

Immobilization of proteins on agarose beads, monitored in real time by bead injection spectroscopy

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A novel approach to real-time monitoring of protein immobilization resulted in the surprising finding that current immobilization protocols are far from optimized.

Summary

This work introduces a novel tool for the examination and optimization of protein immobilization protocols, by measuring the rate and yield of coupling reactions, as they take place on the surface of agarose beads in a well-stirred microreactor. The power of the Bead Injection Spectroscopy (BIS) technique is demonstrated on examples of amino coupling reactions for albumin, ovalbumin, lysozyme, human IgG, ribonuclease A and cytochrome C, using commercially available Aminolink[®] agarose beads. It was found, surprisingly, that currently recommended protocols for reductive amination can be shortened from several hours to several minutes, and that, contrary to literature data, the yield of coupling is dependent on pH and the isoelectric point of the protein. In addition, leakage of immobilized ligands can be measured by direct spectroscopic interrogation of captured beads *in situ*. The methodology presented in this work documents that BIS is a useful tool for quality control of agarose-based chromatographic supports, as well as for the optimization of a wide variety of immobilization chemistries, as used for synthesis of chromatographic supports, immobilization of enzymes, and derivatization of biosensing surfaces.

Introduction

Agarose as a solid support for bioligands or enzymes has gained widespread use in processing biomolecules on an industrial and a laboratory scale. Applications that exploit molecular recognition between sites fixed on a stationary phase and a target biomolecule fall into two broad categories: separation and catalysis. For the purification and assay of biomolecules, affinity chromatography relies on the interaction of the biomolecule with an immobilized ligand, which selectively captures the target protein on the stationary phase, while unwanted species are carried away by the mobile phase. Following selective capture, the desired biomolecule is released by changing the composition of the mobile phase, eluted, and quantified. Since its introduction in 1968¹ more than 30 000 papers have been published on affinity chromatography and it is estimated that more than 60% of all purification protocols are based on affinity separations.² The use of immobilized enzymes has had an enormous impact on biotechnological

processing, while analytical applications utilize immobilized enzymes for selective conversion of target analytes into detectable species.

Immobilization chemistry is the key component for successful production of selective supports, as it must create a stable bond between the bioligand and solid support, while the support itself must remain inert in order to avoid nonselective adsorption. The physical and chemical stability of the link between the protein and the support should be such that leakage of the bioligand is minimized during use, not only during the capture, but also during repeated elution of the target molecules from a column or a biosensing surface. Chemistries and protocols for immobilization of proteins have gradually evolved from “home made” recipes, through trial and error, into industrial standards. Presently, materials such as coupling gels, coupling buffers, and wash solutions are commercially available from several sources, and immobilization protocols have been published in research articles,³ monographs,^{4,5} commercial literature,^{6,7} and in instructions accompanying commercially available kits.⁸ These protocols have been developed based on careful work of high scientific quality, but because of instrumental limitations remain rather empirical and lack information on the kinetics of immobilization process.

Typically, evaluation of protein immobilization techniques has been carried out manually by equilibrating 2 mL columns, filled with activated gel, with buffers, auxiliary solutions, and target proteins,^{1–5} an approach that is labor intensive and not suited for the study of reaction kinetics. Other essential parameters, such as yield of immobilization, binding capacity, bioligand loss, and the influence of pH and eluants, are also assayed manually using reagent-based analytical procedures including colorimetry, electrophoresis, and radioisotopes^{1,2} (e.g. I¹³¹ for ligand leaking study). Since Bead Injection Spectroscopy allows real-time monitoring of protein capture and release directly on agarose and Sepharose beads,^{9–12} it is used here, for the first time, for monitoring protein immobilization, with the aim of demonstrating its potential for exploration and optimization of current immobilization protocols, and for the evaluation of the quality of chromatographic and catalytic supports.

So far, BIS has been carried out in *flow-through mode*, while the assayed solution is perfused through a microcolumn of Sepharose beads. This approach has been used for Bioligand Interaction Assays^{9–12} and even for the study of metabolism of

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live cells immobilized on beads.^{13,14} However, for the study of protein immobilization processes, due to the short contact time between the protein to be immobilized and the solid support, the flow-through mode is not suitable. Since immobilization procedures reportedly require equilibration for several hours, or even overnight,^{3–8} flow-through monitoring of protein immobilization would require very large volumes of protein solutions and extremely slow flow rates.

Batch immobilization on the other hand, can be carried out in a time scale compatible with current immobilization protocols and, therefore, monitoring of reaction progress in a well-stirred microreactor will elucidate conditions that produce optimal reaction yield. Reaction progress is monitored by periodic sampling of beads from a stirred microreactor, followed by their spectroscopic interrogation, which will reveal the rate of conjugation, regardless of how long it will take for solution/surface chemistries to reach equilibrium. Such automated monitoring of immobilization chemistries, carried out in a well-defined, stirred reaction mixture, will allow reliable exploration and efficient optimization of reaction conditions, such as pH, buffer composition, and ligand concentration.

Flow and batch monitoring was carried out in this work using the same apparatus, the core of which is the “lab-on-valve” (LOV) module^{10–12,15} operated in micro-Sequential Injection mode. The system (Fig. 1A) comprises a six position valve and a high precision 1 mL syringe pump, capable of generating very high flow rates (up to $500 \mu\text{L s}^{-1}$) for fill up

and bead discharge, moderate flow rates ($20 \mu\text{L s}^{-1}$) for bead transport, and low flow rates (1 to $5 \mu\text{L s}^{-1}$) needed for bead packing, metering, bioligand capture, bioligand elution, and for long-term stability monitoring. The instrument relies on software control that allows for versatile and reliable sequencing of all events that comprise assay protocols and spectra collection in the UV-VIS region.

From a large variety of chemistries used for protein immobilization, reductive amination has been selected as a model system, due to its simplicity, efficiency, and the stability of the final product. An additional advantage of this reaction is that the details of the immobilization protocol have been published in research^{3–5} and in commercial literature.^{6–8}

Experimental

Apparatus

The unique feature of the BIS- LOV system is the configuration of the flow cell, within which agarose beads are trapped between two optical fibers (Fig. 1B). The beads ($30\text{--}150 \mu\text{m}$ in size) are held in the optical path by two plugs made of PEEK tubing. The length of the optical path is adjusted by moving the ends of optical fibers into the desired positions. For the experiments described here, the optical path was 1 mm and the flow cell volume was $4 \mu\text{L}$. In the flow channel, two plugs were inserted: downstream from the optical path, a plug made of red PEEK tubing (id $125 \mu\text{m}$) held agarose beads in place, while upstream a plug made of green PEEK tubing (id $800 \mu\text{m}$) was used to focus the carrier stream into the center of the packed beads, to define the flow cell volume, and to hold the packed beads within the optical path (color coded PEEK is available from Upchurch Scientific (www.upchurch.com) (products #1533 (green), #1535 (red)). When assembling the flow cell, the green plug is inserted first, then the optical fibers are inserted to define the optical path, and are adjusted to keep the green plug in position (Fig. 1C, point a). Next, the red plug is inserted and secured in position by Teflon tubing that has been cut at an angle (Fig. 1C, point b) to prevent channel clogging. This flow cell geometry allows the passage of beads through the green plug into the flow cell at moderate flow rates (up to $20 \mu\text{L s}^{-1}$), while the red plug allows carrier stream to pass while retaining beads in the optical path at this or lower flow rates. Since agarose beads are elastic, they can be flushed through and around the red plug by a short burst of carrier stream at a very high flow rate ($200 \mu\text{L}$ at $200 \mu\text{L s}^{-1}$). The volume of beads injected and packed into the flow cell is selected by the volume of bead suspension (1:10, beads: buffer) aspirated into the holding coil and injected into the flow cell.

The lab-on-valve module was used as purchased from FIALab Instruments (www.flowinjection.com), without any modification. All connecting tubing, including the holding coil (1 m long) was made of 0.8 mm id Teflon tubing (coded green, Upchurch #152226). The tubing leading from the carrier buffer container to the inlet port of the syringe pump was made of 1 mm id Teflon tubing (Upchurch #1675) to allow the pump to refill at $500 \mu\text{L s}^{-1}$ without bubble formation caused by cavitation. The instrument used was a microSIA-LOV system, controlled by FIALab software for Windows, with an Ocean Optics spectrophotometer, a deuterium lamp

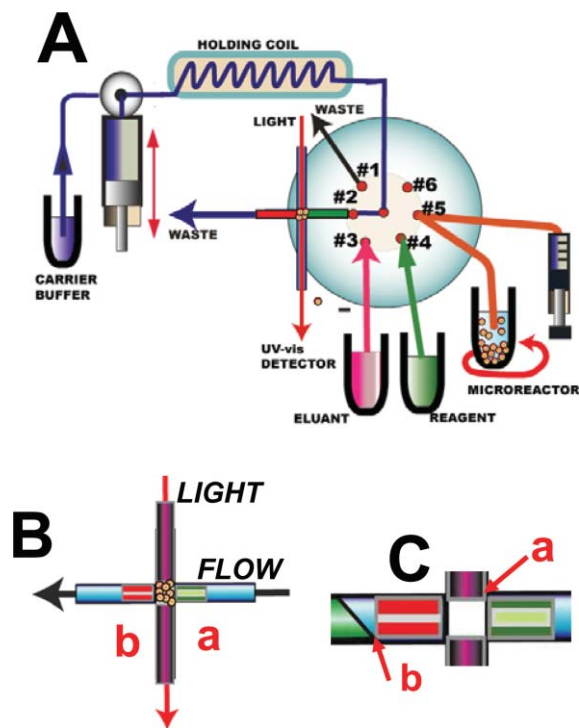


Fig. 1 MicroSequential Injection System configured for Bead Injection Spectroscopy. A. System setup. B. Flow cell configuration. Plug “a” focuses the carrier stream into the center of the packed beads while plug “b” helps retain the beads within the optical path. C. Details of flow cell construction: (a) the optical fibers hold plug “a” in place and (b) the Teflon tubing in the waste channel is cut at an angle to hold plug “b” in place and to prevent clogging.

(Analytical Instruments Systems Inc. AIS Model UV-2), and 600 μm optical fibers, assembled in house using silica/silica optical fibers (Polymicro Technologies, L.L.C. #FVP600660710UVM1), stainless steel tubing (Upchurch #U-139), SMA termination connectors (Ocean Optics #TERMKITSMA-710) and green PEEK tubing, that were secured in the LOV module by standard Upchurch fittings.

Microreactor and composition of reaction mixture

Flat bottom, 4 mL glass vials served as stirred microreactors. The stirring was accomplished by placing the vial into a 10 mL glass beaker that was fixed eccentrically on a turntable (diameter 5 cm) fashioned from a small magnetic stirring table (Micro-V, Cole Parmer Instruments Co.). A rigid tubing (green PEEK) that protruded from the flow-through port of LOV module, reached into the solution and close to the bottom of the glass vial, stirring the contents in accord with the vial's rotational movement.

In all experiments, the reaction mixture was made up by pipetting into the vial 200 μL of agarose beads, followed by 1800 μL of buffer solution. The vial was placed on the stirring table and the automatic monitoring begun. The reaction was initiated by pipetting 200 μL of a protein solution into the bead suspension, during the second bead sampling cycle. All experiments were carried out at room temperature.

Reagents

Agarose beads were washed with water and equilibrated with a reaction buffer that was later drained from sedimented beads immediately prior to their use. Aminolink[®] Coupling Gel supplied with Immobilization kit (Product #44890), Aminolink[®] Plus Immobilization Kit (Product # 44890, Pierce Biotechnology Inc.), and Aminolink[®] Gel (Product # 44894) were all obtained from Pierce Biotechnology Inc. (www.piercenet.com). Sepharose 4B CL was supplied by Sigma (www.sigma-aldrich.com).

Protein solutions were prepared in deionized water as stock solutions of 60 $\mu\text{g } \mu\text{L}^{-1}$ and were diluted with water prior to use to appropriate concentrations. The following proteins were obtained from Sigma for use in this work: (1) bovine serum albumin, (product #A7030); (2) lysozyme (product #L6876), (3) ribonuclease A (product #R6513); (4) ovalbumin (product # A5503); (5) cytochrome C (product # C12037); and (6) human IgG (product #I8640).

Buffer solutions: (1) phosphate buffered saline, pH 7.4: (0.1 M NaCl); (2) borate/phosphate, pH 9.2: (10:1, 0.1 M potassium dihydrogen phosphate/0.1 M sodium tetraborate); (3) pH 10.0: (6 : 4, 0.1 M sodium hydroxide/0.1 M sodium tetraborate); (4) pH 11.0: (1 : 1, 0.1 M sodium hydroxide/0.1 M sodium tetraborate); (5) pH 12.1: (4 : 6, 0.1 M sodium hydroxide/0.1 M sodium tetraborate); (6) phosphate buffer, pH 5; (7) phosphate buffer, pH 4; (8) Pierce coupling phosphate buffer, pH 7.0 (0.1 M sodium phosphate); (9) Pierce coupling citrate buffer, pH 10. The pH of all buffers was monitored by a glass electrode.

Auxiliary reagents: sodium cyanoborohydride was supplied with Pierce Aminolink[®] kits. Sodium P-20 surfactant was obtained from BIAcore (Product #BR-1000-54). All other

chemicals were reagent grade from JT Baker Co. Deionized water was used to prepare all solutions.

Carrier solution: In all experiments, PBS (pH 7.4) containing 0.02% P-20 surfactant was used.

Monitoring protocols

Monitoring of protein immobilization was controlled by the software protocol summarized in Table 1. The microreactor, filled with buffer and bead suspension was placed on the rotary table, and stirred, while the sampling conduit was primed manually by a syringe (Fig. 1A). Each monitoring cycle commenced with filling the automated syringe with a carrier solution, followed by short purging of the flow cell, after which the spectrophotometer baseline reading was zeroed, data collection was initiated, and the monitoring loop began. During each monitoring cycle 150 μL of bead suspension was aspirated from the microreactor into the LOV module. Following the valve switch, beads were gently packed in the flow cell and perfused, at a slow flow rate (10 $\mu\text{L s}^{-1}$), with 400 μL of PBS carrier solution. Next, the beads were expelled from the flow cell to waste by a short burst of carrier solution of a high flow velocity (400 $\mu\text{L s}^{-1}$). Finally, the sampling conduit leading from the flow-through port to the microreactor was purged by 150 μL of carrier stream. This cycle was

Table 1 Software protocol for monitoring of reaction rate

Fill syringe	Load & monitor sample
Valve waste	Valve sample
Syringe pump valve in	Syringe pump flowrate: 200 ($\mu\text{L s}^{-1}$)
Syringe pump flowrate: 400 ($\mu\text{L s}^{-1}$)	Syringe pump aspirate: 200 (μL)
Syringe pump aspirate: 400 (μL)	Syringe pump delay until done
Syringe pump delay until done	Valve flowcell
Syringe pump valve out	Syringe pump flowrate: 10 ($\mu\text{L s}^{-1}$)
Syringe pump delay until done	Syringe pump dispense: 400 (μL)
Valve flowcell	Syringe pump delay until done
Syringe pump empty	Flush sample from flow cell
Syringe pump delay until done	Syringe pump flowrate: 400 ($\mu\text{L s}^{-1}$)
Spectrometer	Syringe pump dispense: 400 (μL)
Spectrometer reference scan	Syringe pump delay until done
Spectrometer absorbance scanning	Flush sampling line^a
Loop start	Valve sample
Loop start: 10 (#)	Syringe pump flowrate: 50 ($\mu\text{L s}^{-1}$)
Analyte new sample	Syringe pump dispense: 100 (μL)
Refill syringe	Syringe pump delay until done
Syringe pump valve in	Empty pump
Syringe pump flowrate: 200 ($\mu\text{L s}^{-1}$)	Valve waste
Syringe pump aspirate: 900 (μL)	Syringe pump flowrate: 400 ($\mu\text{L s}^{-1}$)
Syringe pump delay until done	Syringe pump empty
Syringe pump valve out	Syringe pump delay until done
Syringe pump flowrate: 20 ($\mu\text{L s}^{-1}$)	Loop end
Syringe pump dispense: 20 (μL)	Loop end
Syringe pump delay until done	Spectrometer stop scanning

^a Note: For monitoring equilibrated beads, next line reads "valve waste".

repeated ten times, under control of the software loop script. At the end of the last loop data collection was terminated. Note that the bead reaction mixture (1 : 10, beads : buffer) is aspirated into the flow-through port through a sampling conduit that has a volume of 100 μL and is filled with carrier solution. Therefore, only 50 μL of bead suspension were aspirated into LOV module yielding 5 μL of beads packed into the flow cell. As a result, approximately $\frac{3}{4}$ of beads remained in the reaction mixture after ten sampling cycles. It was critical to adjust the above volumes in concert with the volume of the sampling conduit, in order to sample sufficient amount of beads to pack the flow cell and to washout the bead sampling conduit between sampling cycles to avoid carry-over. Such fine tuning was conveniently accomplished using dyed beads and visual control of the transparent LOV module.

Monitoring of protein linkage stability was controlled by the software protocol summarized in Table 2. Briefly, at the beginning of the monitoring period, the flow cell was purged by carrier solution and the beads to be investigated were loaded into the flow cell, where they were perfused at a slow flow rate (typically 10 $\mu\text{L s}^{-1}$). Next, the spectrometer baseline reading was zeroed (making the beads “invisible”) and data

collection began. A typical monitoring cycle comprised bead perfusion with 200 μL of PBS and injection of 40 μL of an eluant (0.1 M HCl) followed by 600 μL of PBS solution. The monitoring cycle was repeated ten times under control of the software loop script. At the end of the last loop the data collection was terminated, and the beads were purged from the flow cell by three high velocity bursts of carrier solution.

Spectra monitoring was carried out by data collection at 260 nm and 280 nm with the reference wavelength set at 700 nm. The remaining spectrometer channel was tuned to an appropriate wavelength for monitoring of proteins that exhibited absorption maximum in the visible region (such as cytochrome C monitored at 410 nm).

System calibration and response. Signal to background ratio and reproducible discrimination of background are the keys to successful monitoring of protein immobilization on agarose beads. Agarose consists of linear polysaccharide chains that are purposely cross linked to provide mechanical stability and a desired porous structure. Depending on the degree of cross-linking, typical agarose beads contain up to 80% water and the pores between cross linked chains are large enough to allow proteins with molecular weight up to 1 MDa to penetrate, while small molecules move freely through the network. Agarose beads are available from numerous commercial sources, as Sepharose 4B and Sepharose 6B, that is 4% and 6% cross-linked agarose, originally designed and marketed by Pharmacia (Uppsala, Sweden). The absorbance of agarose beads depends on the degree of cross-linking and decreases with increasing wavelength. The absorbance of proteins in their native forms varies widely, mainly depending on the content of tryptophan and tyrosine as well as, to a lesser degree, the content of phenylalanine and histidine.

Since the monitoring of bovine serum albumin (BSA) and of antibodies is, for practical reasons, often carried out at 260 nm and 280 nm¹⁶ these wavelengths were also chosen in this work. In order to quantify the amount of protein actually immobilized on beads, the instrument must be calibrated by protein solutions of known concentrations. First, the minimum volume of sample required to fill the flow cell with undiluted protein solution, when injected into the lab-on-valve (LOV) module, had to be determined. Since the LOV is operated by programmable flow in sequential injection mode, the injected sample plug travels from the injection port (Fig. 1. port #5) first into the holding coil and then, following flow reversal, through the flow cell (volume of 4 μL). During this transport, the front end of the injected zone is diluted by carrier solution, contained in the channel between port #5 and the flow cell (10 μL volume). By injecting increasing volumes of protein solution (6.0 $\mu\text{g } \mu\text{L}^{-1}$ of BSA) a series of typical response curves is obtained (Fig. 2, top), showing increasing peak height until the “steady state” response is obtained. This is the *minimum* sample volume that will fill the flow cell with sample solution *undiluted* by the carrier solution.¹² (It is defined as $D = 1$, the dispersion coefficient being $D = C^{\text{max}}/C^0$ where C^{max} is the concentration at peak maximum and C^0 is the concentration of injected analyte). For the LOV system, the minimum sample volume to reach a plateau is 100 μL , yielding an absorbance at 260 nm of 0.082 for BSA. Note that any *larger* volume would yield the same absorbance. Using this

Table 2 Software protocol for monitoring ligand leakage

Fill Syringe	
Valve waste	Loop start: 10 (#)
Syringe pump valve in	
Syringe pump flowrate:	Refill Syringe
500 ($\mu\text{L s}^{-1}$)	
Syringe pump aspirate: 300 (μL)	Syringe pump valve in
Syringe pump delay until done	Syringe pump flowrate:
	500 ($\mu\text{L s}^{-1}$)
Syringe pump valve out	Syringe pump fill
Syringe pump delay until done	Syringe pump delay until done
Valve flowcell	
Syringe pump dispense:	Mobile phase (PBS)
100 (μL)	
Syringe pump delay until done	Syringe pump valve out
	Syringe pump flowrate:
	5 ($\mu\text{L s}^{-1}$)
	Syringe pump dispense: 300 (μL)
	Syringe pump delay until done
Load & wash beads	
Valve sample	
Syringe pump flowrate:	
100 ($\mu\text{L s}^{-1}$)	
Syringe pump aspirate:	Eluant (HCl) followed by
100 (μL)	
Syringe pump delay until done	Mobile phase (PBS)
Valve flowcell	Valve eluant
Syringe pump flowrate:	Syringe pump flowrate:
40 ($\mu\text{L s}^{-1}$)	10 ($\mu\text{L s}^{-1}$)
Syringe pump dispense:	Syringe pump aspirate: 40 (μL)
200 (μL)	
Syringe pump delay until done	Syringe pump delay until done
Syringe pump flowrate:	Valve flowcell
5 ($\mu\text{L s}^{-1}$)	
	Syringe pump flowrate: 5 ($\mu\text{L s}^{-1}$)
Spectrometer scanning	Syringe pump empty
Spectrometer reference scan	Syringe pump delay until done
Spectrometer absorbance scanning	
Syringe pump flowrate:	Loop end
10 ($\mu\text{L s}^{-1}$)	
Syringe pump dispense:	
100 (μL)	
Syringe pump delay until done	Spectrometer stop scanning
Syringe pump empty	
Syringe pump delay until done	

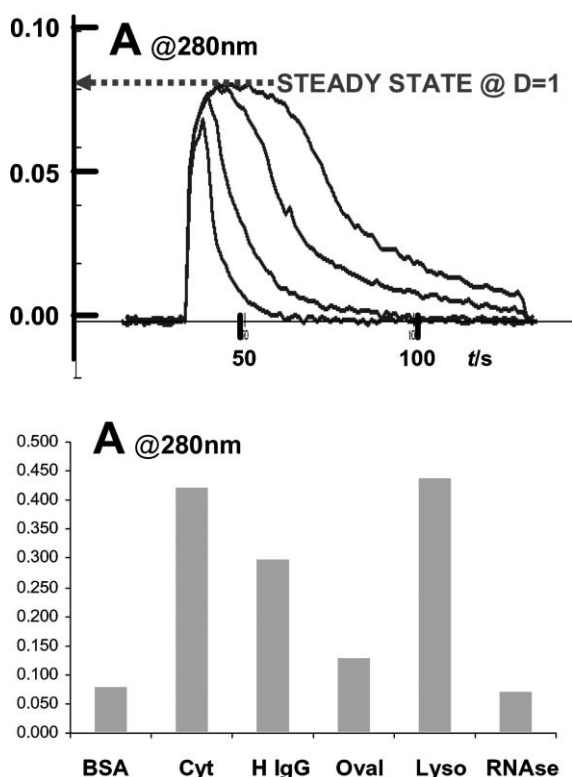


Fig. 2 Calibration of the LOV system. A: Response curves obtained by injecting 25, 50, 100, and 150 μL of bovine serum albumin (BSA, $6 \mu\text{g} \mu\text{L}^{-1}$) solution into the LOV system. Steady state response (undiluted sample solution) was obtained by injecting 100 μL , or larger, volumes of BSA solution that yielded an absorbance at 280 nm of 0.082; optical path: 1 mm. B: Absorbance values at 280 nm for protein solutions ($6.0 \mu\text{g} \mu\text{L}^{-1}$) obtained by injecting 100 μL of bovine serum albumin (BSA), cytochrome C (cyt), human serum albumin (HIgG), ovalbumin (oval), lysozyme (lyso), and ribonuclease A (RNase) into the LOV module.

approach, responses of the LOV system to solutions of BSA, ovalbumin, lysozyme, ribonuclease A, cytochrome C, and human IgG were calibrated (Fig. 2, bottom).

Results and discussion

Aminolink[®] coupling gel is comprised of cross-linked agarose beads, activated to contain a high density of aldehyde groups that react with amine-containing ligands by reductive amination. The first step of reductive amination is the formation of a Schiff base between the amine and the aldehyde groups, a reaction that is believed to be reversible. Following this step, an agent such as sodium cyanoborohydride is used to reduce the labile Schiff base into a stable amide bond that anchors the target ligand irreversibly to the bead surface. Ligand coupling protocols, developed and used throughout the last 15 years,^{3–8} are quite lengthy as they prescribe equilibration of the reaction mixture for 2 hours, followed by 4 hours of storage at room temperature (or overnight storage in a cold room). The preferred reaction mixture for Aminolink[®] consists of 0.1 M phosphate buffer pH 7.0, containing 0.6% of NaCNBH_3 . It is said that reductive amination proceeds quite efficiently between pH 4 and pH 10³ and published tables state that

better than 90% immobilization yield is achieved in this pH range.^{3,6–8} In summary: “excellent yields... can be expected for most proteins, regardless of pH of the coupling reaction or the molecular weight or pI [isoelectric point] of the protein” -Hermanson, Mallia, and Smith (Ref. 4, p 73)

The rate of protein immobilization on Aminolink[®] beads was monitored by BIS using an assay protocol that comprised ten bead-sampling cycles (Table 1). The stirred microreactor, containing 200 μL of beads suspended in 1800 μL of appropriate buffer was first sampled twice, yielding two “rectangular peaks” or “bars” that were used to establish a blank absorbance value for beads without any immobilized protein. Next, *prior* to the third sampling cycle, 200 μL of protein solution ($60 \mu\text{g} \mu\text{L}^{-1}$ in water) were pipetted into the stirred suspension to begin the immobilization reaction and the monitoring continued for the next eight bead sampling cycles. (Fig. 3–5). The unusual peak shapes or “bars” are due to the sequence of (1) rapid loading of beads into the flow cell; (2) perfusion of packed beads by the carrier stream ($400 \mu\text{L} @ 10 \mu\text{L} \text{s}^{-1}$); (3) instant discharge of beads from the flow cell by a burst of carrier ($400 \mu\text{L} @ 400 \mu\text{L} \text{s}^{-1}$). The shape of the

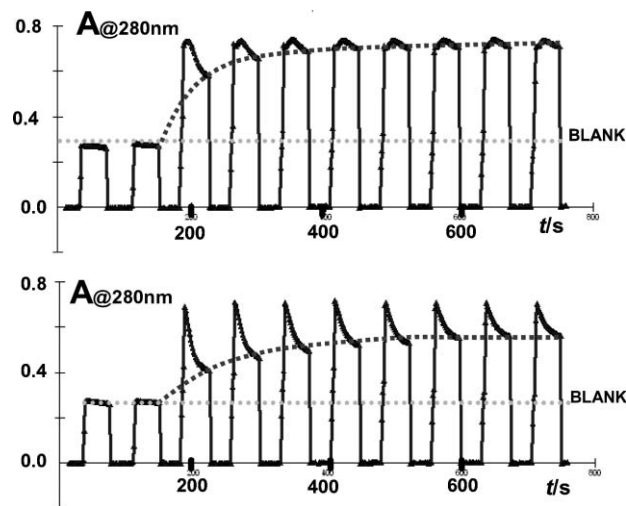


Fig. 3 Influence of pH on the rate of immobilization of lysozyme. Reaction mixture: 1800 μL of buffer containing $6.0 \mu\text{g} \mu\text{L}^{-1}$ lysozyme, was equilibrated with 200 μL of Aminolink[®] beads. Top: pH 10.0; bottom: pH 9.2. For protocol details see text and Table 1.

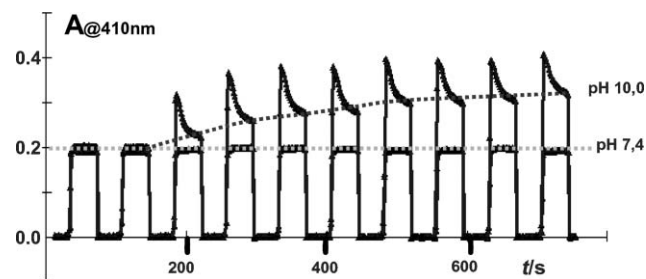


Fig. 4 Influence of pH on the rate of immobilization of cytochrome C. Reaction mixture: 1800 μL of buffer containing $6.0 \mu\text{g} \mu\text{L}^{-1}$ cytochrome C was equilibrated with 200 μL of Aminolink[®] beads. Monitored runs are superimposed to show experimental reproducibility and emphasize influence of pH. For protocol details see text and Table 1.

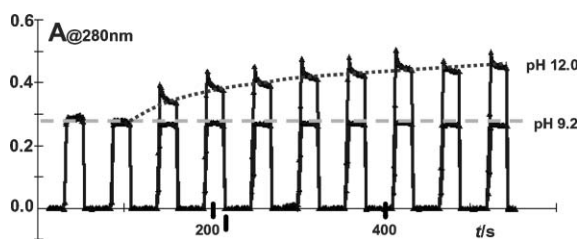


Fig. 5 Influence of pH on the rate of immobilization of bovine serum albumin. Reaction mixture: 1800 μL of buffer containing $6.0 \mu\text{g} \mu\text{L}^{-1}$ BSA was equilibrated with 200 μL of Aminolink[®] beads. Note the difference in pH values compared to Fig. 4 and 5. For protocol details see text and Table 1.

rather unusual “peaked bars” (Fig. 3 bottom and Fig. 4 pH 10) is typical for monitoring of the reaction mixture, where only *part* of the protein has been immobilized. Thus, when an aliquot of a reaction suspension is sampled into the LOV, protein in the solution phase travels through the flow cell resulting in a peak. The plateau at the end of the “peaked bar” corresponds to protein immobilized on the solid phase. Note the stability of baseline and excellent repeatability of all measurement cycles. Response curves for lysozyme (Fig. 3), cytochrome C (Fig. 4) and BSA (Fig. 5) show marked

differences in immobilization rates and immobilized amounts as well as the influence of pH. For lysozyme, at pH 10, the reaction equilibrium has been reached within 5 minutes (Fig. 3, top), while at pH 9.2 (Fig. 3, bottom) the immobilization rate was slower and the yield markedly lower. For BSA, the protein was not immobilized at pH 9.2 and only at pH 12 was BSA gradually captured on the beads.

Immobilization rates for six proteins were monitored using the above procedure, while reaction mixtures were adjusted to pH 7.4, 8.0, 9.2, 10.0, 11.0 and 12.0, (Fig. 6) along with “equilibrium” absorbance values that were obtained by monitoring these reaction mixtures after overnight storage at room temperature. (For equilibrium measurements, the software protocol summarized in Table 1 was used, albeit with only 3 sampling cycles). It is important to note that these immobilization rate curves were obtained with high protein concentrations ($6.0 \mu\text{g} \mu\text{L}^{-1}$), so that the total amount of protein in the reaction mixture (1.2 mg) far exceeded the binding capacity of reactive sites on beads (about 200 μg total). The purpose of using such a large excess of protein, was to choose conditions where the reaction rate would be controlled by pH and the reactivity of individual proteins, not the binding capacity of the solid support or by the diffusion of proteins to binding sites.

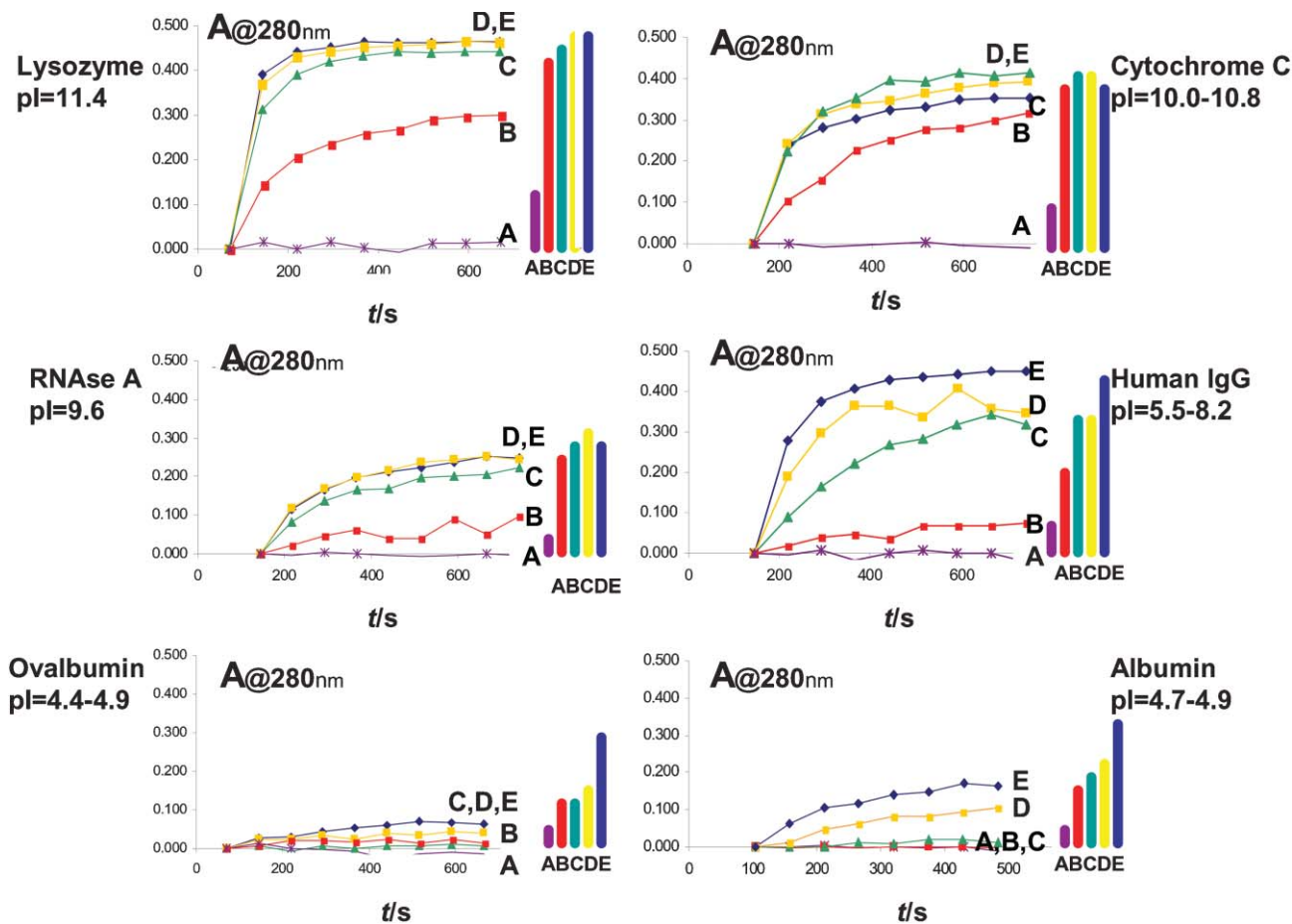


Fig. 6 Summary of immobilization rates and equilibrium yields. Composition of reaction mixture: 1800 μL of buffer containing $6.0 \mu\text{g} \mu\text{L}^{-1}$ of protein equilibrated with 200 μL of Aminolink[®] beads at the following pH values: A, 7.4; B, 9.2; C, 10.0; D, 11.0; E, 12.0. Bars at the right show bead absorbance values obtained after overnight equilibration.

In order to evaluate the immobilization efficiency at conditions closer to traditional immobilization protocols, protein concentrations were lowered to $2.0 \mu\text{g } \mu\text{L}^{-1}$ and the reaction mixture was allowed to react for 24 hours at room temperature. For comparison, Pierce Aminolink[®] kit (phosphate buffer, pH 7.4), Pierce Aminolink[®] Plus kit (citrate buffer, pH 10) and Aminolink[®] gel in borax buffer (pH 10) were used. Pierce immobilization protocols were meticulously followed and reagents supplied with kits were used exactly as prescribed. Equilibrated reaction mixtures were monitored in the same way as described above, and blank bead values were subtracted from immobilized protein absorbances, as indicated by the solid arrows in Fig. 7 (top). Note that response bars for Aminolink[®] Plus blank beads have absorbances almost 30% higher than for Aminolink[®] beads that are 4% cross-linked. This agrees with the manufacturer's statement that Aminolink[®] Plus beads are more cross-linked, indicating the use of Sephadex 6B with 6% cross-linking. In summary, it

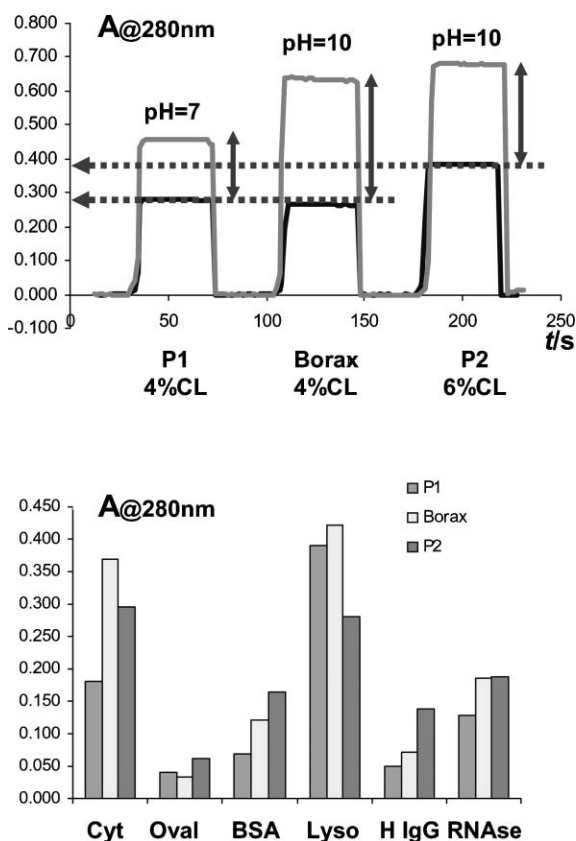


Fig. 7 Immobilization yield at equilibrium for different immobilization protocols. P1, Aminolink[®] kit by Pierce (pH 7.2); Borax, Aminolink[®] beads in borax buffer (pH 10); P2, Aminolink[®] Plus kit by Pierce (pH 10). All proteins, at $2 \mu\text{g } \mu\text{L}^{-1}$ in $1800 \mu\text{L}$ of buffer, were equilibrated for 2 hr with $200 \mu\text{L}$ of beads. Top: superimposed monitoring responses of blank beads and beads equilibrated with cytochrome C. Note the higher blank of 6% cross-linked Aminolink[®] Plus beads (P2) compared to 4% Aminolink[®] beads (P1 and borax). Bottom: yield at equilibrium after bead blank values have been subtracted. Symbols: cyt, cytochrome C; oval, ovalbumin; BSA, bovine serum albumin; lyso, lysozyme; H IgG, human IgG; RNase, ribonuclease A.

follows (Fig. 7, bottom) that the yield of immobilization for various proteins is chiefly influenced by pH. The improved performance of Aminolink[®] Plus is only due to the use of higher pH (citrate buffer, pH 10) and does not differ substantially from immobilization yields obtained with Aminolink[®] at pH 10 (borate buffer). Obviously, higher cross linking does not improve the immobilization yield, although lower yield may also be result of lower binding capacity of Aminolink[®] Plus beads (the total amount of protein in the reaction mixture (0.4 mg) exceeded the capacity of the beads). The immobilization levels obtained at pH 7.4 are somewhat higher than those reported in Fig. 6, probably due to the longer equilibration time.

In order to express the immobilization capacity and immobilization rates in amounts of proteins, rather than in absorbance values, the absorbance data collected has to be corrected by the molar absorptivities of individual proteins, or, more practically, by calibration absorbance values for individual proteins as summarized in Fig. 2. However, an assumption has to be made: namely, that the response, at any given wavelength, for these biomolecules immobilized on agarose follows the Lambert–Beer law in the same way as when in solution. To test the validity of such an assumption, the following experiment was carried out. A series of reaction mixtures were prepared using borax buffer (pH 10) and $200 \mu\text{L}$ of Aminolink[®] bead suspension in such a way that, after the blank beads test (zero protein), the concentration of protein was doubled in each successive reaction mixture. Using the absorbance values summarized in Fig. 2 as a guide, the concentration ranges of cytochrome C, ribonuclease A, lysozyme, BSA, Human IgG, and ovalbumin were selected to yield comparable absorbance values. The reaction mixtures were equilibrated for 24 hours at room temperature before $700 \mu\text{L}$ of supernatant was carefully pipetted off from the sedimented reaction mixture. Both the supernatants and remaining reaction mixtures were monitored by sampling each supernatant or reaction mixture two times (Fig. 8 and 9) using the software protocol with ten cycles (Table 1). For these experiments, the sample vials were manually exchanged after two sampling cycles. In addition, calibrant solutions that simulated initial concentration of proteins in reaction mixture *prior* to immobilization were prepared and monitored by injecting $100 \mu\text{L}$ of these solutions into the LOV module. In this way, absorbances of protein solutions *prior* to equilibration and *after* equilibration with beads (supernatants) were measured in exactly the same way, yielding the absorbance values of proteins *removed* from solution by immobilization (Table 3). This allowed comparison of the absorbance of proteins immobilized on beads with the absorbance of protein removed from the solutions for a range of concentrations and for proteins monitored at various wavelengths (Table 3 and Fig. 10). Note that the light path for all measurements was exactly the same (1.0 mm), that the ratio of bead to solution volume was the same, and that all proteins were immobilized at equilibrium and at the same highest practical pH (pH 10).

It follows from Table 3 and Fig. 10 that immobilization yield decreases from 100% for the lowest concentration of cytochrome C to 1% for lowest concentration of ovalbumin

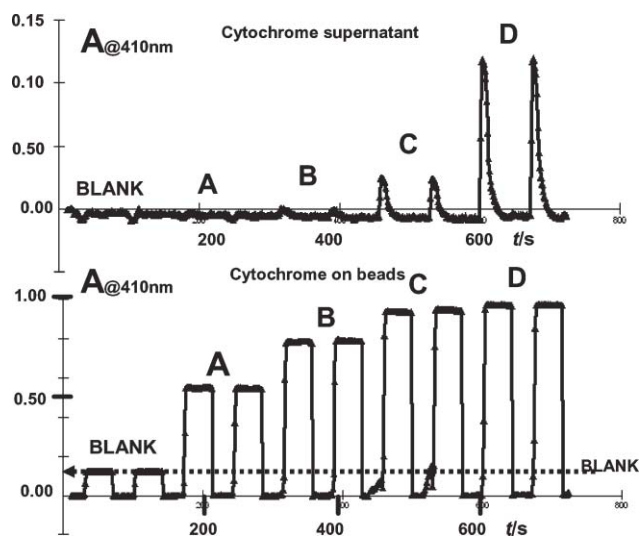


Fig. 8 Absorbance values of supernatant (top) and of beads (bottom) reflect immobilization yield for cytochrome C. All values were measured at equilibrium; all samples were monitored in duplicate. Composition of reaction mixture: 1800 μL of borax buffer (pH 10) was equilibrated with 200 μL of Aminolink[®] beads. Cytochrome C concentrations: blank, 0 $\mu\text{g } \mu\text{L}^{-1}$; A, 0.125 $\mu\text{g } \mu\text{L}^{-1}$; B, 0.250 $\mu\text{g } \mu\text{L}^{-1}$; C, 0.50 $\mu\text{g } \mu\text{L}^{-1}$; D, 1.0 $\mu\text{g } \mu\text{L}^{-1}$. (For evaluation, see Table 3.)

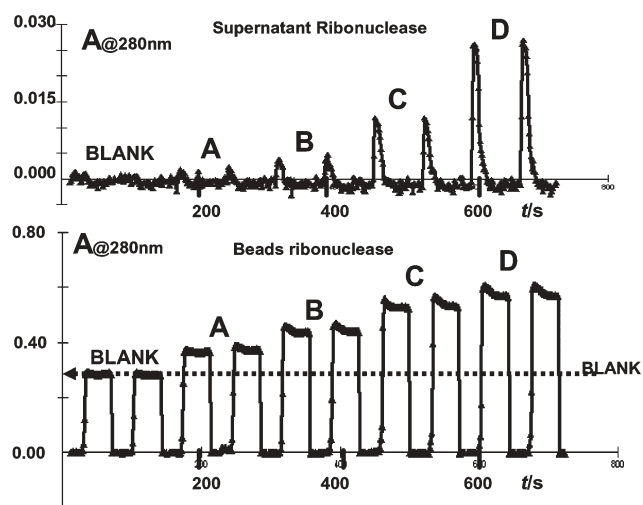


Fig. 9 Absorbance values of supernatant (top) and in beads (bottom) reflect immobilization yield for ribonuclease A. All values were measured at equilibrium; all samples were monitored in duplicate. Composition of reaction mixture: 1800 μL of borax buffer (pH 10) was equilibrated with 200 μL of Aminolink[®] beads. Ribonuclease A concentrations: blank, 0 $\mu\text{g } \mu\text{L}^{-1}$; A, 0.29 $\mu\text{g } \mu\text{L}^{-1}$; B, 0.57 $\mu\text{g } \mu\text{L}^{-1}$; C, 1.15 $\mu\text{g } \mu\text{L}^{-1}$; D, 2.30 $\mu\text{g } \mu\text{L}^{-1}$. (For evaluation, see Table 3.)

Table 3 Comparison of protein absorbance in solution vs. immobilized on beads

Protein	[Protein]/ $\mu\text{g } \mu\text{L}^{-1}$	Abs	Abs	Abs	Abs	Abs	% immobilized
	Initial solution	Initial solution	Supernatant	Init – super	Beads	Ratio ^a	init – super / initial
Cyt C @410 nm	1.00	0.278	0.123	0.155	0.85	3.21	56
	0.50	0.139	0.029	0.110	0.82	1.79	79
	0.25	0.070	0.004	0.065	0.66	1.30	94
	0.13	0.035	0.000	0.035	0.42	1.08	100
RNase @280 nm	2.30	0.047	0.024	0.023	0.28	1.05	48
	1.15	0.023	0.011	0.012	0.25	0.66	53
	0.57	0.012	0.003	0.008	0.15	0.71	71
	0.29	0.006	0.001	0.005	0.09	0.75	83
Lyso @260 nm	0.50	0.062	0.017	0.045	0.36	1.64	73
	0.25	0.031	0.009	0.022	0.28	1.07	72
	0.13	0.015	0.003	0.012	0.18	0.91	79
	0.06	0.008	0.002	0.006	0.11	0.76	78
IgG @280 nm	1.20	0.072	0.053	0.019	0.25	1.00	27
	0.60	0.036	0.027	0.009	0.17	0.74	26
	0.30	0.018	0.013	0.005	0.10	0.62	26
	0.15	0.009	0.007	0.002	0.06	0.46	22
BSA @280 nm	8.00	0.124	0.111	0.013	0.25	0.68	10
	4.00	0.062	0.056	0.007	0.21	0.41	10
	2.00	0.031	0.027	0.004	0.14	0.37	13
	1.00	0.016	0.015	0.001	0.10	0.09	4
Oval @280 nm	12.0	0.206	0.193	0.013	0.13	1.30	6
	6.00	0.103	0.101	0.003	0.08	0.41	3
	3.00	0.052	0.050	0.001	0.06	0.29	3
	1.00	0.026	0.025	0.000	0.05	0.11	1

^a Note: Abs ratio is the ratio of the Abs of protein removed from solution (Abs) to the Abs of protein on the beads, times a factor of 13.2. The factor takes into account a 200 L aliquot of beads, with 66% packing efficiency, and a preconcentration factor of 10, which results from the protein in 2000 L of solution being captured on 200 L of beads.

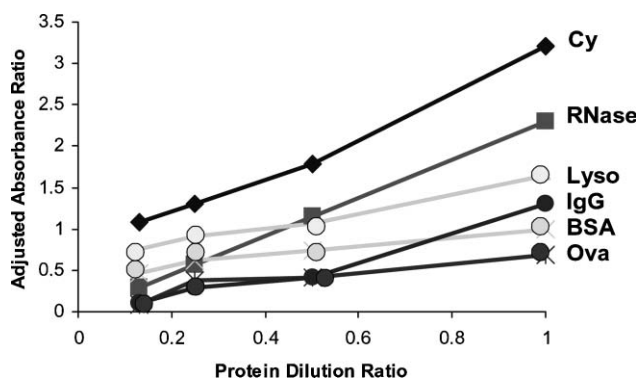


Fig. 10 Absorbance ratios as a function of protein dilution ratio. The graph summarizes the ratio of the absorbance of protein removed from solution to the absorbance of protein immobilized on beads *versus* protein dilution in solution (see Table 3). The data confirm that the absorbance monitored on beads is proportional to the amount of protein immobilized on beads. Cy, cytochrome C; RNase, ribonuclease A; Lyso, lysozyme; IgG, human IgG; BSA, bovine serum albumin; Ova, ovalbumin.

and it is dependent on the pI and concentration of the proteins. The absorbances measured on beads, for all proteins and wavelengths, show the same trend. As the protein concentration in each mixture increases exponentially, the absorbance of protein removed from solution decreases in a linear fashion when measured in bead phase (Fig. 10). Therefore, there is not a linear relationship between absorbances measured in solution and on the beads. Note that this observation has not been influenced by the binding capacity of the beads, since the graph shows the ratio of absorbances *removed* from solution to absorbances measured on the beads. Since light loss by absorbance and light scattering are quite different when measuring a bead-filled flow cell compared to a solution-filled flow cell, it might be possible to alleviate this problem by optimizing the light output and spectrophotometer settings. In spite of this drawback, the monitoring of bead absorbance proved to be a convenient and reliable tool for monitoring of protein immobilization since bead absorbances increase with the amount of immobilized protein (Table 3, columns 6 and 7).

Because immobilized ligands are almost always used repeatedly, their useful lifetime will be affected by the rate of leakage. The rate of ligand leaking is usually determined by means of radiolabelled,² or fluorescence tagged ligands, with subsequent measurement of their content in collected eluates. Since BIS in *flow-through* mode has been applied, in the past, to the monitoring of protein capture and elution,^{9–12} it was easily adopted here for the monitoring of ligand leakage by designing a monitoring loop that simulates a chromatographic protocol by perfusing a bead layer, trapped in the flow cell, with a mobile phase (Table 2). Elution by 0.1 M hydrochloric acid was purposely chosen to simulate the harshest possible elution conditions and the monitoring cycle was repeated ten times in order to simulate repeated use of immobilized protein. Since the leakage monitoring experiment requires as little as 10 μL of packed beads, it was feasible to test all materials produced in the course of this investigation. Representative examples of the monitoring of ligand leakage from Aminolink[®] support, prepared by various protocols and at reaction equilibrium using several proteins, are shown in Fig. 11. Leakage rates are depicted by absorbances at 280 nm, allowing the mass of lost protein to be estimated from data summarized in Table 3. Ribonuclease A immobilized at pH 10 from a solution initially containing $2.3 \mu\text{g} \mu\text{L}^{-1}$ RNase (initial absorbance value of 0.28) shows a decrease after 10 elution cycles (Fig. 11) and remains under the detection limit, $\text{Abs} = 0.003$. This signal represents less than 1% loss due to ligand leakage. Indeed, leakage of immobilized ligands from Aminolink[®] beads was found to be at that very low level for *all* investigated proteins and immobilization procedures, confirming an excellent quality of this support. It was surprising, however, to find that the use of NaCNBH_3 had no positive effect on leakage rate. Actually, monitoring of proteins, such as BSA in Fig. 11, showed a slight *increase* in absorbance during the ligand leakage monitoring sequence. A possible explanation for this anomaly is that NaCNBH_3 makes the agarose beads more elastic, so that beads gradually become more densely packed into the flow cell. This effect is, of course, far too small to have any practical consequences. Yet, the surprising result of this finding is that the use of the highly toxic NaCNBH_3 appears to be quite unnecessary.

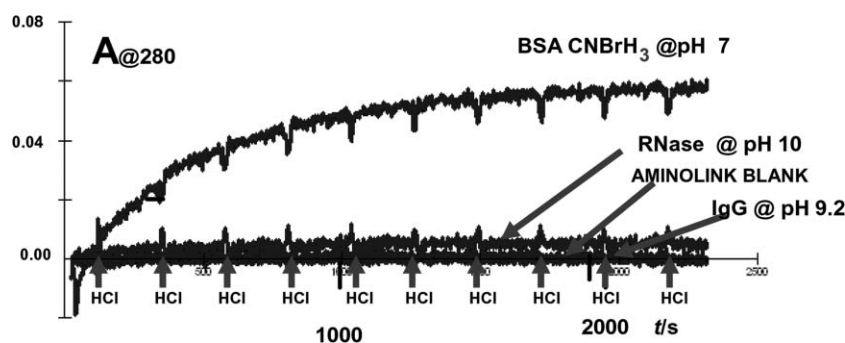


Fig. 11 Ligand loss of proteins immobilized on beads and captured in the flow cell monitored over ten elution cycles. Mobile phase: PBS; eluant: 40 μL of 0.1 M HCl injected ten times. BSA, bovine serum albumin immobilized at pH 7 according to Aminolink[®] protocol using NaCNBH_3 . RNase immobilized at pH 10, IgG immobilized at pH 9.2, both without using NaCNBH_3 . Aminolink blank, Aminolink[®] beads without any protein. Note that the absorbance scale is zeroed after loading a bead column, resulting in an initial reading of zero for each experiment. (Protocol: Table 2)

Conclusion

There are two aspects of this work: reporting a novel approach to real time monitoring of protein immobilization by Bead Injection Spectroscopy and the surprising findings that this technique has allowed us to discover. In contrast to established protocols, accepted for a number of years,³⁻⁸ it was found that the rate and amount of immobilized protein depends on pH and on protein pI. Furthermore, with some proteins, such as lysozyme, cytochrome C and human IgG, immobilization equilibrium is reached in alkaline solutions within 10 minutes rather than on a scale of several hours. While literature data suggest that almost 90% immobilization yield can be achieved at pH 4–7,³⁻⁸ the response curves obtained by BIS monitoring (Fig. 4) show that below pH 7 almost no protein is being immobilized, and for proteins with pI < 5 alkaline solutions have to be used. It should be reiterated that data in Fig. 3 and 4 were obtained with a large excess of protein compared to available bead capacity in order that pH will be the limiting factor influencing the rate of immobilization. In real life applications, however, much lower concentrations, of often rare proteins, need to be immobilized. In such a scenario, the negative effect of low pH values will be even more pronounced (Table 3 last column). One can only speculate how many unsuccessful attempts to immobilize proteins on agarose or other supports, including biosensing surfaces, were due to the use of a media with too low pH. As to the evaluation of commercial immobilization protocols, we strived for the fairest possible evaluation by choosing citrate buffer (pH 10) from Aminolink[®] Plus kit rather than phosphate buffer (pH 7) that is also included in the package. Results confirm (Fig. 6 and 7) that, while Aminolink[®] is indeed an excellent reactive support, pH is the main factor influencing the rate and yield of protein immobilization and that the use of NaCNBH₃ has no impact on the yield or stability of immobilized protein.

Periodic sampling of the reaction mixture containing suspended beads turned out to be a feasible and effective approach to monitoring the immobilization rate in real time. This simple approach is not only time and labor saving, but also very economical in terms of reagent consumption and waste generation. While the manual mapping of an immobilization procedure typically uses one bead column (2 mL of bead suspension) and several hours to obtain a single data point, an automated run that yields reaction rate at a chosen pH requires only 200 μ L of beads. The ligand leakage rate monitoring is even more economical. These attributes make BIS monitoring a valuable tool for optimization and investigation of immobilization protocols applicable to a wide range of immobilization chemistries.

Future work will focus on improvement of the way in which protein distribution between beads and supernatant can be

monitored simultaneously, and how the reaction mixture can be prepared automatically. For the first goal, the planned approach is to construct a LOV with two flow cells in series: the first one for monitoring the bead layer, the second one for monitoring the protein content of the supernatant solution. Next, the entire assay protocol will be automated; bead suspension will be metered automatically into the reaction mixture and protein solution will be automatically added at an appropriate time. This should eliminate irreproducibilities associated with manual pipetting of bead suspension.

Finally, a note for flow injection enthusiasts: while this work introduces new features, “bars” and “peaked bars” instead of usual peaks, we hope that it will also inspire applications far beyond monitoring of protein immobilization!

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