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Notes & Tips

High-throughput and multiplexed regeneration buffer scouting for affinity-based interactions

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ABSTRACT

Affinity-based analyses on biosensors depend partly on regeneration between measurements. Regeneration is performed with a buffer that efficiently breaks all interactions between ligand and analyte while maintaining the active binding site of the ligand. We demonstrated a regeneration buffer scouting using the combination of a continuous flow microspotter with a surface plasmon resonance imaging platform to simultaneously test 48 different regeneration buffers on a single biosensor. Optimal regeneration conditions are found within hours and consume little amounts of buffers, analyte, and ligand. This workflow can be applied to any ligand that is coupled through amine, thiol, or streptavidin immobilization.

Surface plasmon resonance (SPR)[1] is a widely applied and accepted method to study protein–protein interactions in real time. Affinity between an immobilized ligand and a soluble analyte is measured when a reversible binding is established between the two interacting compounds. One of the key parameters in a successful SPR experiment is an adequate regeneration of the sensor surface after analyte binding. Regeneration is considered to be efficient and robust when all bound analyte is removed from the surface, activity of the surface is maintained after each cycle, and it can be performed in a reproducible way. Binding between analyte and ligand is established, for example, through electrostatic forces, hydrophobic interactions, or hydrogen bonds. A regeneration buffer can be chosen depending on the type of interactions that take place. However, multiple types of interactions between ligand and analyte can play a role and may influence each other, complicating the selection of a proper regeneration buffer.

Systematic screening of sensors for optimal regeneration conditions has been previously performed with the Drake–Klakamp methodology [1]. Alternatively, a multivariate cocktail approach may be applied [2–4]. Although the Drake–Klakamp methodology is a strategic approach, it requires multiple experiments because only one regeneration buffer is screened at a time, either manually or in an automated fashion. Thus, finding the best regeneration buffer may take many experiments with different buffers to be tested sequentially. The cocktail approach, on the other hand, screens mixtures of one type of buffer, reducing the number of experiments for initial screening. However, this may lead to complicated regeneration buffer systems or extensive further fine-tuning to find the best regeneration buffer, increasing the number of experiments to be performed again. In addition, both strategies may require multiple new sensors if the tested regeneration buffer destroys the ligand activity.

Here we demonstrate the use of a continuous flow microspotter (CFM) in combination with an SPR imaging instrument to rapidly screen many regeneration buffers simultaneously. The CFM has been developed to uniformly array proteins on a biosensor surface [5]. Here the microfluidics channels in the CFM are used for independent multiplexed surface regeneration, resulting in a buffer scouting with up to 48 different regeneration buffers that can be tested using a single sensor surface. The multiplexed regeneration buffer screening is illustrated in this article using a lectin–glycoprotein interaction as an example where multiple

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[1] *Abbreviations used:* SPR, surface plasmon resonance; CFM, continuous flow microspotter; SBA, soybean agglutinin; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; RU, resonance units.

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interactions between lectins and glycans need to be effectively broken and simple regeneration buffers are often not applicable.

Soybean agglutinin (SBA) was purchased from Vector Laboratories (Burlingame, CA, USA). Acetic acid, sodium hydroxide, urea, *ortho*-phosphoric acid, calcium chloride dihydrate, magnesium chloride, glycin, ethanol, and hydrochloric acid were of analytical grade and purchased from Merck Millipore (Amsterdam, The Netherlands). Formic acid, Tween 80, RBS neutral, manganese chloride, zinc chloride, ethanolamine, Hepes, sodium chloride, glycerol, fetuin, ethylenediaminetetraacetic acid (EDTA), sodium carbonate, ethylene glycol, sodium dodecyl sulfate (SDS), Chaps, Triton X-100, and guanidine hydrochloride were of analytical grade and purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Ultrapure water was produced in-house using a Millipore Milli-Q system.

Easy2Spot SensEye sensors were supplied by IBIS Technologies (Enschede, The Netherlands). Experiments were performed on an IBIS MX96 (IBIS Technologies) and a CFM (Wasatch Microfluidics, Salt Lake City, UT, USA).

A selection of 42 buffers for regeneration screening was made based on the subclasses of regeneration buffers: acidic (A), basic (B), chelating (C), detergents (D), ionic (I), and nonpolar (U) water-soluble solvents [2,3]. Buffers of choice were 1% formic acid (A1-a), 10% formic acid (A1-b), 10 mM HCl (A2-a), 100 mM HCl (A2-b), 25 mM phosphoric acid (A3-a), 100 mM phosphoric acid (A3-b), 10 mM glycin–HCl (pH 2.0) (AB1-a), 100 mM glycin–HCl (pH 2.0) (AB1-b), 10 mM glycin–HCl (pH 2.0)/20 mM EDTA (ABC), 10 mM glycin–HCl (pH 2.0)/1% SDS (ABD), 10 mM glycin–HCl (pH 2.0)/1 M NaCl (ABI), 10 mM glycin–HCl (pH 2.0)/1% ethanol (ABU), 10 mM HCl/1% SDS (AD1), 25 mM phosphoric acid/1% SDS (AD2), 25 mM phosphoric acid/1% SDS/20 mM EDTA (ADC), 10 mM HCl/3 M $MgCl_2$ (AI1-a), 10 mM HCl/1 M NaCl (AI1-b), 25 mM phosphoric acid/3 M $MgCl_2$ (AI2-a), 25 mM phosphoric acid/1 M NaCl (AI2-b), 25 mM phosphoric acid/1 M NaCl/20 mM EDTA (AIC), 10 mM HCl/1% ethanol (AU1), 25 mM phosphoric acid/1% ethanol (AU2), 10 mM glycin–NaOH (pH 10.0) (B1), 10 mM Hepes–NaOH (pH 8.5) (B2), 10 mM NaOH (B3-a), 100 mM NaOH (B3-b), 200 mM Na_2CO_3 (pH 11.0) (B4), 10 mM Hepes/20 mM EDTA (BC1), 10 mM Hepes/20 mM EDTA/1 M NaCl (BCI), 25% ethylene glycol (D1-a), 50% ethylene glycol (D1-b), 1% SDS (D2-a), 20% SDS (D2-b), 0.3% Chaps (D3), 0.3% Triton X-100 (D4), 3 M guanidine chloride (I1), 3 M $MgCl_2$ (I2), 1 M NaCl (I3), 4 M urea (I4), 4 M KCl (I5), 10% ethanol (U1-a), and 20% ethanol (U1-b).

An Easy2Spot SensEye was cover coupled with SBA lectin. In cover coupling, the entire surface of a sensor is exposed to the same ligand solution to create a homogeneously immobilized surface. A baseline of 1 min was recorded on the preactivated Easy2Spot sensor, followed by an injection of 100 µl of a 4-µM SBA solution in 50 mM sodium acetate (pH 4.5)/0.05% Tween 80. An association time of 10 min was programmed, enabling covalent coupling of SBA lectin to the sensor surface. No dissociation, regeneration, or wash steps were programmed. Next, the sensor was deactivated with ethanolamine (pH 8.5) for 8 min.

A 500-µg/ml fetuin solution in Hepes-buffered saline (pH 7.2) (20 mM Hepes, 150 mM NaCl, 0.05% [w/v] Tween 80, 1 mM $ZnCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$) was injected onto the cover-coupled SBA sensor. An injection volume of 100 µl was used. A baseline of 2 min was followed by an association phase of 15 min until sufficient binding of fetuin was measured (~1000 resonance units [RU]). No dissociation, regeneration, or wash steps were applied. Hereafter the sensor was directly transferred to the CFM for regeneration.

One of the selected regeneration buffers per well was pipetted into 48 wells of a 96-well plate, with a total volume of 150 µl per buffer. The SBA cover-coupled sensor with fetuin bound to it was placed in the CFM printer, which was set up to print 42 different regeneration buffers. In addition, 6 channels of the CFM were used as reference and contained Milli-Q water. A pre-buffer gap

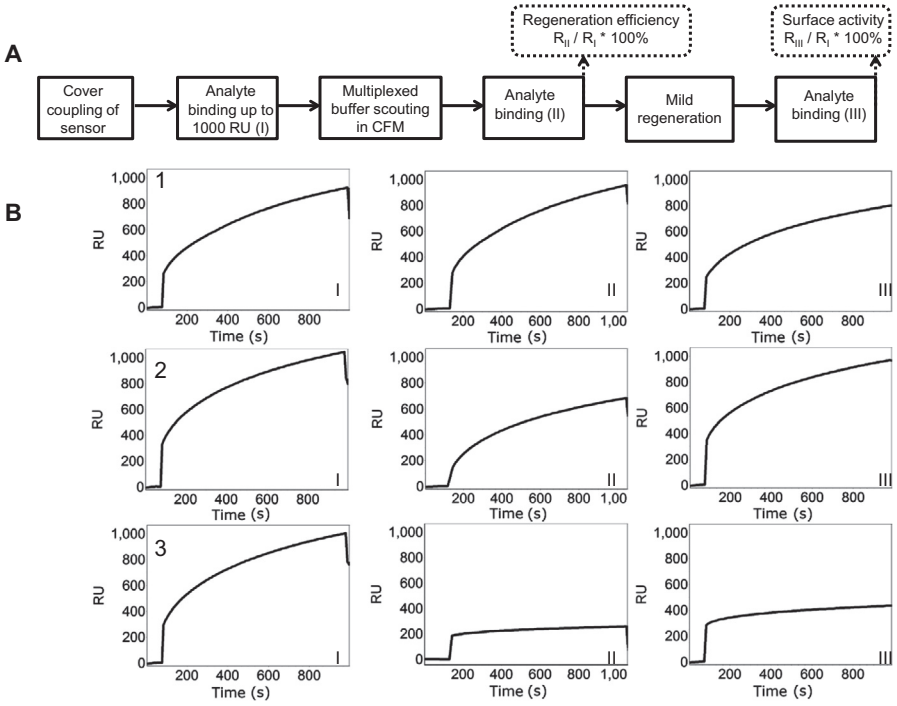

Fig.1. (A) Workflow of the multiplexed regeneration buffer scouting with CFM and SPR imaging. (B) Sensorgrams of regeneration efficiency and surface activity in a multiplexed buffer screening. Sensorgram I: Binding of analyte (fetuin) to ligand (SBA lectin) on a newly prepared surface. Sensorgram II: Binding of analyte to ligand after regeneration with one of the buffers in the screening. Sensorgram III: Binding of analyte to ligand after mild regeneration of surface with 25 mM phosphoric acid. (1) High regeneration efficiency and high surface activity after regeneration with 25 mM phosphoric acid/3 M $MgCl_2$. (2) Poor regeneration efficiency but high surface activity with 10 mM glycin–HCl (pH 2.0)/1% ethanol. (3) Poor regeneration efficiency and poor surface activity with 10 mM HCl/1% SDS (see text for further explanation).

of 25 μl between system buffer (Milli-Q) and regeneration buffers through each of the 48 channels was programmed. A print time of 5 min with regeneration buffers was used, followed by a post-print wash of 2 min with Milli-Q water. Directly after regeneration, the sensor was transferred to the IBIS MX96 instrument.

A baseline of 2 min, followed by an association time of 15 min with 500 μg/ml fetuin solution and 5 min dissociation, was performed. The injection volume was 100 μl. A mild regeneration with 25 mM phosphoric acid in two steps was applied, followed by a wash step. The analysis cycle was finished with a calibration cycle according to the IBIS calibration method. In short, calibration is performed with mixtures of glycerol in running buffer and Milli-Q water at different levels. The RU value at each spot on the sensor is measured for all mixtures. All experiments on the MX96 were performed at a temperature of 25 °C.

Data were acquired in iSPR software, and data analysis was performed in SPRint (IBIS Technologies). Calculations were performed in GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA).

Scouting of regeneration buffers in multiplexed format is demonstrated with the interaction between SBA lectin as a ligand and the glycoprotein fetuin as the analyte according to the workflow in Fig. 1A. The entire surface was covalently coupled with SBA lectin, and activity of the surface was verified by binding with fetuin (Fig. 1B, sensorgram I). A total of 48 spots were regenerated with one of the buffers by exposing the SBA–fetuin complex to the buffers using the microfluidics of the CFM. Following multiplexed regeneration, fetuin was injected again to reestablish binding to SBA lectin (Fig. 1B, sensorgram II) for determination of regeneration efficiency. The entire surface was regenerated with a mild regeneration buffer (25 mM phosphoric acid), followed by another fetuin injection to determine surface activity (Fig. 1B, sensorgram III).

Regeneration efficiency and surface activity were calculated based on report points at 15 min of association rather than comparing baseline levels. Baseline levels are usually evaluated to determine regeneration efficiency, but due to transfer of the sensor from MX96 to CFM and vice versa, the sensor cannot be replaced with millidegree resolution and baselines cannot be compared. Therefore, we chose to determine regeneration parameters from report points after 15 min with the same analyte concentration after baselines were zeroed. Three scenarios were then observed: (i) high surface activity and proper regeneration, (ii) high surface activity but insufficient regeneration, and (iii) poor surface activity (Fig. 1B).

A poor surface activity was measured when the third sensorgram (Fig. 1B3) did not reach a similar response level compared with responses on a new surface in the first sensorgram. The ligand was destroyed; hence, the regeneration buffer was not a suitable candidate. Surface activity was calculated based on the signal after a 2-fold regeneration. First, a regeneration cycle was performed with one of the buffers in the CFM, followed by a mild regeneration with 25 mM phosphoric acid in the IBIS MX96. Responses at 15 min in measurement III (after mild regeneration) were divided by responses at 15 min in measurement I (new surface) (Fig. 1B) and expressed as percentages. Surface activity of the ligand was determined for each of the tested regeneration buffers (Supplementary Fig. 2A), and those buffers resulting in less than 70% active ligand were considered to be too harsh regeneration conditions. The ligand, SBA, was damaged too much by SDS-containing buffers (ABD, AD1, AD2, ADC, D2-a, and D2-b), 10 and 100 mM glycin–HCl (pH 2.0) (A2-a and A2-b), 10 and 100 mM NaOH (B3-a and B3-b), and 200 mM Na_2CO_3 (pH 11.0) (B4). Elimination of regeneration buffers that destroy the ligand (i.e., surface activity <70%) resulted in a subset of mild regeneration buffers.

Regeneration efficiency was then evaluated for the mild regeneration buffers. Regeneration efficiency was expressed as the level of binding that was measured after regeneration in the CFM relative to the level of binding on a newly prepared surface. Responses at 15 min in measurement II (after CFM regeneration) were divided by responses at 15 min in measurement I (new surface) (Fig. 1B) and expressed as percentages. Mild regeneration buffers either were too mild or were capable of sufficient removal of analyte. Too mild buffers removed insufficient amounts of bound analyte (Fig. 1B2), as indicated by sensorgram II that does not reach the same response level. Mild buffers that were capable of removing sufficient amounts of bound analyte (Fig. 1B1) resulted in similar responses in all three sensorgrams and were considered to be good regeneration conditions for the SBA–fetuin interaction. Regeneration efficiencies for all mild regeneration buffers were calculated, and results of triplicate measurements are shown in Supplementary Fig. 2B. Best regeneration efficiencies were measured with 25 mM phosphoric acid/3 M $MgCl_2$ (AI2-a) and 25 mM phosphoric acid/1% ethanol (AU2) for the SBA–fetuin complex.

In summary, we have demonstrated a new regeneration scouting workflow capable of testing 48 different regeneration buffers simultaneously in affinity-based biosensor applications. The full experimental workflow can be performed within 3 h and triplicate measurements within a single working day. The CFM is ideally suited for this buffer scouting, adding a novel application to the use of the instrument. Furthermore, the workflow is an economic alternative because it consumes minute amounts of ligand, analyte, and buffers. In addition, all tests can be performed on a single sensor even when harsh regeneration conditions are to be tested.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2014.03.011.

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