Captavidin: a new regenerable biocomponent for biosensing?

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Functionalisation of a biosensor's sensing surface with the appropriate biorecognition elements is essential for the correct performance of the biosensor. In this paper, we investigate by Surface Plasmon Resonance (SPR) if captavidin, a recently described biotin-binding regenerable protein, could be used to bind and release biotinylated biocomponents for the development of regenerable biosensors. In our experiments, biotinylated antibodies were successfully subjected to up to nine serial capture–release events from the captavidin-functionalised surface. Up to three consecutive captures were possible when a protein target had been subsequently added. On the other hand, biotinylated bacteria were also efficiently captured and released from the gold surface, suggesting also the suitability of captavidin for the development of whole-cell regenerable biosensors. Our results indicate that captavidin is a promising regenerable molecular tool that could be used during biosensor optimisation and validation, and that captavidin-modified surfaces could be fine-tuned into truly reusable sensors.

1. Introduction

Biosensor performance and specificity strongly depend on the successful functionalisation of the sensing surface with appropriate capture biocomponents.[1] A variety of bio-functionalisation strategies have been described to date, ranging from simple and fast random physisorption to a variety of strategies for directed anchoring.[2–7] The use of biotin-binding proteins is one of the preferred approaches when searching for biomolecule orientation and optimal performance.[8–10] Among others, avidin incorporation onto the sensing surface generates highly efficient and specific substrates; binding of the biotinylated biomolecule of choice is straight-forward, and a variety of biotinylation reagents and ready-to-use biotinylated molecules are commercially available. Avidin's Achilles' heel is precisely the high affinity for its biotin target and thus the difficulty to regenerate and reuse the avidin-modified surfaces. In this way, sensor bio-functionalisation leads to disposable instead of reusable devices, increasing considerably the final production costs.

For this reason, researchers have looked for biomolecule regeneration strategies as well as for reusable molecules/components. For example, a variety of conditions, such as acidic or basic solutions, surfactants, chaotropic ions, denaturing agents, organic solvents, or mixtures of them have been tested for antibody (Ab) regeneration.[11–15] Nevertheless, these treatments frequently cause a certain level of Ab denaturation and damage, and should be carefully evaluated for each target–ligand couple. In a different approximation, protein A/G has been exploited as a capture element for immunoglobulins.[5,16] Protein A binds to the IgG Fc (fragment crystallizable) region at neutral pH, thus orienting the molecule to expose its target-binding site, and releases it when treated at acidic pH.

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Avidin is a tetrameric glycoprotein obtained from egg white, has a molecular weight of about 67 000 Da, and an isoelectric point of approximately 10.[8–10,17–19] Each subunit binds a biotin molecule with dissociation constant, K_d, of around 1.3×10^{-15}. Avidin–biotin binding is very specific, occurs very fast, and is the strongest non-covalent biological interaction described between a protein and its ligand ($K_a \approx 10^{15}$ M^{-1}). Once formed, the complex is extremely stable and remains unaffected under a wide range of pH and temperature, and in the presence of organic solvents and other denaturing agents. Thus disruption of the assembly entails harsh conditions that mostly result in avidin denaturation and permanent lost of function.

Captavidin, on the other hand, is a biotin-binding protein derived from avidin, in which the tyrosine in the biotin-binding site has been nitrated.[20] This modification permits dissociation of the captavidin–biotin complex at pH 10.0, that takes place at pH 4.0 with a $K_a \approx 10^9$ M^{-1}. This characteristic has been exploited by the provider to develop regenerable reagents, such as captavidin conjugated to agarose and acrylamide, applicable to affinity chromatography and the isolation of biotinylated components.[21] For these reasons, we raised the question of whether captavidin could be exploited for the production of regenerable sensing surfaces. Only one publication reports the use of this molecule for biosensing,[22] but captavidin is not exploited as a surface-bound regenerable component but is used among other ligands to evaluate the performance of a cantilever.

In this work, we use Surface Plasmon Resonance (SPR) to show that captavidin can be immobilised onto a sensing surface, which can then be successfully subjected to up to 9 serial capture and regeneration steps using a biotinylated antibody. Alternatively, the surface could be used for the serial capture of biotinylated bacteria, applicable to the development of whole-cell microbial biosensors. Besides, a captavidin/biotin-Ab-modified sensor could be used to detect 3 consecutive protein target captures. Our results indicate that captavidin is a promising regenerable molecular tool that could be used during biosensor

optimisation and validation, and that captavidin-modified surfaces could be fine-tuned into truly reusable sensors.

2. Experimental

2.1 Reagents and biocomponents

Phosphate buffered saline (PBS, 0.01 M, pH 7.4) was obtained in tablets from Invitrogen (Barcelona, Spain). The regeneration buffer consisted on carbonate-bicarbonate buffer 0.05 M, pH 10 (Sigma, Barcelona, Spain). Captavidin was from Molecular Probes (Invitrogen). Bovine serum albumin (BSA), avidin, biotinylated and unmodified anti-rabbit antibodies (B-aRbt and aRbt), and biotinylated rabbit Ab (B-Rbt) were purchased from Sigma. The unmodified rabbit Ab (Rbt) was from GeneTex (San Antonio, USA).

2.2 Surface Plasmon Resonance (SPR) instrumentation

SPR was carried out using the portable SPR device Biosuplar 400 and gold sensors from Analytical μ-systems (Sinzing, Germany). This is a small-size portable apparatus that has been shown to be very useful for the performance of preliminary laboratory tests, such as the work presented in this paper. Nevertheless, the lack of a precise temperature control system generates a certain degree of baseline instability and signal shift along the experiment. The incorporation of a thermally insulated chamber with temperature control and/or a fluidic system would have produced improved sensorgrams.

The experiments were performed at room temperature. Data are expressed in milli-degrees (m°) and indicate angle shift.

2.3 Surface functionalisation

Gold chips were washed with acetone for 5 min and serially rinsed with water, ethanol, and water again before being placed in the equipment cell. Unless otherwise stated, biocomponents were dissolved in PBS, manually injected on the SPR sensor using a 1 ml syringe, and incubated for 20 min at room temperature. Each incubation was followed by wash with PBS for at least 5 min in order to remove non-specifically or poorly bound molecules.

Gold functionalisation was carried out by biomolecule random physisorption. The sensor gold surface was coated with captavidin 10 μg/ml, followed by blocking with 1% (w/v) BSA so as to prevent later non-specific binding of undesired components (Fig. 1a). The functionalised sensor was extensively washed until signal stabilisation was achieved and then immediately used for the capture of biotinylated targets. Although this strategy is described to generate poorly structured and reproducible surfaces, we have obtained good results for antibody immobilisation onto gold substrates.[2,4] Furthermore, physisorption provides an extremely fast and easy approach. This was especially important in this work, mainly due to the setting of the SPR equipment used. In this respect, the cell design demands for extensive manipulation of the chip, and thus on-line rather than pre-assembling functionalisation was preferred. Once assembled, the experiment had to be performed within a working day.

2.4 Capture of biotinylated antibodies using captavidin-functionalised sensors

The above described sensor was incubated with biotinylated Ab dissolved to 10 μg/ml in either 50 mM citrate-phosphate buffer pH 4.0 (the buffer recommended by the provider) or PBS pH 7.4. Two different Ab were alternatively used: B-aRbt Ab and B-Rbt Ab. Unmodified Ab (not biotinylated aRbt and Rbt Ab) were used in parallel as a negative control for non-specific adsorption. When required, target protein (unmodified Rbt Ab 5 μg/ml in PBS) was captured for 20 min (Fig. 1b).

2.5 Capture of biotinylated bacteria using captavidin-functionalised sensors

The sensor, functionalised with captavidin as described before, was subjected to serial capture–regeneration using biotinylated *Escherichia coli*, prepared as follows: 10 ml of *E. coli*, grown overnight in LB broth, was centrifuged at 16 000 *g* for 5 min, washed 3 times with chilled PBS, and incubated for 1 h in ice with 1 mg/ml of Sulfo-NHS-XX-Biotin (Invitrogen). The biotinylated cells were then washed 3 more times with chilled PBS to remove free biotin, resuspended in PBS supplemented with 10% sterile glycerol, and preserved in 500 μl aliquots at −20 °C until used. Prior to bacteria immobilisation, residual glycerol was eliminated by washing 3 times with PBS. The final concentration of bacteria was 10^7 CFU/ml. Non-biotinylated *E. coli*, 10^8 CFU/ml, was used in parallel as a negative control for non-specific adsorption.

2.6 Surface regeneration

The regeneration procedure consisted of incubation in freshly made carbonate-bicarbonate buffer pH 10.0 for 15 min, as recommended by Invitrogen.

3. Results and discussion

3.1 Captavidin as a regenerable biocomponent for the serial capture and release of biotinylated antibodies

According to the provider's description, captavidin is a regenerable molecule able to bind biotinylated molecules at pH 4.0 and release them at pH 10.0. Bolivar *et al.* have recently shown that a similar home-made avidin derivative, called in their work nitroavidin, could be immobilised onto an SPR chip, and the modified sensor could be submitted to a single capture and release step of a biotinylated component.[23] In order to study its potential applicability in biosensing we investigated by SPR if captavidin could (i) be regenerated when physically immobilised onto a sensing surface, and (ii) survive serial biotin binding and regeneration cycles.

Gold chips were functionalised with captavidin and submitted to serial incubations with biotinylated Ab (B-aRbt, 10 ug/ml in citrate-phosphate buffer pH 4.0) and regeneration buffer (Fig 1a). As shown in Table 1, up to 6 consecutive captures were possible, although each incubation generated a slightly lower signal and regeneration efficiency than the previous one. The differences in signal observed between the first and succeeding B-aRbt captures were attributed to the first regeneration step, which presumably caused rearrangement of the surface-bound proteins, as well as desorption of poorly trapped BSA molecules. Thus, a regeneration step was added to the functionalisation protocol immediately after BSA blocking in subsequent experiments. Incubation of a non-biotinylated Ab instead (aRbt),

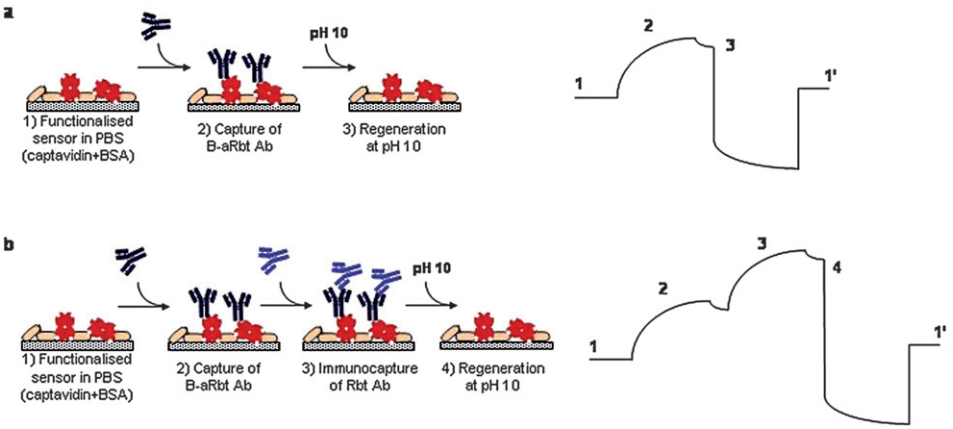

Fig. 1 Graphical illustration of the assay formats described in the text. On the right hand side, expected SPR graphs. (a) The SPR sensor, functionalised with captavidin and blocked with BSA, is submitted to serial capture and release of a biotinylated Ab. (b) The SPR sensor, functionalised with captavidin and blocked with BSA, is submitted to capture a biotinylated Ab and subsequent immunocapture of a protein target (in this case, Ab developed in rabbit).

Table 1 Successive biotin-Ab capture/regeneration steps at different capture pH. Increase/decrease in signal (expressed in m°) generated on a captavidin-modified SPR sensor by 6 successive B-aRbt Ab capture/regeneration steps carried out at pH 4.0 or pH 7.4. In brackets, percentage of signal compared to the first capture

		Successive B-aRbt Ab capture/regeneration steps					
		1	2	3	4	5	6
Ab capture at pH 4	Capture signal increase	$37_{(100)}$	$41_{(112)}$	$31_{(85)}$	$29_{(78)}$	$21_{(57)}$	$24_{(65)}$
	Regeneration signal decrease	−90	−43	−36	−32	−18	−16
Ab capture at pH 7	Capture signal increase	$38_{(100)}$	$38_{(101)}$	$33_{(85)}$	$35_{(91)}$	$34_{(89)}$	$28_{(72)}$
	Regeneration signal decrease	−65	−37	−39	−27	−28	−35

generated increases in signal below 11m° – within the equipment noise – and no significant decrease in signal after the regeneration steps. This confirmed that the capture of biotinylated Ab was specific and that the regeneration procedure was not promoting non-specific adsorption.

According to the supplier's instructions, captavidin–biotin capture should be carried out at pH 4.0. Nevertheless, biomolecule integrity and Ab performance are better preserved at physiological pH. This moved us to investigate if captavidin–biotin capture could be done at neutral pH rather than pH 4.0. Table 1 and Fig. 2a summarize the results obtained for 6 consecutive B-aRbt captures carried out at either pH 4.0 or pH 7.4. The signal changes related to both Ab capture and surface regeneration were very similar for the first three capture/regeneration steps, independently of the capture pH. Subsequent captures proved to be less efficient for those surfaces treated under acidic conditions. For example, the increase in signal generated by the fifth Ab capture was above 85% of the signal registered by the first capture at neutral pH, compared to less than 60% at pH 4.0. Capture was thus carried at pH 7.4 in the rest of experiments. In the absence of regeneration steps between consecutive Ab injections only the first capture produced a significant response (53m° compared to 0–8m°; Fig. 2b), confirming that serial capture of B-aRb is possible only after surface regeneration.

Fig. 2c and 2d show a similar experiment, this time assaying 5 μg/ml of Ab (instead of 10 μg/ml). In this case, up to 9 consecutive capture/regeneration steps were achieved (Fig. 2c). Under these conditions, the first addition of Ab does not saturate the captavidin-modified surface. This is evident in the negative control experiment where consecutive incubations with B-aRb, with no regeneration steps in-between, generate signals which are lower at each step but detectable for the first 4 captures (17–36m°; Fig. 2d). If this control surface was submitted, following the 5 consecutive B-aRb captures, to a regeneration step and a new Ab capture, capture efficiency was recovered (Fig. 2d). In the same way, a captavidin-modified SPR chip subjected to 6 serial incubation/regeneration steps with non-biotinylated Rbt Ab (10 μg/ml), generated nearly undetectable signals, but efficient capture of B-aRb Ab following the sixth regeneration step (data not shown). This confirmed that incubation in the carbonate-bicarbonate buffer did not have a deleterious effect on captavidin integrity or performance *per se*.

3.2 Captavidin in the serial capture and release of biotinylated bacteria

In order to test if captavidin could also bind and release larger and more complex targets, biotinylated bacteria were assayed in place of the biotinylated antibodies. With this purpose, *E. coli* was biotinylated, diluted in PBS to a concentration of 10^7 CFU/ml, and submitted to serial capture and regeneration steps as described above. Three consecutive capture/release events were successfully carried out, with recorded signals

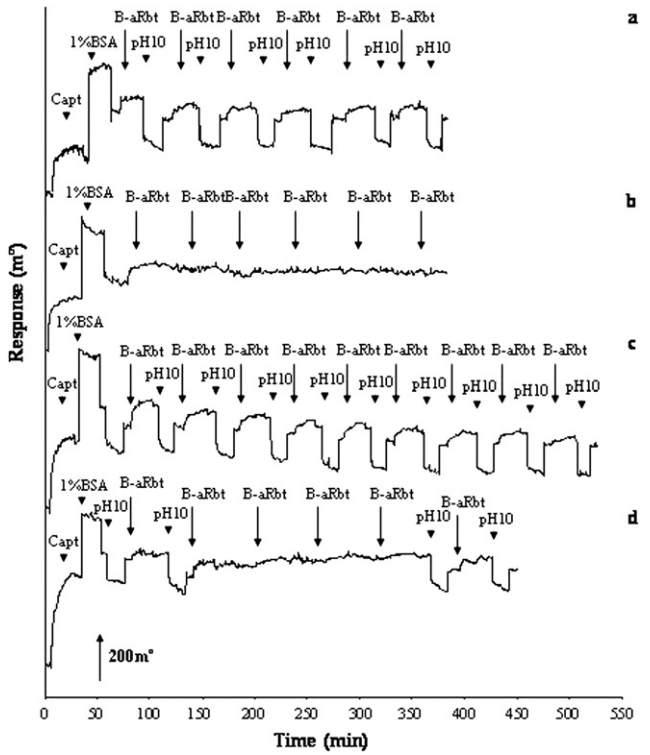

Fig. 2 Regeneration of captavidin-functionalised sensor surfaces with carbonate buffer (pH 10.0) after subsequent captures of B-aRbt Ab at concentrations (a) 10 μg/ml and (c) 5 μg/ml, compared to the capture of similar concentrations on non-regenerated surfaces (b and d). Each incubation was followed by washing with PBS.

of 28, 42, and 27m°, and complete signal recovery following regeneration (data not shown). On the contrary, the control experiment, carried out in parallel by assaying non-biotinylated *E. coli* ten times more concentrated (10^8 CFU/ml) generated signals of 4.6, 12, and 9m°. These results indicate that captavidin could also be used for the serial capture and release of complex targets such as microorganisms, useful, for example, for the development of whole-cell sensors.

The signals registered following the capture of whole bacteria, although low compared to the signals generated by antibody capture, are in accordance with previous reports.[24] This is due to the fact that the evanescent wave formed under SPR conditions decays as it penetrates the solution and usually shows a maximal penetration of 300 nm. Therefore, only the refractive index changes occurring closer from the gold surface than 300 nm will cause a signal change. Considering the average size of an *E. coli* cell (2–6 × 0.7–1.5 μm),[25,26] only a small portion of the captured bacteria is close enough to the sensor surface as to contribute to producing a response signal.[24] On the other hand, the relatively large deviation observed between measurements could account for the limited sensitivity of the portable SPR equipment used, but also for the shape and complexity of bacterial cells. For example, as *E. coli* consists of long rods, capture of bacteria lying flat on the surface will cover more surface, and thus generate higher signals, than bacteria 'standing up' on the sensor surface.

3.3 Captavidin *versus* avidin and the molecular basis of the regeneration event

The utilisation of avidin generated capture SPR signals twice as high as the ones generated by captavidin. This is consistent with a decrease in affinity induced by the molecular modification introduced in captavidin (affinity constant, K_a, 10^{15} and 10^9 M^{-1} for avidin and captavidin respectively), and is also consistent with data previously published by Bolivar *et al.*[23] The attempts to release avidin by regeneration at pH 10.0 were completely unsuccessful (data not shown). Furthermore, incubation of an avidin-modified sensor with the regeneration buffer induced complete inactivation of the capture protein. These data suggest that the modification that allows captavidin regeneration reduces in fact its sensitivity to basic pH, presumably preserving the integrity of the biotin-binding site.

Our results indicated that the decrease in surface performance registered after serial capture/regeneration steps was related to captavidin partial regeneration. Each regeneration step seemed to release only a proportion of the biotinylated molecules captured, leaving on the surface a decreasing number of free/functional captavidin units. This was confirmed by performing a new experiment, where a biotinylated target (B-Rbt Ab in this case) was captured onto a captavidin-modified sensor. Unmodified aRbt (which recognises and binds the B-Rbt) was added both immediately and following a regeneration step. Although lower, the later incubation generated a detectable signal, demonstrating that part of the biotinylated Ab remained on the surface. Accordingly, the long-term regenerability of the captavidin-modified sensor will depend on the concentration, characteristics and degree of biotinylation of the biotinylated molecule captured. For example, a biotinylated molecule captured simultaneously by several captavidin molecules will presumably be less efficiently removed from the surface.

3.4 Captavidin in protein immunosensing

Reusing a functionalised sensing surface might be extremely interesting when a prototype equipment is to be tested but only a limited number of sensor chips are available, or when chip-to-chip variability wants to be avoided. True sensor regeneration should then involve, not just repeated capture of the binding component (in this case, B-aRbt), but also serial capture and detection of a target component. For this reason, we evaluated the performance of the captavidin-modified sensors in the serial capture and regeneration of B-aRbt Ab (5 μg/ml) when it had been submitted to capture of its target, Rbt Ab (Fig. 1b). As shown in Fig. 3, three consecutive detections of the protein target were successfully performed (44.1, 32.9, and 26.9m° respectively). In contrast, regeneration efficiency seemed poor compared to regeneration of just biotinylated Ab. This translated into signal drift, with increased signal background for each successive biotin capture (65.5m° for the first B-aRbt Ab capture, but 96.5 and 93.4m° for the second and third). This phenomenon was partly due to a bulk effect, where the target-Ab assembly impels steric hindrance on the biotin surroundings and/or interferes in the diffusion

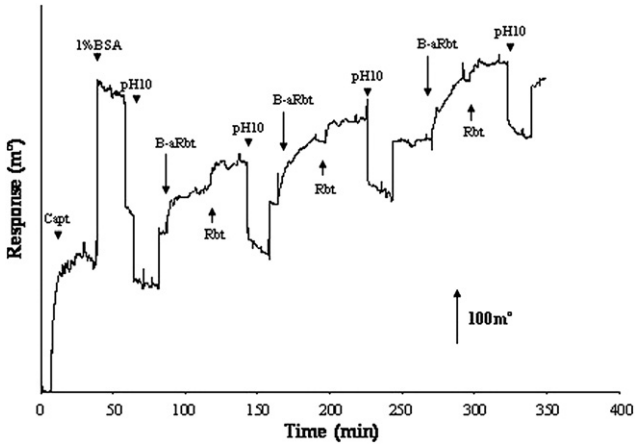

Fig. 3 Effect of the carbonate buffer (pH 10.0) regeneration procedure on a captavidin–B-aRbt (5 μg/ml) functionalised sensor surface after exposure to three subsequent protein target (Rbt Ab, 5 μg/ml) capture–release events.

of the regeneration buffer towards the surface. Consistently, serial injection of small volumes of the carbonate buffer along the regeneration time generated improved surface regeneration (data not shown), suggesting that the use of a flow system might additionally improve regeneration efficiency. On top of this, not released B-aRbt and target protein assemblies could promote subsequent affinity binding of a number of B-aRbt, generating an accumulative effect along the procedure.

Attempts to improve target release, and so surface reusability, were made by modifying the regeneration procedure as summarized in Table 2. The best results were obtained when the surface was submitted to a short wash with 50 mM HCl, followed by incubation at pH 10.0 for 15 min and a brief blocking with BSA (Table 2a). Longer expositions to the acid induced surface damage that translated into enhanced levels of non-specific adsorption and/or dismissed capture efficiency (Table 2b). Incubation with only HCl and no carbonate (data not shown), or with HCl after carbonate regeneration appeared to damage captavidin integrity (Table 2c). In all cases, a blocking step with BSA was necessary in order to avoid subsequent biomolecule non-specific adsorption and compensate the damage induced by the improved regeneration procedure on the surface protein cover.

Table 2 Effect of different regeneration protocols on successive captures/regenerations of biotin-Ab and target protein. The captavidin SPR sensor was serially submitted to capture of B-aRbt Ab (5 μg/ml), binding of its protein target (Rbt Ab, 5μg/ml), and regeneration as follows. (a) HCl 50 mM for few seconds, carbonate pH 10.0 for 15 min, and blocking with 1% BSA for 5 min. (b) HCl 50 mM for 5 min, carbonate pH 10.0 for 5 min, and blocking with 0.1% BSA for 10 min. (c) Carbonate for 15 min, HCl 50 mM for few seconds and blocking with 1% BSA

	Signal increase/decrease (m°)		
	B-aRbt Ab	Target (Rbt Ab)	Regeneration
(a)			
1st capture	42.8	18.9	−127.0
2nd capture	29.2	27.6	−46.7
3rd capture	42.5	27.4	−57.7
Target alone	—	7.9	—
(b)			
1st capture	59.7	31.5	−216.7
2nd capture	36.7	13.1	−72.4
3rd capture	19.4	10.5	−34.7
Target alone	—	15.6	—
(c)			
1st capture	51.7	19.2	−178.5
2nd capture	24.6	16.7	−78.9
3rd capture	24.0	19.5	−65.8
Target alone	—	22.7	—

Regeneration efficiency might be limited in cases where each single target unit can be simultaneously bound by several Ab on the surface. Our results suggest that surface regenerability could improve in cases where a target of smaller size and/or exposing on surface a lower number of epitopes were targeted, such as drugs, peptides or small proteins, or detection using monoclonal Ab. Work is currently in progress as to implement captavidin-functionalisation to microfabricated optical and electrochemical sensors which, taking advantage of non-regenerable functionalisation strategies, have already been successfully used for immunodetection and for whole-cell sensing of water contaminants.[27–29] Due to the high cost of microfabricated devices, sensor regenerability/reusability would compensate their production costs and facilitate their application to the study of real samples.

4. Conclusion

In this paper we used SPR to demonstrate that captavidin can be exploited for the production of regenerable and reusable sensing surfaces. In this context, the captavidin-modified sensors have been successfully subjected to up to 9 serial capture and regeneration steps using a biotinylated antibody, and for the serial capture of biotinylated bacteria applicable to the development of whole-cell microbial biosensors. Besides, a captavidin/biotin-Ab-modified sensor could be used to detect 3 consecutive protein target captures. Our results indicate that captavidin is a promising regenerable molecular tool that could be used during biosensor optimisation and validation, and suggest that captavidin-functionalised surfaces could be finely tuned into truly regenerable/reusable sensing surfaces for at least certain ligand–target systems.

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