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Stability of Peptide in Microarrays: A Challenge for High-Throughput Screening

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1. Introduction

Microarrays are becoming a common tool in biology for screening large numbers of samples. However, the relevance of such an approach depends on the reproducibility of measurements that is directly linked to the stability of the probes grafted on the chip especially when many cycles of regeneration are performed. Indeed, regeneration of microarray chips is of great interest in improving the throughput and reducing the costs. The impact of treatments performed to remove bound ligands in order to reuse the chip depends partially on the grafted probe and on the characteristics of the probe-ligand interaction. Thus DNA microarrays are considered to be stable as oligonucleotides are highly stable molecules and hybridization reaction depends very little on the conformation of the partners; so, multiple regeneration/rehybridization procedures can be carried out without major loss of signal intensity (Benters et al., 2002, Donhauser et al., 2009). Stability of the probes is much more difficult to achieve when proteins are used. Indeed, such molecules are very complex and heterogeneous, thus there are no general rules to account for their behaviour upon different regeneration steps. Moreover, protein-protein interactions are highly susceptible to partner conformation. This is particularly true in the case of antigen (Ag) - antibody (Ab) binding, and several papers mention a loss of signal after the second or third regeneration step (Barton et al., 2008, Yakovleva et al., 2003). Peptide microarrays are a good alternative, as peptides are shorter and their stability less dependant on their tri-dimensional structure. Thus they are often used for antibody profiling (Cherif et al., 2006, Halperin et al., 2011, Neuman de Vegvar et al., 2003). However, there is no study dealing with the stability of such microarrays during a large samples screening (>20). Furthermore, a conformational change is not the only parameter which can impact on ligand binding. In this chapter, our aim was to analyze the evolution of the signals during samples screening and to determine which parameters are involved in the decay of the chip efficiency: grafting method, saturation step, probe itself or probe-ligand interactions, presence of protease activity in the sample. We use a microarray system based on pyrrole electropolymerization

for probe immobilization and surface plasmon resonance imaging (SPRi) for ligand detection. The biological model consists in antigen-antibody interactions, where probes are peptides used as antigens and ligands are antibodies (Ab) contained in serum samples. Our data suggest that modification of the peptide conformation is the main parameter involved in the decrease of the signal observed upon successive uses of the chip. This conformational change leads to both a progressive reduction of the signal due to a decrease of the peptide reactivity with Ab, and the selection of Ab with the highest affinity. This phenomenon can be evaluated and must be taken into account in the analysis of the data resulting from samples screening.

2. Materials and methods

2.1 Reagents

Polyvinylpyrrolidone (PVP, MW 360 kDa) was obtained from Sigma-Aldrich (St Quentin Fallavier, France). Poly(L-Lysine)-PEG (PLL(20KDa) grafted with PEG(2 Kda) having 3.5 Lys units/PEG chains = PLL-PEG) was purchased from SurfaceSolutionS (Zurich, Switzerland).

Peptides were synthesized by Altergen (Bischheim, France) with a pyrrole-modified NH₂ terminus as previously described (Villiers et al., 2009). Two peptides are derived from the structural protein core of hepatitis C virus of the genotype 1b (HCV), one from ovalbumine, one from hen egg lysozyme, nine from hepatitis D virus (HDV) of various genotypes. Full sequences can be found on the ExPASy server (<http://ca.expasy.org/>) under different accession numbers (Table 1).

Peptide	Original protein	Accession number	Amino acids number
C131	HCV	P26663	131-150
C20	HCV	P26663	20-40
Ova75	Ovalbumine	P01012	75-96
HEL101	Lysozyme	P00698	101-120
HDV1	HDV	P0C6M9	155-172
HDV2	HDV	P0C6M9	174-195
HDV3	HDV	P0C6M9	189-211
HDV4	HDV	P0C6M9	65-80
HDV5	HDV	P0C6M9	1-18
HDV6	HDV	Q70E23	155-172
HDV7	HDV	A1IVP7	155-172
HDV8	HDV	Q70E23	174-195
HDV9	HDV	A1IVP7	174-195
HDV10	HDV	Q70E23	189-211 (aa 195: X=W)
HDV11	HDV	A1IVP7	189-211

Table 1. Peptides used in the study

Rabbit immune serums against different peptides were prepared by NeoMPS (Strasbourg, France). Human serums from healthy donors as non immune serum (NIS) were purchased from the Etablissement Français du Sang (La Tronche, France). Human serums from HDV infected patients were provided by Drs E. Gordien and S. Brichler (Hôpital Avicenne, Paris,

France) and Dr P. Morand (Centre Hospitalo-Universitaire, Grenoble, France). Serums were stored at -20°C.

2.2 Materials

Glass prisms coated with a 50 nm gold layer were obtained from Genoptics-HORIBA Scientific (Chilly-Mazarin, France). Electrodeposition was performed using an Omnigrid Micro robotic arrayer (Genoptics-HORIBA Scientific). Surface Plasmon Resonance (SPR) signals were monitored using a surface plasmon resonance imager (SPRi-Plex from Genoptics-HORIBA Scientific). Measurements were performed using SPRi dedicated software (Genoptics-HORIBA Scientific). Sample injections were ensured by a 231XL sampling injector coupled to a 832 temperature regulator (Gilson, Roissy, France).

2.3 Peptide immobilization on gold

Peptides (100µM) were grafted at least in triplicat on the gold surface of the biochip by electrochemical copolymerization of pyrrole-peptide conjugates using a solution containing 20 mmol/L pyrrole, 100 µmol/L of pyrroled peptides and 10% glycerol in phosphate buffer (50 mmol/L). The polymerisation step was performed by a short 100 ms electrical pulse (2 V) between the counter electrode located in the needle of the microarrayer and the prism gold layer (Cherif et al., 2006, Villiers et al., 2009). Another grafting method based on electro-deposition of diazonium-peptide adducts (Corgier et al., 2009) was also used, as indicated in the text. The prism was rinsed with distilled water and saturated at room temperature for 2h using various mediums as indicated in the text. After washing with distilled water, the prism was positioned in the SPRi-Plex and used immediately.

2.4 SPRi interaction monitoring

All reactions were carried out at room temperature, in phosphate-buffered saline (PBS)/0.01% Tween 20. The flow rate in the chamber was 37 µL/min. Reflectivity was measured at 810 nm, at a fixed incidence angle ($55^\circ < \theta < 56^\circ$). After injection of serum (500 µL, 1/50 or 1/200 as indicated in the text), the biochip surface was rinsed with running buffer (10 min) to remove unbound ligands and specific binding was quantified by measuring the change in reflectivity (ΔR) obtained after 10 min washing. The chip was regenerated using 0.1 M HCl-Glycine (pH 2.3) solution for 10 min and stabilized in the running buffer (10 min). Every twelve injections, a cleaning step was performed by injection of 1% SDS (sodium dodecyl sulphate) in water for 10 min followed by running buffer for 20 min. Saturation with NIS (1/25 in PBS), PLL-PEG or PVP as indicated in the text was realized after each cleaning step.

3. Results and discussion

3.1 Is a grafted peptide sufficiently stable to allow a multiple re-use of the chip?

To assess the stability of the peptide chip during samples screening, we immobilized C131 peptide using pyrrole electropolymerization and performed multiple injection/regeneration cycles using non immune serum (NIS) with periodical injections of anti-C131 serum to monitor the reactivity with the grafted probe. SDS cleaning was realized every twelve injections, as described in §2.4. The SPR (Surface Plasmon Resonance) signal obtained for each injection was monitored. Results from eight independent experiments are presented in Fig. 1.

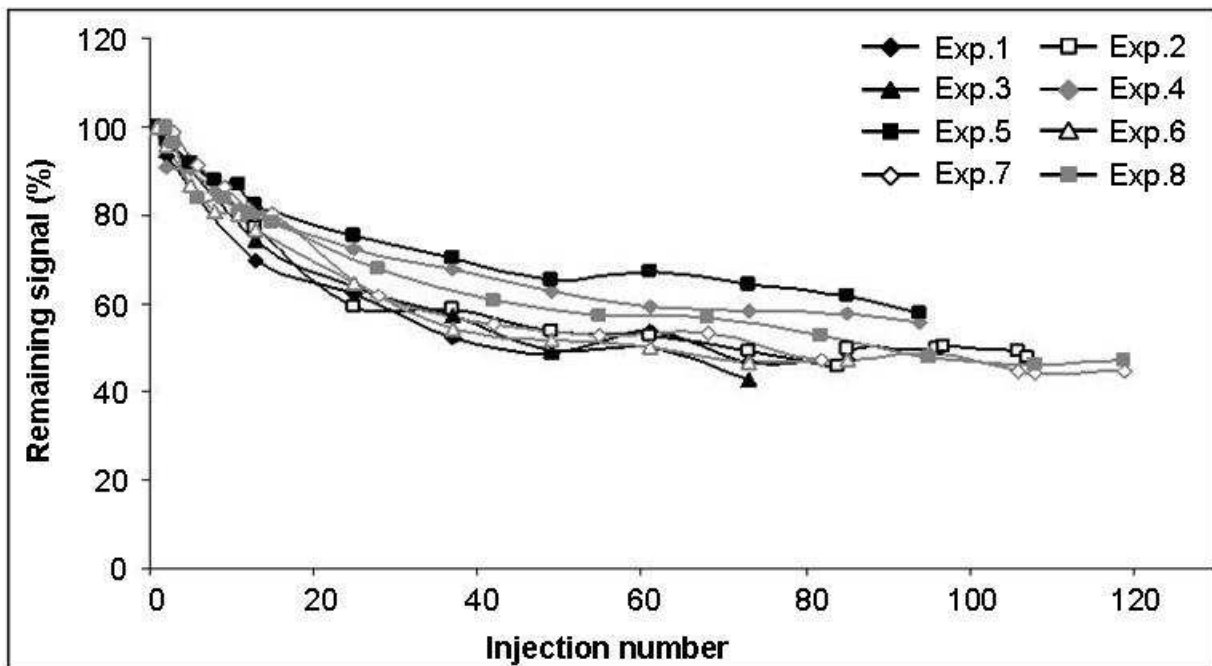


Fig. 1. Peptide chip stability. Eight independent experiments were realized as follows: C131 was immobilized in triplicate on the gold chip surface via pyrrole electropolymerization. Successive injections of non immune serum (1/50) were performed, followed by HCl-Glycine regeneration. Anti-C131 serum (1/200) was periodically injected (every 12 injections) and SPR signal was quantified. Results were standardized according to the signal obtained for the first injection (100%).

We observed a progressive decrease in the SPR signal during the experiments. This decay is very reproducible from one experiment to another and presents a biphasic profile with a rapid drop during the first 12 injections followed by a slighter decrease upon the subsequent injections. Such a loss of efficiency could limit the use of peptide chip for samples screening, therefore it is important to identify the parameters involved in this phenomenon to limit its impact.

3.2 What can influence grafted peptide stability?

SPR signal is directly related to probe-ligand binding which depends on many parameters at both probe and ligand levels. Various hypotheses are summarized in Fig. 2.

SPR signal depends on the quantity and the quality of both probe and ligand. In our experiments, ligands were Ab from a rabbit anti-serum which were aliquoted and kept at 8°C in the autoinjector rack. Thus it is unlikely that any changes in the Ab content of the samples occur.

The problem is more complex when we consider the probe. First, the decrease of the binding capacity could be due to a reduction in the number of available binding sites (epitopes) for the injected Ab during the set of injection/regeneration cycles. This could be due to either a gradual release of the grafted peptide from the chip surface or a proteolysis of the peptide due to the presence of proteolytic activity in the injected samples. The quality of the probe is also an important parameter for Ab binding as it must have a proper conformation and a good accessibility. Thus, if the regeneration step is partially inefficient, remaining Ab may

prevent de novo binding, either directly or indirectly by steric hindrance. Various sets of experiments were performed in order to determine which parameters are involved in the chip efficiency decay.

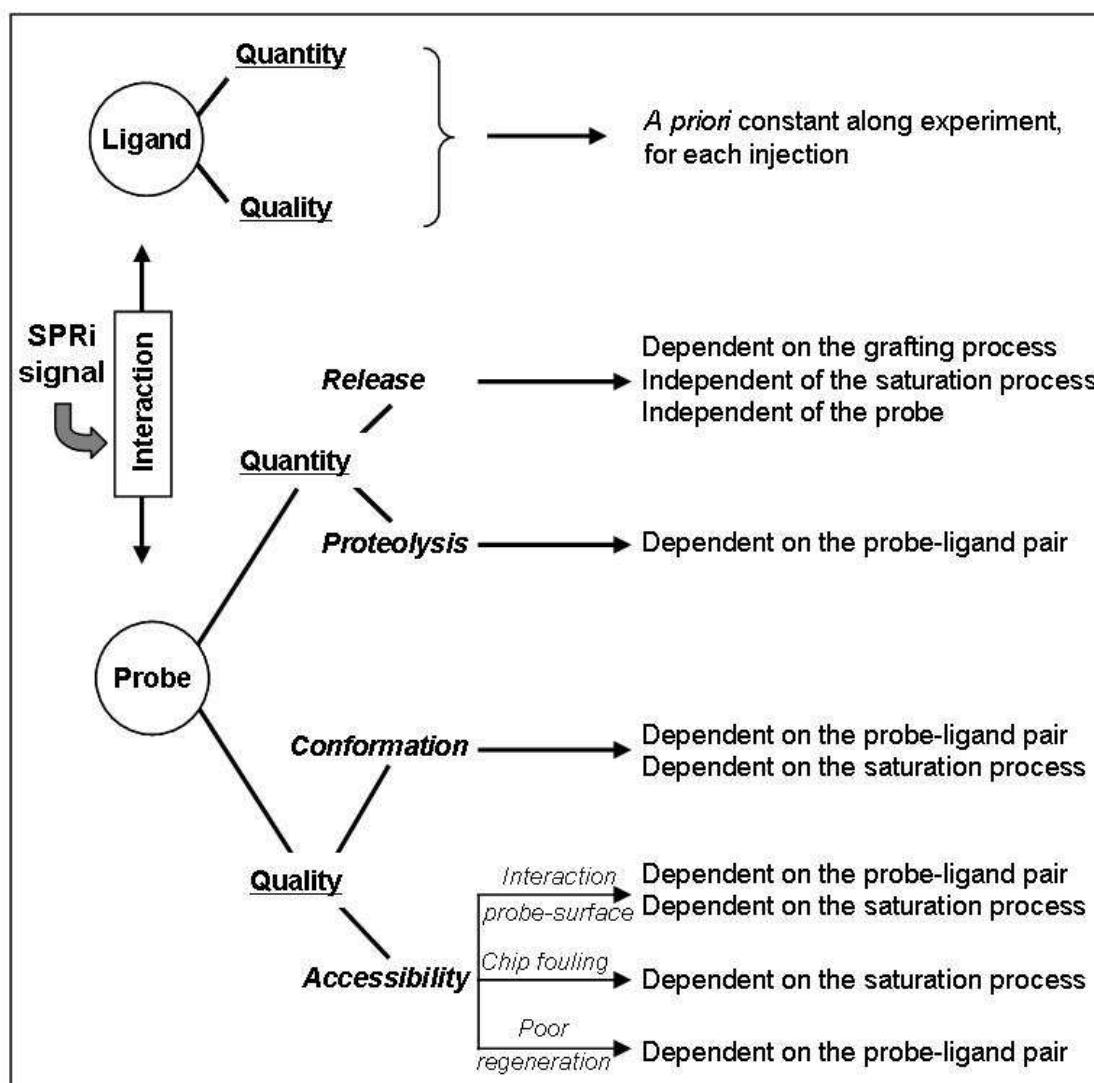


Fig. 2. Parameters involved in the signal loss: tree of possibilities

3.3 Influence of the grafting process

If probe release occurs, this may be related to the nature of the link between the probe and the chip surface, i.e. to the grafting process. To evaluate this hypothesis, C131 peptide was immobilized using either electropolymerization of pyrrole-peptide conjugates or electrodeposition, as a monolayer, of diazonium-peptide adducts and the signals obtained upon successive anti-C131 serum injections were recorded in both cases.

As shown in Fig. 3, the stability of the SPR signal was much better when C131 was immobilized via diazonium, but the change in reflectivity is much lower (likely due to lower probe density on the surface), impairing the sensitivity of the system. At first sight, this observation fits with a probe release in the case of pyrrole electropolymerization protocol, but this eventuality will be discussed later on in light of the other results (§ 4).

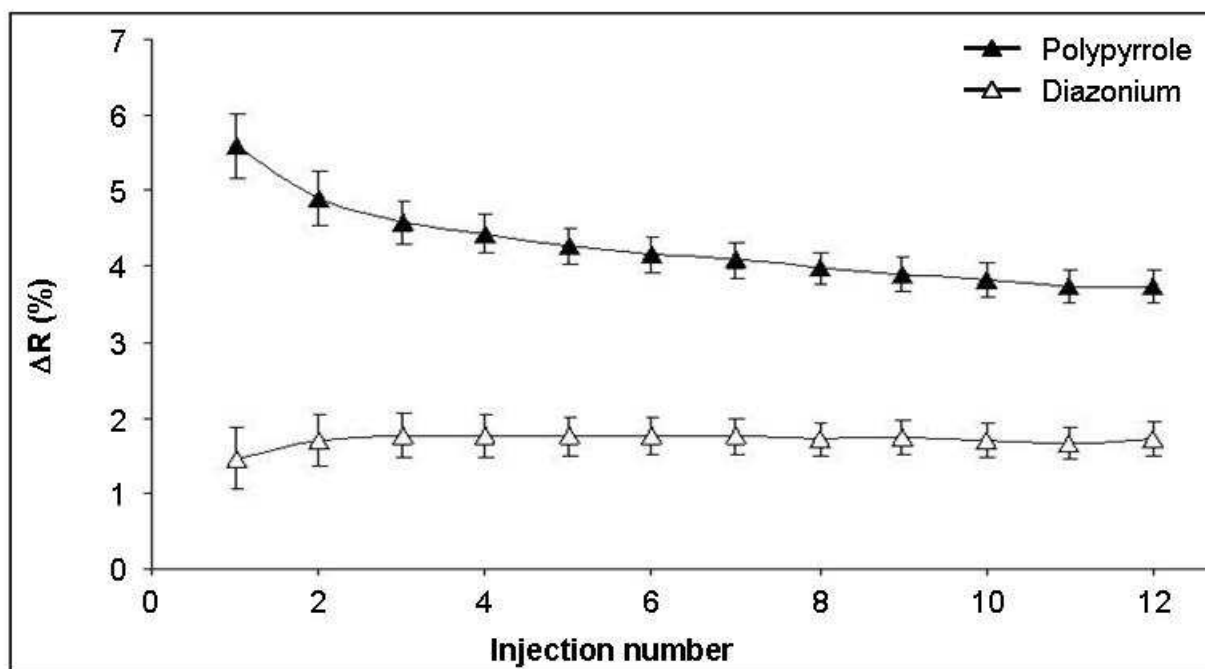


Fig. 3. Influence of the grafting process on the evolution of SPR signal (change in reflectivity = ΔR). C131 peptide was immobilized in triplicate on the gold chip surface via either pyrrole electropolymerization or diazonium electrodeposition. Successive injections of NIS (1/50) with periodical injections of anti-C131 serum (1/200) were performed, followed by HCl-Glycine regeneration.

3.4 Efficiency of the regeneration step

As mentioned above, a potential decrease in the number of binding sites available on the probe could be due to a partially inefficient regeneration process. In these conditions, still bound Ab would lead to a progressive increase in baseline level. To assess this point, we analyzed SPR signal after each regeneration step.

Results presented in Fig. 4 demonstrate that there is no significant increase in the SPR signals measured after each regeneration, attesting that no Ab stays behind the washing step. Therefore, the loss of signal observed for the specific peptide/Ab binding cannot be attributed to incomplete regeneration of the chip.

3.5 Influence of the saturation process

Probe accessibility is a key parameter to ensure a good interaction with injected ligands. This point is especially important when the probe is a small molecule (peptide). Indeed, the molecules used to saturate the chip surface to avoid non-specific interactions could impact on epitope accessibility, either by masking the binding sites or by interfering with peptide conformation through molecular interactions. Furthermore, the conformation of the peptide can evolve during the experiment, affecting the epitope reactivity. Moreover, an incomplete saturation could lead to a progressive fouling of the surface and, thus, to a gradual loss of available binding sites. To saturate chip surface, we usually use non-immune serum (NIS, 1/25 dilution) which leads to non-specific adsorption of many proteins on chip surface, probably predominantly albumin (MW = 65800 Da) as it represents about 60% of total

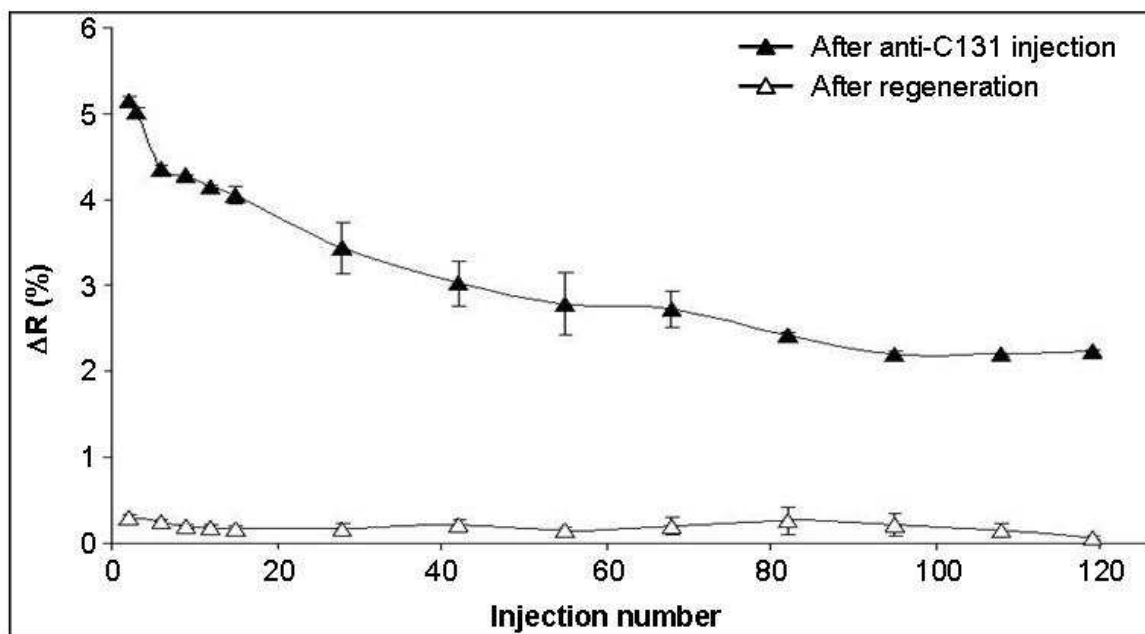


Fig. 4. Efficiency of the regeneration process. C131 peptide was immobilized in triplicate on the gold chip surface via pyrrole electropolymerization. Successive injections of NIS (1/50) with periodical injections of anti-C131 serum (1/200) were performed, followed by HCl-Glycine regeneration. SPR signal was quantified after anti-C131 injection and after the regeneration step.

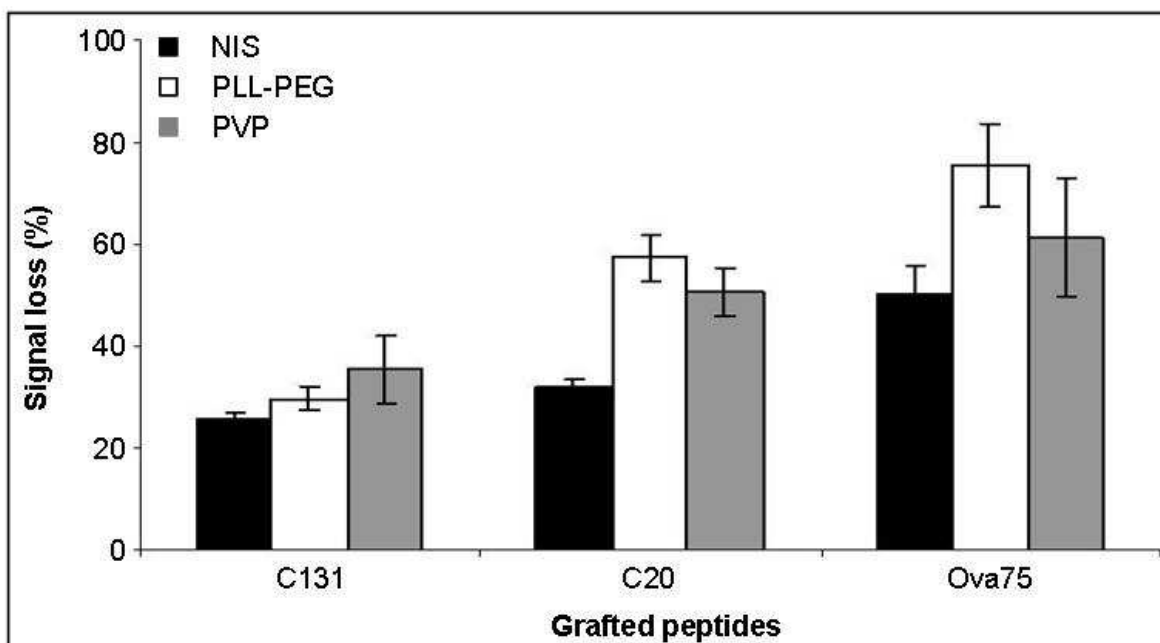


Fig. 5. Influence of the saturation process and of the peptide sequence on SPR signal loss. C131, C20 and Ova75 peptides were immobilized in triplicate on the gold chip surface via pyrrole electropolymerization. Saturation of the chip surface was ensured using either NIS or PLL-PEG or PVP. Successive injections of anti-C131, anti-C20 and anti-Ova75 serums (1/50) were performed, followed by HCl-Glycine regeneration. SPR signal loss after 74 injections.

serum proteins. To determine whether the saturation process is involved in the loss of chip efficiency, we performed the same type of experiments with two anti-fouling molecules: Poly (L-Lysine)-PolyEthyleneGlycol (PLL-PEG) and Polyvinylpyrrolidone (PVP) instead of NIS (0.5mg/mL and 1% p/v respectively). Signal evolution was analyzed in each case. As shown in Fig. 5, signal loss on C131 spot is similar, whatever the saturation process used. As interactions between peptide and anti-fouling molecules could also depend on the physico-chemical characteristics of the peptide, we wondered whether signal loss depends on the grafted probe. Two others peptides (C20 and Ova75) were immobilized on the chip and their corresponding rabbit anti-serums were injected in the same conditions than anti-C131.

As shown in Fig. 5, signal loss depends both upon saturation process and grafted peptide sequence. However, the general shape of the signal decay curve seems to be related with the peptide (Fig. 6).

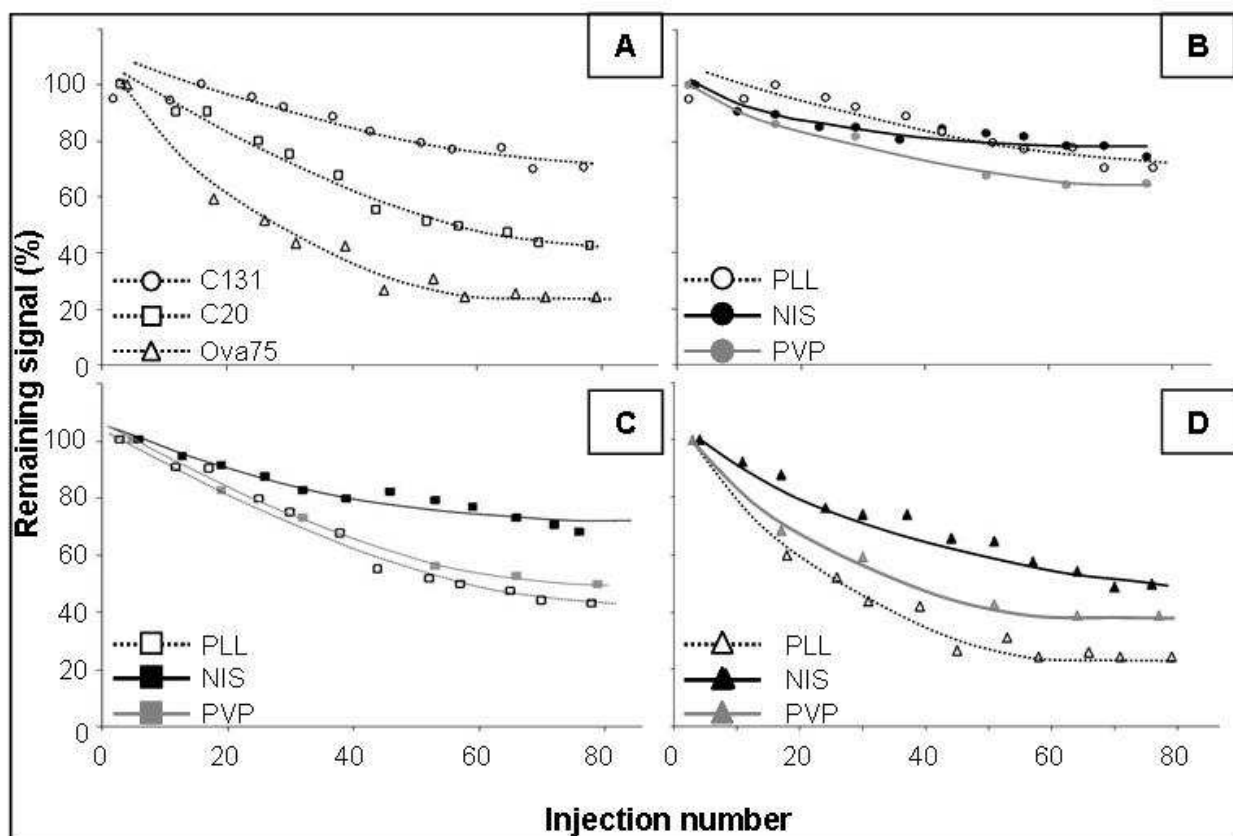


Fig. 6. Evolution of the SPR signal along the experiment. C131, C20 and Ova75 peptides were immobilized in triplicate on the gold chip surface via pyrrole electropolymerization. Saturation of the chip surface was ensured using either NIS or PLL-PEG or PVP. Successive injections of anti-C131, anti-C20 and anti-Ova75 serums (1/50) were performed, followed by HCl-Glycine regeneration. Remaining SPR signal obtained A) on the different spots upon anti-C131, anti-C20 and anti-Ova injections on a chip saturated with PLL-PEG B) on C131 spots after anti-C131 injection on chip saturated with NIS or PLL-PEG or PVP C) on C20 spots after anti-C20 injection on chip saturated with NIS or PLL-PEG or PVP D) on Ova75 spots after anti-Ova injection on chip saturated with NIS or PLL-PEG or PVP.

Altogether, these results indicate that the saturation process impacts on the amplitude of the signal loss while the shape of the signal decay curve is more likely dependant on the peptide. The impact of the saturation process may be related to the physico-chemical properties (hydrophobicity, charge, etc) of both the saturating molecules and the peptides.

3.6 Influence of the ligand

Until now, our analyses were always performed using the same Ab for a given Ag, which did not allow checking the influence of the peptide from that of the Ab. In the next experiment, we compare the signal obtained on C131 and C20 spots after injections of anti-C131 and anti-C20 serums. In each case (anti-C131 and anti-C20) two different serums issued from the same rabbit, but collected at two different days (D=39 and D=66) were tested. As observed in Fig.7, the second sample led to a more stable signal.

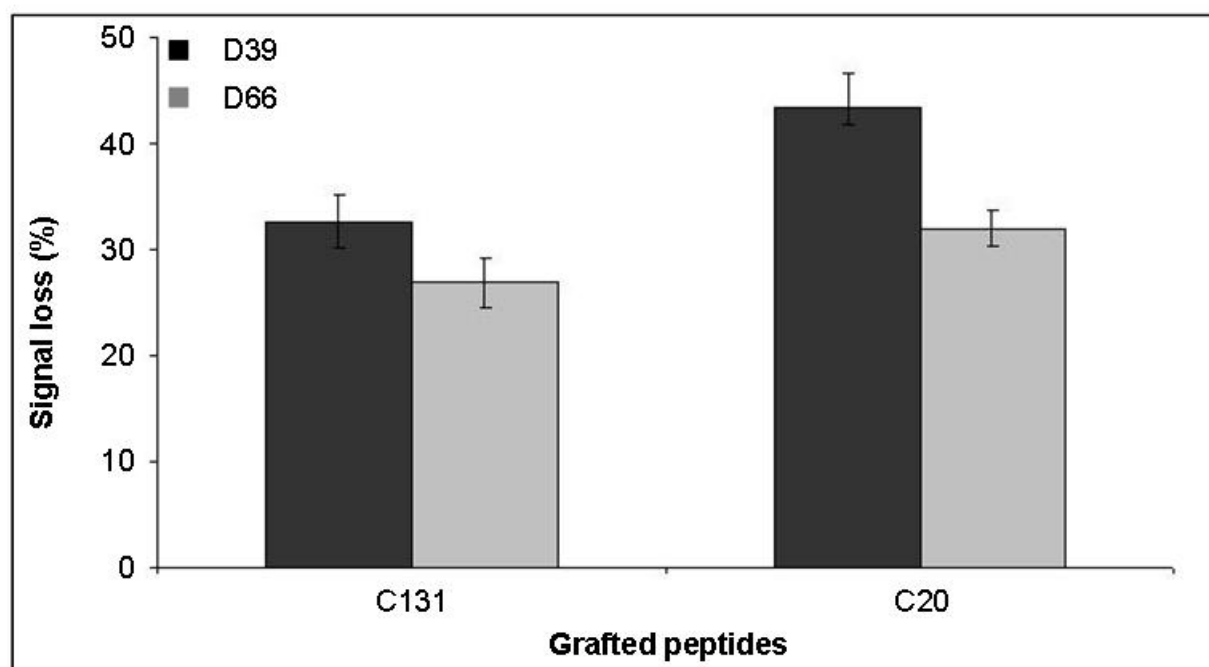


Fig. 7. Influence of the ligand on SPR signal loss. C131 and C20 peptides were immobilized in triplicate on the gold chip surface via pyrrole electropolymerization. Saturation of the chip surface was ensured using NIS. Successive injections of anti-C20 and anti-C131 serums (1/50) were performed, followed by HCl-Glycine regeneration and signal loss after 74 injections was quantified. In each case (anti-C131 and anti-C20) two different serums issued from the same rabbit, but collected at two different days (D=39 and D=66) were injected.

These results could be related to a difference in the affinity of the ligands for the probe. Indeed, it is well known that Ab affinity usually increases gradually during the immune response, which is referred to affinity maturation process (Berek & Ziegner, 1993). Thus, it is likely that D66 sample contains anti-C20 Ab with higher affinity for C20 than D39. But we cannot exclude that, despite the small size of the antigen (20aa), the epitopes recognized by D66 Ab differ from those recognized by D39 Ab, which would impact on the overall characteristics of peptide/Ab interaction.

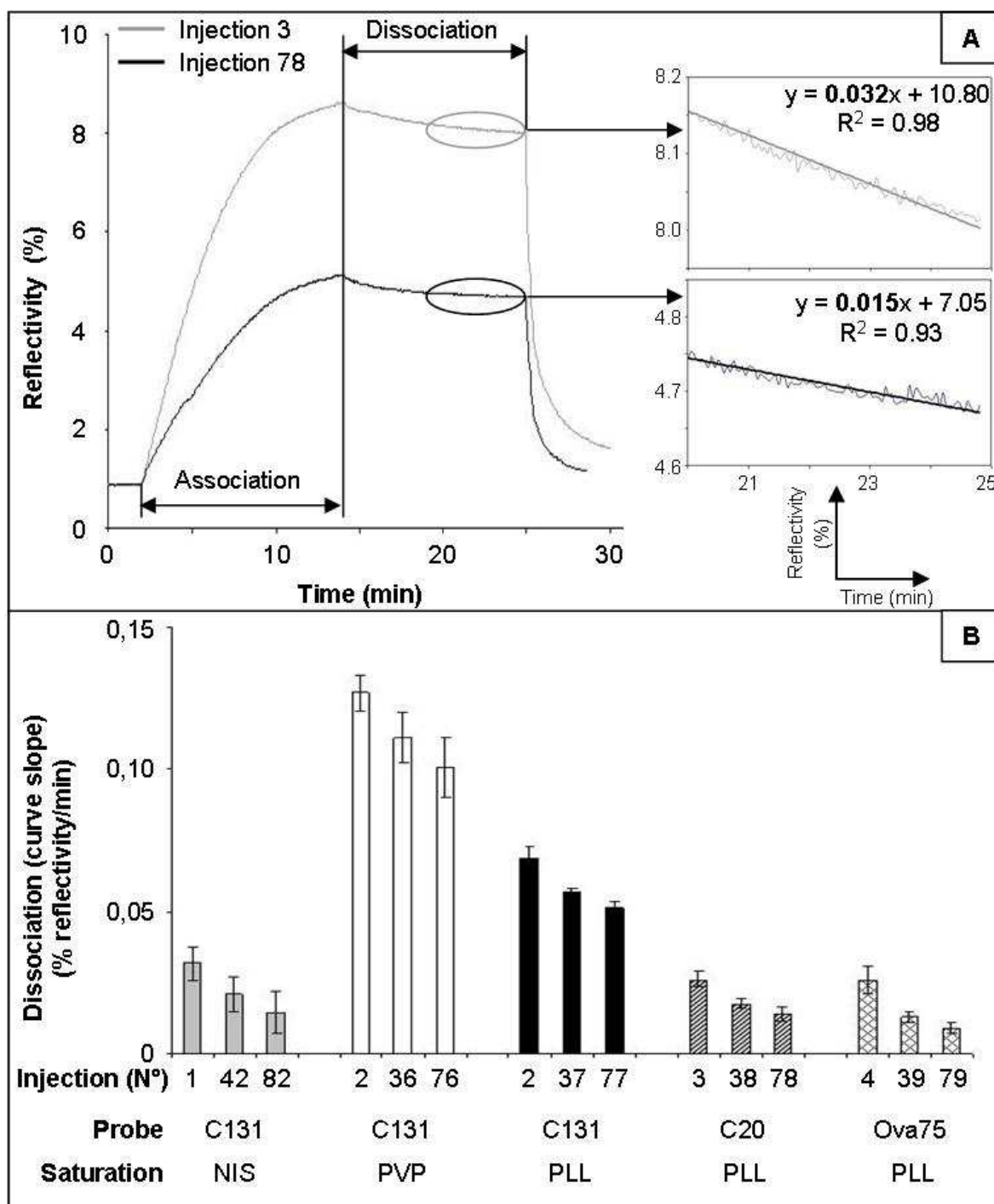


Fig. 8. Ab-peptide binding affinity. C131, C20 and Ova75 peptides were immobilized in triplicate on the gold chip surface via pyrrole electropolymerization. Saturation of the chip surface was ensured using either NIS or PVP or PLL-PEG. Successive injections of anti-C131, anti-C20 and anti-Ova serums (1/50) were performed, followed by HCl-Glycine regeneration. A) Sensorgrams obtained on C20 spots after the third and the 78th injection of anti-C20 serum (PLL-PEG saturation). Insert: detail of the dissociation curves. B) Quantification of antibody dissociation for the different peptides, from the slope of the dissociation curves.

3.7 Is there a change in the affinity of the ligand (Ab) for the probe (peptide) between the first and the last injection?

Affinity is a key parameter in Ab-Ag interaction which could impact on the signal observed upon Ab binding. Actually, Ab binding on the grafted peptides was quantified by measuring the change in reflectivity obtained after washing, a step during which ligands can dissociate, accordingly to their affinity for the probes. Moreover, it is well known that Ag conformation influences Ab binding (Fieser et al., 1987). Thus, a modification of peptide conformation during the experiment could lead to a change in the Ag-Ab affinity. As SPRi technique allows label-free and real-time detection of biomolecular interactions, it gives access to affinity parameters. But, as ligands in our experiments consisted in polyclonal antibodies, the dissociation curves obtained during the washing step after serum injection correspond to a mean value resulting from all the individual dissociation constants of the various Ab. In this case, it is not possible to determine classical affinity parameters, but the slope of the dissociation curve is representative of the overall affinity. In order to analyze the evolution of peptide/Ab affinity during an experiment, the slopes of the dissociation curves were calculated using the last 6 min of washing (Fig. 8A).

As shown in Fig. 8B, the slope of the curve corresponding to the dissociation of Ab-peptide complexes decreased during the experiment. Thus the diminution of the quantity of Ab bound to the peptides (signal loss) is associated with an increase in the affinity of the interaction, whatever the probe/ligand pair used or the saturation process.

These results suggest that successive injection/regeneration cycles lead to a slight conformational change in the probe. At the beginning of the experiment, peptide conformation is suitable for the binding of different Ab with a large range of affinity. As the experiment progress, Ab having lower affinity for the peptide can no longer bind, due to modification in peptide conformation.

3.8 Effect of glycerol and protease inhibitors

It is well known that the stability of protein is enhanced by various molecules among which polyols (Lee & Kim, 2002, Vagenende et al., 2009). In the aim of reducing conformational change in the grafted probes, glycerol (0.1%) was added to both the running buffer and the regeneration solution. As shown in Fig. 9, the presence of glycerol in the running solutions improved the signal stability in the case of Ova75, but not in the case of C131. It seems that the protective effect of glycerol occurs only on the less stable peptides.

As injected samples are complex biological mediums (serums), we wondered if some peptide degradation could occur, due to protease activities. So, we performed the same experiments after addition of protease inhibitors in the samples (cocktail set VII, CalbBiochem, 1/100). We did not observe any improvement of chip stability (Fig. 9), suggesting that probes proteolysis was not responsible for the loss of signal during the experiments.

3.9 Improvement possibilities of the system

Various strategies can be implemented to improve the quality of the data resulting from samples screening on a peptide chip. First, addition of glycerol in the solutions can improve peptide stability, depending on the peptide (§3.8). Second, we observed that signal loss was usually biphasic, with a rapid drop during the first injections followed by a slighter decrease

upon the subsequent injections. Thus we suggest performing 10 - 12 blank injections/regeneration cycles before sample analysis to limit the conformational change between the first and the last sample of interest. Finally, we recommend to include a spot with a control peptide on the chip and to perform periodical injections of a control sample. The reduction of signal measured on this control spot can be modelled using a polynomial curve (Fig. 10A), which can be used to determine a correction factor for the others injections.

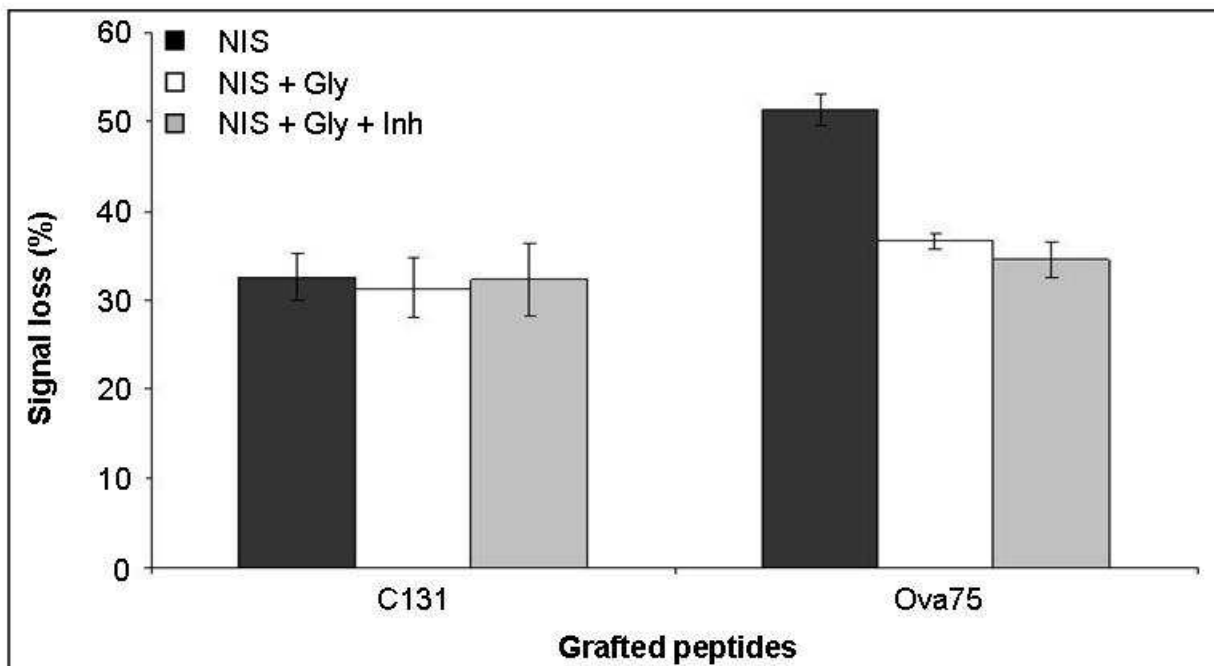


Fig. 9. Effect of glycerol and protease inhibitors on SPR signal loss. C131 and Ova75 peptides were immobilized in triplicate on the gold chip surface via pyrrole electropolymerization. Saturation of the chip surface was ensured using NIS. Successive injections of anti-C131 and anti-Ova75 serums (1/50) were performed, followed by HCl-Glycine regeneration and signal loss after 76 injections was quantified. + Gly: presence of glycerol 0.1% in the running buffer and regeneration solution. + Inh: addition of protease inhibitors in the serum samples.

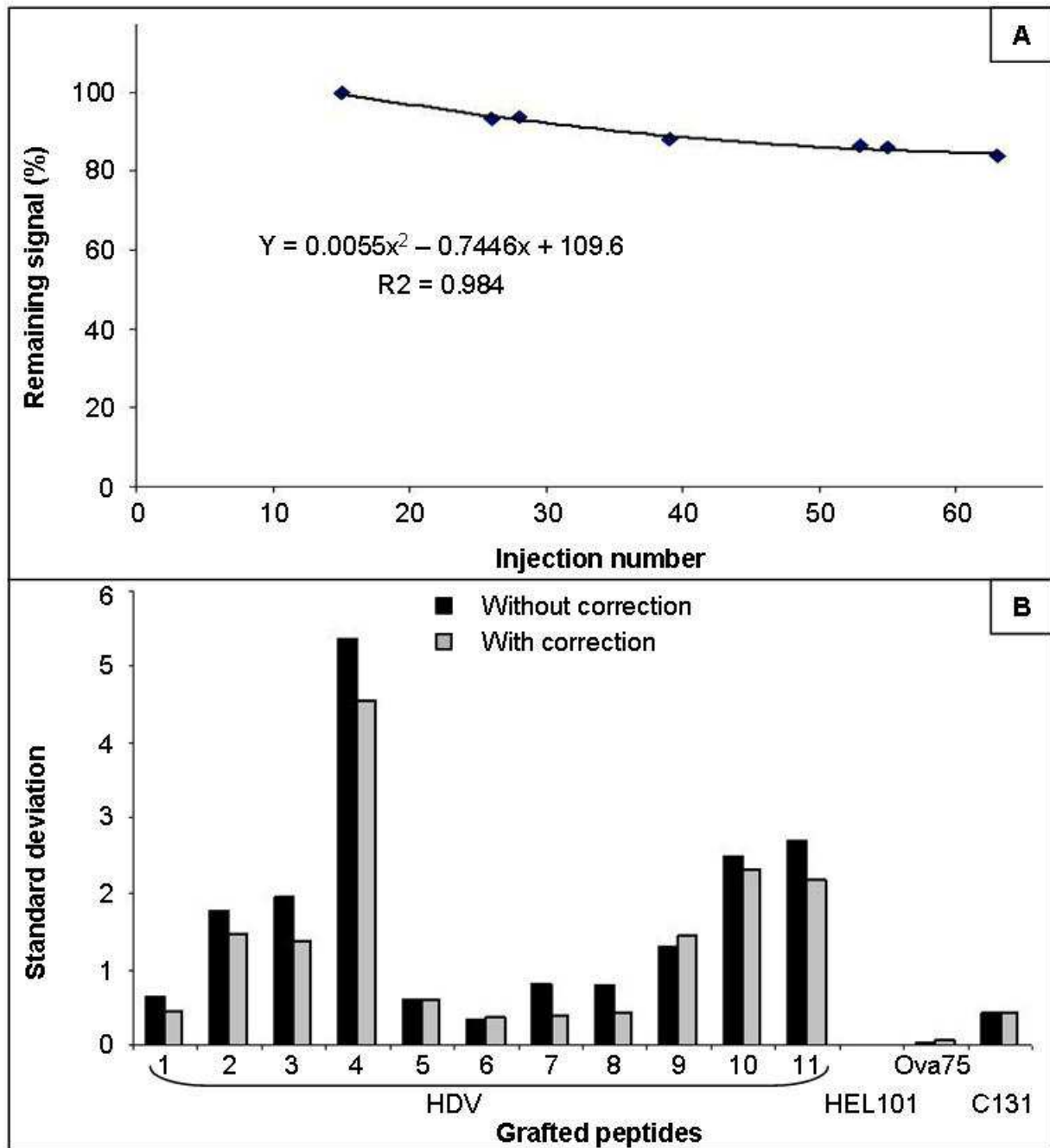


Fig. 10. SPR signal correction: 14 peptides were immobilized in triplicate on gold chips surface via pyrrole electropolymerization. Saturation of the chip surface was ensured using NIS and glycerol was added to running solutions. Successive injections of serums from HDV infected or healthy donors (1/50) were performed, followed by HCl-Glycine regeneration. Rabbit anti-C131 serum was periodically injected. A) Remaining signal obtained on C131 spot upon anti-C131 serum injections. B) Standard deviation of the SPR signal obtained before and after signal correction, for three independent sets of injections.

To validate this correction procedure, we realised the following experiments: 14 peptides (11 from HDV, 1 from HEL, 1 from Ova and the control peptide C131) were grafted in triplicates on 2 chips. A set of 17 serums (14 from HDV infected patients and 3 from healthy donors) was injected twice on the first chip and once on the second chip. Serums were in a different random order within each set. Rabbit anti-C131 serum was periodically injected (at least every 12 injections) to establish a control curve for signal decrease. The sum of the SPR signals obtained for each peptide was calculated for each injection set, before and after application of the correction, as well as the standard deviation (Fig. 10B). Indeed, as signal loss depends on the peptide/ligand pair, this correction is not optimal, but nevertheless improves the results.

4. Conclusion

Among factors susceptible to be involved in signal loss during sample screening on peptide chip, we can exclude probe proteolysis as protease inhibitors had no significant effect on peptide chip stability. Nevertheless, it is recommended to check for protease activities in samples before running large screening. The accessibility to peptides does not seem to limit the signal: steric hindrance and/or chip fouling due to partial regeneration or poor chip surface saturation would lead to an increase in base line, which is not the case.

The influence of peptide grafting process suggests a release of the probes during the experiments. However, in this case, the reduction of signal should be independent of the peptide, which is actually not the case. It is worth to notice that SPR signal is much lower when diazonium protocol was used. We suggest that this is due to a weaker grafting efficiency, leading to fewer immobilized peptide on the chip. Thus, SPR signal is lower and the competition for Ab binding favours high-affinity Ab. As discussed in § 3.7, high-affinity Ab keep their capacity to bind their epitopes even after epitope conformational changes, thus leading to a more stable signal. This is supported by the slope values obtained for the dissociation curves ($12 \pm 2.6 \times 10^{-3}$ %reflectivity/min and $4 \pm 0.9 \times 10^{-3}$ %reflectivity/min for ppy and diazonium respectively, first C131 injection).

Altogether, our results suggest that the main reason for the loss of SPR signal during the experiments is a change in the conformation of the grafted peptides, probably induced by the successive injection/regeneration cycles. This conformational change impairs peptide recognition by some Ab, selecting the Ab possessing a high affinity for the probe. The signal loss, due to a decrease in Ab amounts that bind to peptides, depends on both the peptide (according to its conformational stability) and the Ab (according to the targeted epitope via its accessibility upon conformational change of the peptide). Saturation process can also impact peptide stability, depending on the nature of peptide-chip surface interactions, some of them being able to induce epitope masking. Involvement of peptide conformational change in signal loss is strengthened by the improvement of the signal stability observed for some peptides when glycerol was added in the running solutions.

This study points out several ways to both reduce and correct the signal loss occurring on peptide chip upon multiple injection/regeneration cycles.

5. Acknowledgment

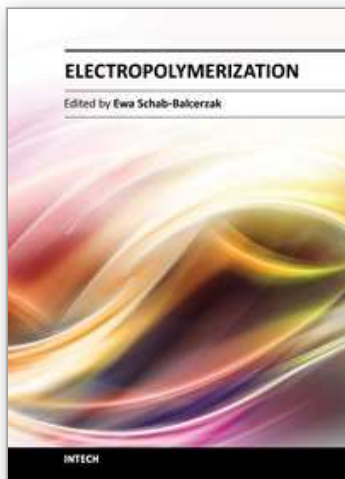
This work was supported by the “Agence Nationale de Recherches sur le Sida” (ANRS), by the “Fonds Unique Interministériel (FUI) Biotherapeutic and Alphavac” and the “Région Rhones-Alpes (Cluster Infectiologie)”.

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Electropolymerization

Edited by Dr. Ewa Schab-Balcerzak

ISBN 978-953-307-693-5

Hard cover, 214 pages

Publisher InTech

Published online 22, December, 2011

Published in print edition December, 2011

In recent years, great focus has been placed upon polymer thin films. These polymer thin films are important in many technological applications, ranging from coatings and adhesives to organic electronic devices, including sensors and detectors. Electrochemical polymerization is preferable, especially if the polymeric product is intended for use as polymer thin films, because electrogeneration allows fine control over the film thickness, an important parameter for fabrication of devices. Moreover, it was demonstrated that it is possible to modify the material properties by parameter control of the electrodeposition process. Electrochemistry is an excellent tool, not only for synthesis, but also for characterization and application of various types of materials. This book provides a timely overview of a current state of knowledge regarding the use of electropolymerization for new materials preparation, including conducting polymers and various possibilities of applications.

How to reference

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Marie-Bernadette Villiers, Carine Brakha, Arnaud Buhot, Christophe Marquette and Patrice N. Marche (2011). Stability of Peptide in Microarrays: A Challenge for High-Throughput Screening, *Electropolymerization*, Dr. Ewa Schab-Balcerzak (Ed.), ISBN: 978-953-307-693-5, InTech, Available from:
<http://www.intechopen.com/books/electropolymerization/stability-of-peptide-in-microarrays-a-challenge-for-high-throughput-screening>

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