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Full Length Research Paper

An approach towards optimal usage of immobilized sensor chips in Surface Plasmon Resonance based biosensor

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In recent decades, there was a surge of interest in Surface Plasmon Resonance (SPR) biosensor based bimolecular interaction analysis. The unique characteristics of this technique made it possible to measure real time unlabelled bimolecular interactions with great sensitivity. However, the major challenge in SPR is providing the ability to re-use the surface of the chip. The main goal of this study was to address the problem faced in establishing an ideal regeneration condition and removing non-covalently bound analyt without disturbing ligand. Considering four different types of proteins including virus, hormone, cells and antibody, a comprehensive regeneration protocol for protein-protein interaction was developed and compared with common regeneration methods. The presented protocol screened five multi-ingredient stock solutions that represented the five most common chemical properties such as acidic, basic, ionic, chelating and non-polar water soluble solvent solutions employed as regeneration agents. Upon three cycles of screening, it was found out that enveloped virus–antibody complexes could be effectively regenerated via a combination of acidic and chelating solution whilst non-enveloped viruses needed a basic solution for successful regeneration. Both insulin-antibody and cell-enveloped virus complexes could be detached efficiently using acidic solutions. Regenerations using non-polar water soluble solvents presented a harsh reaction, whilst ionic solutions were too mild. Thus, incomplete regeneration occurred. In summary, this study will serve as a platform of reference for multiple regenerations for a cluster of protein-protein complexes.

Key words: Surface Plasmon Resonance (SPR), regeneration, virus, insulin, monoclonal antibody, cell.

INTRODUCTION

Regeneration is a critical step in Surface Plasmon Resonance (SPR) assay development and it is unfeasible to have a reproducible and practical biochip without a successful regeneration (Quinn et al., 1999). Generally in a typical SPR experiment, ligand is immobilized on the surface of a chip which is followed by passing analyt over ligand automatically. Consequently, analyt-ligand interaction is monitored in real time. In the last step, a regeneration solution is injected over analyt-ligand in order to break their specific binding. Successful regeneration displaced analyts whilst ligands remained active, allowing numerous cycle runs on the same chip. Non-optimised regeneration led to mounting a baseline level, inactivation of ligand or scrap off of ligand from the chip surface (Andersson et al., 1999; FonfrÃ-a et al., 2007). Accordingly, chip's life time was effected.

Despite regeneration imperatives, hardly any related optimisations were reported. Previously, Anderson et al. (1999) developed a regeneration protocol (Ro) for purified enzyme and surface proteins. As bimolecular bonds can be affected by physiochemical factors like ionic strength, solvents, temperature and pH, altering such conditions posed impact on protein-protein bonds

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Table 1. Studied ligand (Lg)_analyt(A) complexes.

Ligand	analyt
PrV	PrV/gC-mAb
PrV/gC-mAb	PrV
Vero cells	PrV
CSFV	CSFV/E2-mAb
Insulin	Insulin/mAb
IBDV	IBDV/PAb

disassociation. Hence, appropriate cocktails were designed for screening ideal disassociation condition in bimolecular systems.

This study performed regeneration modification and optimisation for viral and hormone based bimolecular systems, as well as cells including immobilized Pseudorabies virus (PrV) interacting with monoclonal antibody (mAb), immobilized Classical Swine Fever virus (CSFV) coupled with its mAb, immobilized Infectious Bursal Disease *virus* (IBDV) associated with Polyclonal Antibody (PAb), immobilized insulin–mAb interacting with insulin, immobilized prV antibody (PrV/mAb) associated with PrV and immobilized Vero cell interacting with PrV. Table 1 represents all ligand (Lg) and analyts (An) for easy consideration. The aim of this study was to develop a practical approach in optimising regeneration protocol and to facilitate rapid assay development and consequently, a realistic approach adapted for rapid assay development with no restriction to analyt purity and taking advantage of optimised regeneration protocol. The basic compound recommended by Anderson et al. (1999) was also taken into consideration.

MATERIALS AND METHODS

Ligands and analyts

All ligands, insulin mAb (Abcam, Uk), PrV/gC-mAb (Axyll, USA), PrV (strains established in Virology Laboratory Faculty Of Veterinary Medicine UPM), IBDV-mAb(Abcam, Uk), Vero cell line and CSFV/E2-mAb (VLA, UK) were immobilized onto carboxylated surface of chip via established amine coupling method (Lrofas et al., 1995). Consequently, respective analyt for each ligand was insulin, PrV, PrV/gC-mAb, IBDV and CSFV. All antibodies were commercially available and all virus stocks were propagated according to established protocol (Zeenathul, 2004). The insulin samples were obtained from islet cell secretion.

Regeneration optimization for various bimolecular systems

It was previously reported that R**O** protocol was referred as a platform for modification (Andersson et al., 1999). Five different groups of solutions were considered for preparation of 12 cocktails. Stock solutions included basic (B), acidic (A), ionic (I), chelating (C) and non-polar water soluble solvents (U). The content of each stock solution is listed in Table 1. The mixture trend shown in Table 2 involved 12 cocktails that were scouted over ligand-analyt; each cycle was in triplicates. Subsequently, from each group of stocks, one unit was mixed with two units of water (W), or two different units were mixed with one unit of water (Table 3).

Regeneration evaluation for efficiency

Regeneration scouting was accomplished by diluting each analyt in HBS buffer, and then with injection of treated analyt over immobilized ligand for 2 min at a flow rate of 10µl/min. The pre and post injection baseline of analyt were recorded. Then, each regeneration cocktail was injected at a flow rate of 20 µl/min for 30 s over the analyt-ligand complex. This was aimed at reducing the analyt level to 10% or lesser from the original baseline. Once this was achieved, a second round of scouting was employed by analyt injection and followed by other cocktail injections orderly. Regeneration efficiency (R_e) for each cocktail in each bimolecular system was estimated using the formula provided by Anderson (1999). Figure 1 is an example of our regeneration evaluation process.

R_e = (analyt loss) / (analyt level) ×100%

Higher R_e indicates higher efficiency of regeneration solution. In each bimolecular system, at least one cocktail was expected to have R_e equal to or higher than 90%. If in any bimolecular system such cocktail was not attained, another set of optimisation was carried out. According to situation, second round of optimization was performed only for two bimolecular systems by selecting two compounds corresponding higher R_e in each system and by mixing together as the trend shown in Table 4.

Statistical analysis

One-way analysis of variance was used to determine the significance of variation among the cocktails with three replications. Subsequently, the Duncan New Multiple Range Test (DNMRT) was applied for comparison of R_e means of the cocktails. All analyses were performed by SAS computer package (SAS Institute Inc., 2005).

RESULTS AND DISCUSSION

Analysis of variance for all analyt-ligand complexes indicated significant differences among the regeneration cocktails. The cocktails which possessed R_e more than 100 or lesser than 0 were excluded. In the first trial, at least one cocktail with $R_e \geq 90\%$ was found for IBDV(A)_PAb(Lg), Prv/gC-mAb(A)_P-PrV(Lg), PrV(A)_ Vero Cell(Lg) and Insulin (A)_Insulin/mAb(Lg) complexes. Table 5 shows all the R_e values. For both PrV(A)_PrV/ gC-mAb(Lg) and CSFV(A)_CSFV/E2-mAb(Lg) complexes, R_e obtained was between 75 and 90 ($90 \geq R_e \geq 75$) which is not ideal. Hence, the second cycle of optimization was carried out to reveal the best cocktail combination for PrV(Lg)_PrV/gC-mAb(A) and CSFV(Lg)_ CSFV/E2-mAb(A) complexes. Figure 1 represents the typical sensorgram obtained from regeneration optimization procedure.

It was highlighted that only $R_e \geq 90\%$ was considered as an ideal regeneration solution. In cases were more than one solution comprised $R_e \geq 90\%$, the one nearest to 100 was assumed as ideal and the other one as

Table 2. Content of stock solutions used for cocktail preparation adopted from Andersson et al. (1999).

Cocktail	Composition
Acidic (A)	Equal volumes of oxalic acid, H_3PO_4, formic acid, and malonic acid, each at 0.15 M, adjusted to pH 5.0 with 4 M NaOH
Basic (B)	Equal volumes of ethanolamine, Na_3PO4, piperazine, and glycine, each at 0.20 M, adjusted to pH 9.0 with 2 M HCl
Ionic (I)	KSCN (0.46 M), $MgCl_2$ (1.83 M), urea (0.92 M), guanidine- HCl (1.83 M)
Non polar water soluble solvents (U)	Equal volumes of DMSO, formamide, ethanol, acetonitrile, and 1-butanol
Chelating (C)	20 mM EDTA

Table 3. Mixture trend of cocktails.

Screening cocktail		
Two units of water (W) + One unit of (B, C, I and W)	**One unit of W + One unit of A + One unit of (B, I, U and C)**	**One unit of water + One unit of C + One unit of (I and B)**
BWW	ABW	BCW
AWW	AIW	ICW
CWW	ACW	
IWW	AUW	
UWW	BUW	

acceptable or an alternative regeneration solution. High R_e (approximately $90 \geq R_e \geq 65$) was only considered for those systems that could not reach ideal conditions at first trial.

Disassociation of IBDV (A)_IBDV/PAb(Lg) was obtained up to level of 96% using BCW. In this system, cocktails AWW and UWW were not appropriate due to their harshness. Breaking the PrV/gC-mAb(A) bound from PrV(Lg) and PrV(A) bound from Vero cell(Lg) were effective with AWW. AUW was a harsh cocktail for P-PrV(A)_PrV/gC-mAb(Lg), CSFV(A)_CSFV/E2-mAb(Lg), PrV/gC-mAb(A)_PrV(Lg) and PrV(A)_Vero cell(Lg) complexes, while it had low effectiveness in disassociating insulin(A)_insulin/mAb(Lg) and IBDV(A)_IBDV/PAb(Lg). However, AIW had harsh effect on CSFV(A)_CSFV/E2-mAb(Lg) only.

By using optimized protocol in continuous experimentation, it was possible to use the established chip with the highest activity up to 250 cycles for analyt-ligand interactions. Example of cycle estimation for chip immobilized with PrV/gC-mAb is as follows:

Optimization and interaction analysis of PrV/gC-mAb (Lg) with 4 (An) × 3 (dilution of each analyt) × 9 (replication) = 108
Detection of 10 samples × 3 (replication) = 30
Sensitivity analysis, 7 dilutions × 3 (replication) = 21
Specificity optimization and analysis, 6 sample × 6 (replication) = 36
Antiviral analysis and optimization, 3 samples × 9 (replication) = 27
Mutant analysis and optimization, 4 samples × 9 (replication) = 36
Total cycles equals to 258

The result obtained from second optimization trial shows that solution mixture with A, C and W with “F” combination style had the highest R_e for both PrV(A)_PrV/gC-mAb and CSFV(A)_CSFV/E2-mAb(Lg). The ‘C’ and ‘B’ combination styles were too harsh for both systems (Table 6).

In a study conducted by Heding et al. (1996), 100 mM HCl was used for regeneration, whilst in our study, acidic solution caused precipitation on the surface of chip. It was considered that the type of acid and pH value differed.

Regeneration solution could split antigen-antibody bond which could be either electrostatic (EL), van der Waals

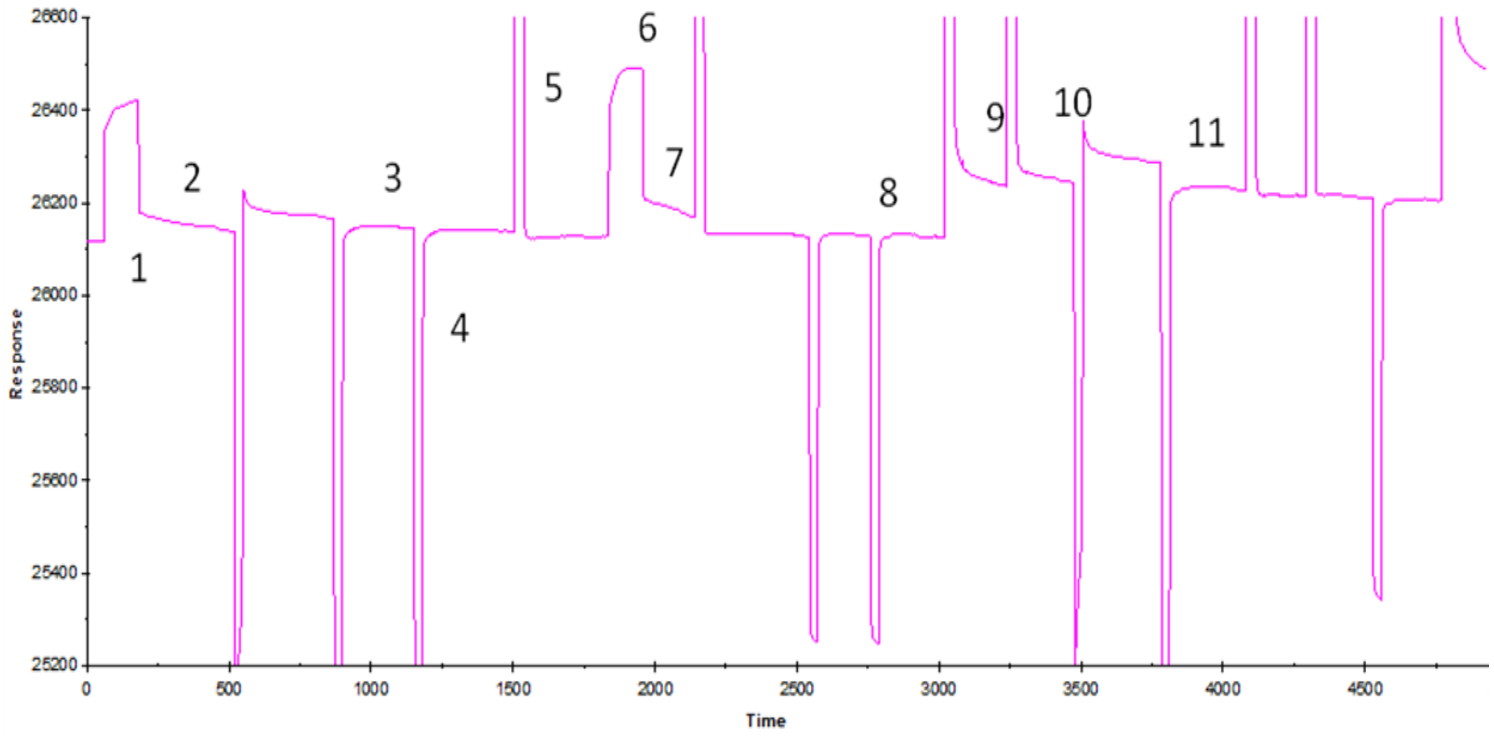

Figure 1. Example of typical sensorgram obtained from a regeneration evaluation process. As numbers indicated, first analyt was injected over ligand (1) regeneration solution (2). Since the baseline after first regeneration did not reach nearby baseline before injection of analyt, second, third and forth regeneration solutions were injected; (3), (4) and (5). After injection of fifth regeneration solution, only 10% of analyts remained, therefore, injection of more analyt was required (6), followed by injection of more regeneration solutions; (8), (9), (10) and (11). As shown in the figure, after injection of (9) and (10), unexpectedly increase in baseline was observed while they were supposed to decrease baseline.

Table 4. Combination style of second optimization trail.

Combination style	Main component 1 (%)	Main component 2 (%)	Water (%)
A	25	75	0
B	75	25	0
C	100	0	0
D	50	50	0
E	25	50	25
F	50	25	25
G	25	25	50

For some complexes, ideal regeneration conditions in first regeneration optimization trial was not achieved therefore two main components comprising higher Re from first regeneration optimization were mix together as the trend shown in order to perform second trial of optimization.

forces (LW), Lewis acid-base (AB) or hydrogen bond (Dumetz et al., 2007). The intention of applying a variety of physicochemical conditions such as ionic strength, pH, content of organic solvent or other solute was for the kind of bonds contributing to the antigen-antibody interaction (Van Oss, 2000). However, temperature variation could have influence on analyt-ligand interaction; it was kept constant at 25°C throughout the experiments. The mechanism of ligand-analyt disassociation was not in the scope of this study. However, the repulsive or attractive nature of salts in protein-protein interactions (breaking ionic bond), the disruptive effects of solvents and the effect of pH on protein solubility supported the findings of this study (Buxbaum, 2007; Dumetz et al., 2007). The harshness of non-polar water soluble solvents on IBDV(A)_IBDV/PAb(Lg) complex, and similarly AWW and

Table 5. Mean values for regeneration efficiency (Re) of analyt-ligand complexes.

Cocktail	R_e mean ± SE					
	PrV(A)_PRV/gC-mAb(Lg)	CSFV(A)_CSFV/E2-mAb(Lg)	IBDV(A)_IBDV/PAb (Lg)	PrV/gC-mAb(A)_PrV(Lg)	P-PrV(A)_Vero Cell(Lg)	Insulin(A)_Insulin/mAb(Lg)
Aww	75.80b* ± 1.66■	71.60 ^{ab} ± 1.15■	NA	98.54 ^{a} ± 0.63▲	96.57 ^{a} ± 1.50▲	-11.40 ^{hi} ± 1.57●
Bww	58.10 ^{d} ± 6.60	29.10 ^{e} ± 2.27	77.68 ^{c} ± 4.01	55.20 ± 1.91	62.37 ^{c} ± 3.25	22.30 ^{e} ± 0.67
Cww	60.00 ^{d} ± 2.76	68.02 ^{b} ± 3.45	84.20 b ± 2.37	78.49 ^{b} ± 1.58	60.63 ^{c} ± 2.15	80.84 ^{b} ± 0.60
Iww	64.10 ^{dc} ± 0.98	6.35 ^{f} ± 0.46	-7.72 ± 1.15 ^{f}●	72.43 ^{bc} ± 2.52	63.74 ^{c} ± 1.49	-8.30 ^{h} ± 2.03●
Uww	31.10 ^{f} ± 0.69	-1.05 ^{f} ± 0.77●	NA	40.43 ^{e} ± 2.24	32.34 ^{e} ± 0.59	-15.70 ^{i} ± 1.11●
ABw	44.20 ^{e} ± 2.03	42.27 ^{d} ± 3.06	55.70 ^{d} ± 1.52	59.67 ^{d} ± 1.26	73.99 ^{b} ± 1.90	-2.00 ± 56 ^{g}●
ACw	84.20 ^{a} ± 5.3■	79.58 ^{a} ± 2.02■	89.82 ^{b} ± 2.07	91.61 ^{a} ± 3.11▲	71.85 ^{b} ± 2.96	74.79 ^{c} ± 2.18
AIw	19.3 ^{g} ± 0.67	NA	-10.66 ^{f} ± 1.92●	27.07 ^{f} ± 1.10	18.68 ^{f} ± 2.05	19.30 ^{ef} ± 1.18
Auw	NA	NA	29.36 ^{e} ± 1.64	NA	NA	16.40 ^{f} ± 1.34
BCw	71.28 ^{bc} ± 1.87	67.34 ^{b} ± 1.35	96.84 ^{a} ± 52.11▲	59.07 ^{d} ± 3.41	50.21 ^{d} ± 2.14	61.81 ^{d} ± 2.28
ICw	60.59 ^{d} ± 2.10	57.24 ^{c} ± 2.52	52.11 ^{d} ± 2.16	70.35 ^{c} ± 1.18	75.14 ^{b} ± 1.59	90.89 ^{a} ± 2.04▲

NA, Not Available; SE, standard error of means; *, means followed by the same letter in the same column are not significantly different at p≤0.05 based on DNMRT; ▲= Ideal; ■=high; ●= precipitation; AWW, one unit of acidic solution with two units of water; BWW, one unit of basic solution with two units of water; CWW, one unit of chelating solution with two units of water; IWW, one unit of ionic solution with two units of water; UWW, one unit of non-polar water soluble solvents with two units of water; ABW, one unit of acidic solution, one unit of basic solution and one unit of water; ACW, one unit of acidic solution, one unit of chelating solution and one unit of water; AIW, One unit of acidic solution, one unit of ionic solution and one unit of water; AUW, one unit of acidic solution, one unit of non-polar water soluble solvents and one unit of water; BCW, unit of basic solution, one unit of chelating solution and one unit of water; ICW, unit of ionic solution, one unit of chelating solution and one unit of water.

AUW on PrV(An)_PrV/gC-mAb (Lg), CSFV(A)_ CSFV/E2-mAb(Lg), PrV/gC-mAb (An)_PrV(Lg) and PrV (An) _Vero cell (Lg) could be due to disruptive effect of solvents and pH on proteins as described by Dumetz et al. (2007). This includes the observation on AIW towards CSFV (An) _CSFV/E2-mAb(Lg) complex.

During regeneration, scouting an increase in baseline was observed in certain cases possibly due to either precipitation of regeneration solution, attractive effects of such solution on analyt-ligand interaction, or interaction of analyt with that solution. For instance, using IWW for regeneration of IBDV(An)_IBDV/PAb(Lg) and insulin(An)_ Insulin/mAb(Lg) initiated increased baseline up to 8%. UWW and ABW had same effect on insulin(An)_Insulin/mAb(Lg). Similarly, UWW had increasing baseline effect on CSFV(An)_ CSFV/E2-mAb(Lg). Regardless of being ligand or analyt, both CSFV and PrV showed higher sensitivity towards AIW and AUW when compared to IBDV. One of the plausible explanation could be that the envelope nature of these viruses is more susceptible than the latter non- envelope hardy virus (Heinzel et al., 2010).

Previously, regeneration of ligand - analyt in which one of the agents is virus or virus particle was reported via different range of buffers and solutions. For instance, injection of MES buffer at pH 6 followed by PBS at pH 8 (Casasnovas and Springer, 1995), HCl (Van Cott et al., 1992 ; Wilson et al., 2006), NaOH or H_3P0_4 (Van Cott et al., 1992), mild acidic buffer at pH 6 to 6. 25 (Xing et al., 2000) was employed. None of the earlier mentioned studies showed that the position of interactant (playing role as analyt or ligand) was considered as a factor for choosing suitable regeneration solution. However, in this study, acidic solution was only ideal for regeneration of PrV/gC-mAb(A)_PrV(Lg) and PrV(An)_Vero cell(Lg), and strongly not recommended for IBDV(An)_IBDV/PAb(Lg) system. The findings of this study show that not only the natures of interactants are factors in regeneration but also their position (analyt or ligand) played an important role in regeneration efficiency. Once both PrV and CSFV were placed as analyt, acidic solution was not strong enough to disassociate bounded mAbs. In this case, a combination of acidic and chelating (second trial of optimization) was perfect in order to achieve optimum

Table 6. Mean values measured for regeneration efficiency of analyt-ligand complexes in second optimization.

Cocktail style	R_e mean ± SE	
	PrV-mAb	CSF-mAb
A	75.48^{b} ± 1.06	81.37^{b} ± 4.07
B	NC	NC
C	NC	NC
D	83.47^{b} ± 0.51	87.29^{ab} ± 1.53
E	70.08^{c} ± 0.87	67.90 ± 3.46
F	95.66^{a} ± 0.51	93.78^{a} ± 1.71
G	69.85^{d} ± 1.61	72.69^{c} ± 3.68

NC= Not considered. Means followed by the same letter in the same column are not significantly different at p≤0.05 based on DNMRT.

regeneration (Table 5). Complete regeneration of IBDV/PAb(Lg)_IBDV(An) required a combination of both basic and chelating solutions.

Previously, Anderson and his co-workers recommended for regeneration of antibody-antigen a combination of ionic and chelating solution (ICW) (Andersson et al., 1999), while such combination was most effective only for disassociation of insulin (An)_mAb(Lg) in this study. However, in most cases, ACW had Re higher than 70%, although it was not the optimal solution. After establishment of the regeneration conditions, the life time of chip was calculated counting each cycle of sample running over immobilized ligands. Approximately, the life time of our developed chips with an optimized regeneration solution was about 150 to 250 cycles. Chip could be used above this range but with less efficiency. Based on the findings of the study, reusability of chips increased based on the achievability of optimum regeneration condition for each examined biomolecular system.

Conclusion

According to the findings of this study, disassociation of envelop virus- mAb (PrV & CSFV) needed more effort than non-enveloped (IBDV) virus-mAb. The dis-association pattern of PrV from immobilized mAb (ligand) was not alike in terms of simplicity compared to when PrV acted as ligand and vice versa. Generally, both envelope viruses (as ligand) were regenerated via combination of acidic and chelating solution in which acidic solution contributed more while for dissociation of mAb-PrV, no significant difference was observed between acidic solutions or combination of acidic and chelating solutions. Acidic solutions could efficiently break cell-*PrV* and mAb-*PrV* bonds and could also serve as an acceptable regeneration choice for the latter (ACW, R_e ~ 91). Subsequently, in case of having envelope virus as one of the interactant partners, acidic solution must be firstly screened prior to combination of acidic and chelating agents. Instead a combination of basic and chelating solution was highly recommended when dealing with non-enveloped virus. However, other proteins such as insulin needed a combination of ionic and chelating solutions for effective regeneration.

The results indicate that analyt played an important role in regeneration process. This study shows the effectiveness of basic, acidic, ionic and chelating solutions on optimal regeneration processes. Based on the nature of interacting agents, type of regeneration solution and its combination with other effective reagents differed. Besides, non-polar water soluble solvents were not suitable in that they cause damage to the chip. Consequently, they could reduce chip life time and were considered to be of economical concern.

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