunoglobulin precipitation with ammonium sulfate since it is a simple, inexpensive and widely used method. The second was immunoglobulin and albumin removal columns. After immunoglobulin precipitation from serum the samples were analyzed using the developed immunosensor and an SPR response of 307.25 m° was registered (Fig. 5B). This was a 62.62% (2.68 times) decrease in the response as opposed to the untreated serum sample proving that IgG binding was at least a partial cause of the high non-specific responses. Native protein G has both immunoglobulin and albumin binding sites[29] but the BSA specificity has been removed from the widely used recombinant protein G. Nevertheless, the developed immunosensor was tested for BSA binding but no binding was observed (data not shown), which supported the hypothesis that IgG binding was the main problem of high non-specific responses.

Since ammonium sulfate precipitation is quite a crude IgG extraction method, to achieve higher extraction efficiency the serum samples were pre-treated with ProteoExtract® Albumin/IgG Removal Kit. A large bulk shift due to differences in the refractive indices of buffer and sample solutions was observed despite IgG and BSA extraction. The bulk shift was more pronounced at the beginning of the dissociation phase and could be observed as a steep drop in the SPR sensogram which could be circumvented by the use of the report points. The SPR response after pre-treating the serum samples with albumin/IgG removal columns was 56.41 m°. This was a 93% (14.6 times) decrease in the non-specific binding as opposed to the untreated serum samples. This result proved that IgG binding was the main cause of the increased SPR signals. In addition, these results verified the potential antibody displacement problem in immunosensors based on affinity immobilization.

Despite a significant decrease in non-specific binding, the obtained response in not spiked serum samples was very close to that obtained using 600 M hGH in PBS (61.69 m° in the measurement channel). For this reason the samples were subjected to repeated BSA/IgG extraction which yielded an SPR response of 11.94 m°. This value showed a 98.55% (68.83 times) decrease in the non-specific response and enabled measurements in spiked serum samples (Fig. 5B). The non-specific signal could be reduced additionally by subtracting the response registered in the reference channel from those obtained in the measurement channel. This way the SPR response in the pre-treated serum samples decreased to 6.43 m°.

Eventually, the immunosensor was exposed to human serum spiked with 600 nM hGH. An SPR signal of 35.57 m° was registered and converted to 0.29 ng cm^{-2} surface mass density. This analytical signal was approximately 1.6 times lower in comparison to the response to the same hGH concentration (600 nM) in buffer solution (Fig. 5C).

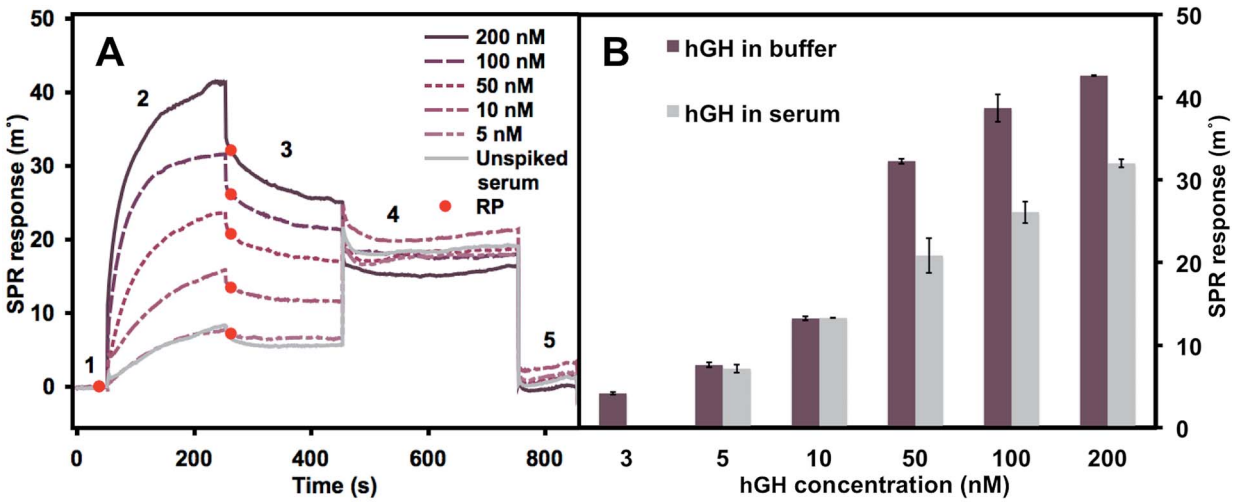

Fig. 6 The responses of the developed SPR immunosensor to a range of hGH concentrations in human serum samples (A) and comparison of SPR immunosensor responses to hGH in PBS and human serum samples (B).

To test the immunosensor responses to other hGH concentrations and compare with those obtained in PBS, serum samples spiked with 5–200 nM hGH were investigated (Fig. 6A). The obtained SPR responses increased with increasing hGH concentrations. The lowest hGH concentration that could be detected in spiked serum was 5 nM hGH yielding an SPR response of 7.18 m°. However, in contrast to measurements in PBS, where 3 nM hGH could be detected with an SPR response of 4.17 m°, a 6.43 m° SPR response could be observed in the case of unspiked serum. This response could most likely be attributed to the nonspecific binding of some proteins still remaining in serum, including some IgG molecules. Nevertheless, the signals could be regenerated to the baseline and the immunosensor could be used for multiple measurements. Overall, the obtained responses were up to 35% lower compared to those in PBS, especially at hGH concentrations higher than 10 nM (Fig. 6B).

Conclusions

This study has shown that sensing molecule orientation, stability, surface mass density and activity are critical to the sensitivity and applicability of the protein G mediated immunosensing surfaces. It has been demonstrated that considering these quantities is a decisive step in the successful development of a reusable SPR immunosensor based on this surface modification technique. Although protein G mediated antibody immobilization increased the direct SPR immunosensor signal, it was also prone to dissociation, which prevented multiple measurements, negatively affected the analytical signal, and was a platform for non-specific binding of serum immunoglobulins. Protein G-antibody complexes could be stabilized by cross-linking but it was also essential to take into account the cross-linking conditions. They had direct influence on the immobilized antibody molecule activity and surface mass density. These parameters had to be optimized to yield desired analytical signals and prevent detrimental steric hindrances. The performance of the developed immunosensor for hGH detection was tested in human serum samples, in which high non-specific binding of serum immunoglobulins to the sensor surface might occur. The presented findings put emphasis on evaluating the composition of the real samples to be analysed. Protein G mediated antibody immobilization might be more efficient in samples lacking naturally occurring immunoglobulins. Alternatively, a simple serum pre-treatment procedure enabling measurements in human serum samples has been proposed. All in all, protein G mediated antibody immobilisation is an attractive surface preparation technique for SPR immunosensor development and the findings presented in this study could help to exploit its full potential. This sensor surface preparation method can also be adapted to other antibody types or different systems, for example, a combination of monoclonal antibodies or polyclonal antibodies. Finally, the SPR responses could be further improved and the LOD could be lowered by employing signal amplification.

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References

1 A. P. F. Turner, *Chem. Soc. Rev.*, 2013, **42**, 3184–3196.
2 I. Baleviciute, Z. Balevicius, A. Makaraviciute, A. Ramanaviciene and A. Ramanavicius, *Biosens. Bioelectron.*, 2013, **39**, 170–176.
3 Z. Balevicius, A. Ramanaviciene, I. Baleviciute, A. Makaraviciute, L. Mikoliunaite and A. Ramanavicius, *Sens. Actuators, B*, 2011, **160**, 555–562.
4 B. Liedberg, C. Nylander and I. Lunström, *Sens. Actuators, B*, 1983, **4**, 299–304.
5 A. Makaraviciute, T. Ruzgas, A. Ramanavicius and A. Ramanaviciene, *Anal. Methods*, 2014, **6**, 2134–2140.
6 D. R. Shankaran, K. V. Gobi and N. Miura, *Sens. Actuators, B*, 2007, **121**, 158–177.
7 F. Yu, S. Yu, L. Yu, Y. Li, Y. Wu, H. Zhang, L. Qu and P. D. B. Harrington, *Food Chem.*, 2014, **149**, 71–75.
8 Y. Lou, T. He, F. Jiang, J.-J. Shi and J.-J. Zhu, *Talanta*, 2014, **122**, 135–139.
9 J. Buijs, D. D. White and W. Norde, *Colloids Surf., B*, 1997, **8**, 239–249.
10 C. A. Haynes and W. Norde, *J. Colloid Interface Sci.*, 1995, **169**, 313–328.
11 J. Martinez-Perdiguero, A. Retolaza, L. Bujanda and S. Merino, *Talanta*, 2014, **119**, 492–497.
12 L.-Y. Zhang and Y.-J. Liu, *Anal. Chim. Acta*, 2014, **806**, 204–209.
13 A. Kausaite-Minkstimiene, A. Ramanaviciene, J. Kirlyte and A. Ramanavicius, *Anal. Chem.*, 2010, **82**, 6401–6408.
14 A. Makaraviciute and A. Ramanaviciene, *Biosens. Bioelectron.*, 2013, **50**, 460–471.
15 B. K. Oh, B. S. Chun, K. W. Park, W. C. Lee, W. H. Lee and J. W. Choi, *Mater. Sci. Eng., C*, 2004, **24**, 65–69.
16 J. H. Park, Y.-P. Kim, I.-H. Kim and S. Ko, *Food Control*, 2014, **36**, 183–190.
17 L. Vroman, A. Adams, G. Fischer and P. Munoz, *Blood*, 1980, **55**, 156–159.
18 L. Callipo, G. Caruso, P. Foglia, R. Gubbiotti, R. Samperi and A. Lagana, *Anal. Biochem.*, 2010, **400**, 195–202.
19 G. Bergstrom and C. F. Mandenius, *Sens. Actuators, B*, 2011, **158**, 265–270.
20 M. Page and R. Thorpe, *The Protein Protocols Handbook*, ed. J. Walker, Humana Press, 1996, pp. 721–722.
21 J. Buijs, J. W. T. Lichtenbelt, W. Norde and J. Lyklema, *Colloids Surf., B*, 1995, **5**, 11–23.
22 W. Schramm and S.-H. Paek, *Anal. Biochem.*, 1992, **205**, 47–56.
23 H. Neubert, E. S. Jacoby, S. S. Bansal, R. K. Iles, D. A. Cowan and A. T. Kicman, *Anal. Chem.*, 2002, **74**, 3677–3683.

24 B. Lu, M. R. Smyth and R. Okennedy, *Analyst*, 1996, **121**, R29–R32.
25 T. H. J. Heutmekers, M. G. E. G. Bremer, W. Haasnoot and M. W. F. Nielen, *Anal. Chim. Acta*, 2007, **586**, 239–245.
26 R. G. Hamilton, *Clin. Chem.*, 1987, **33**, 1707–1725.
27 R. A. Manz, A. E. Hauser, F. Hiepe and A. Radbruch, *Annu. Rev. Immunol.*, 2005, **23**, 367–386.
28 H. A. Krebs, *Annu. Rev. Biochem.*, 1950, **19**, 409–430.
29 C. R. Goward, L. I. Irons, J. P. Murphy and T. Atkinson, *Biochem. J.*, 1991, **274**, 503–507.