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Surface-Plasmon-Resonance-Based Chemical Proteomics: Efficient Specific Extraction and Semiquantitative Identification of Cyclic Nucleotide-Binding Proteins from Cellular Lysates by Using a Combination of Surface Plasmon Resonance, Sequential Elution and Liquid Chromatography–Tandem Mass Spectrometry

Natasja F. C. Visser, Arjen Scholten, Robert H. H. van den Heuvel, and Albert J. R. Heck*[a]

Chemical proteomics is a powerful methodology for identifying the cellular targets of small molecules, however, it is biased towards abundant proteins. Therefore, quantitative strategies are needed to distinguish between specific and nonspecific interactions. Here, we explore the potential of the combination of surface plasmon resonance (SPR) coupled to liquid chromatography–tandem mass spectrometry (LC-MS/MS) as an alternative approach in chemical proteomics. We coupled cGMP molecules to the SPR chip, and monitored the binding and dissociation of proteins from a human lysate by using sequential elution steps and SPR. The eluted proteins were subsequently identified by LC-MS/MS. Our approach enabled the efficient and selective extraction of low-abundant cyclic-nucleotide-binding proteins such as cGMP-dependent protein kinase, and a quantitative assessment of the less- and nonspecific competitive binding proteins. The data show that SPR-based chemical proteomics is a promising alternative for the efficient specific extraction and quantitative identification of small-molecule-binding proteins from complex mixtures.

Introduction

Affinity chromatography or chemical proteomics offers a way of identifying the targets of small molecules, such as signalling molecules or enzyme inhibitors, that is complementary to the in vitro measurements of affinities. In this approach, the small molecule is linked to a solid support and is used to enrich the cellular binding proteins, which are then typically identified by mass spectrometry. Chemical proteomics, for instance, has been successfully used to identify the unexpected cellular targets of kinase inhibitors such as SB203580, which was shown to inhibit RICK more potently than its known target, p38α[1] and to identify potential competitive targets of gefitinib.[2] A limitation of affinity chromatography, however, is that it is biased towards more abundant proteins. Proteins that are enriched for encompass the sought-after low-abundant proteins with a high affinity for small molecules, but also the more abundant proteins with a lower affinity for the molecule, and worse, highly abundant proteins that have affinity for the solid support matrix. Therefore, in chemical proteomics, strategies are needed to distinguish between specific and less-, or nonspecific binders. In one such an approach, differential stable isotope labelling was combined with modification-based affinity purification to follow the EGFR phosphotyrosine proteome in-time,[3,4] in nonstimulated and stimulated cells. Only when the detected isotope ratio of the retrieved peptide–ion pairs from both cell lysates was higher than 1.5, was an extracted protein categorized as specific.

Yamamoto et al.[5] investigated nonspecific and specific binding by performing sequential pull-down experiments on the same protein pool. By comparing the first and second pull-down, selective, low-abundant binders, which were mainly

[a] *Dr. N. F. C. Visser, Dr. A. Scholten, Dr. R. H. H. van den Heuvel, Prof. Dr. A. J. R. Heck*
Department of Biomolecular Mass Spectrometry
Bijvoet Center for Biomolecular Research and
Utrecht Institute for Pharmaceutical Sciences, Utrecht University
Sorbonnelaan 16, 3584 CA Utrecht (The Netherlands)
Fax: (+31) 30-2518219
E-mail: a.j.r.heck@chem.uu.nl

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extracted in the first pull-down due to their high binding constant could be distinguished from the less-specific, more abundant binding proteins, which were retrieved in all pull-down steps. A single pull-down can also be combined with sequential elution steps, which forms a precleaning procedure during which non- and less-specific proteins are dissociated by displacement while the specifically bound proteins remain on the beads.[6]

An alternative tool, which has not been extensively explored yet in the field of chemical proteomics is the combination of surface plasmon resonance (SPR) with mass spectrometry (MS). SPR can be used as an affinity-based extraction technique in which the extraction process can be followed in real time, without the need to label proteins.[7–10] More importantly, SPR provides quantitative information about the amount of material bound to the sensor. When SPR is combined with MS, quantitative and qualitative information can be combined, and a powerful technique is obtained that is suitable for the determination of intact proteins[11–14] as well as digests.[15–18] Although beads and SPR generally result in comparable extraction,[7,9,17] it has been hypothesized that the nonspecific binding on SPR discs is reduced due to the hydrophilic and nonporous characteristics of the sensor, and the high level of functional groups attached.[17]

Similar to other affinity purifications, nonspecific binding is a major challenge when more complex protein sources like cell lysate or plasma are presented to the SPR sensor.[12,15,19] Although different approaches have been used to reduce the nonspecific binding in SPR, including various surface chemistries,[19] and more homogeneous immobilization of the ligand,[10,20] extensive washing of the sensor surface is the most common approach.[8] To distinguish between more and less-specific binding components Roman et al.[21] collected and fractionated the eluent during dissociation. The components retrieved in the first fractions had a lower affinity for the immobilized "bait" than the components found in the later fractions. As an alternative approach, SPR with sequential elution steps can be used, which results in both the reduction as well as the characterization of nonspecific and less-specific binding proteins.

We previously introduced a sequential elution methodology for the specific extraction of cGMP (cyclic guanosine monophosphate)-binding proteins from a HEK293 cell lysate. Cyclic nucleotides such as cAMP (cyclic adenosine monophosphate) and cGMP are intracellular second messenger molecules that play an important role in key physiological responses such as cardiac contractility, transcriptional regulation, proliferation and differentiation.[6,22] The main intracellular target of cGMP is cGMP-dependent protein kinase (PKG), but it is also known to regulate phosphodiesterases and cyclic-nucleotide-gated cation channels.[22]

PKG is a homodimeric protein.[23] Each 76 kDa monomer contains a catalytic domain, with a Mg^{2+}/ATP binding pocket and the substrate recognition site, a regulatory domain, which harbours two tandem cGMP binding pockets, and the autoinhibitory domain.[24] When cGMP binds to the regulatory domain, the conformation of the PKG dimer changes, which leads to kinase activity.[25] A related kinase, cAMP-dependent protein kinase (PKA) shows similar features, however, its regulatory and catalytic domains reside on separate polypeptides. The second messenger that activates PKA is cAMP. Although PKA preferentially binds cAMP over cGMP with a 50-fold selectivity and PKG binds cGMP, rather than cAMP with a more than 100-fold selectivity, cross reactivity has been reported.[26,27] Next to direct binding, the indirect binding of proteins to cGMP or cAMP can also occur, for example, A-Kinase-anchoring proteins (AKAPs) bind via the regulatory domain of PKA to cAMP.[28] Although a few examples have been reported,[29,30] the indirect binding of proteins via PKG to cGMP, that is, G-kinase-anchoring proteins (GKAPs), have been less well defined.

In this report, we explore the potential of the coupling of SPR and LC-MS/MS as an alternative approach in chemical proteomics, by using the cGMP secondary signalling molecule as bait. Our data show that SPR–MS can be used as a qualitative and semi-quantitative tool for the identification of small-molecule-binding proteins directly from cellular lysates. The approach allows for the differentiation between nonspecific, less-specific and specific binding, as well as a reduction in the bias towards highly abundant, less-specific binding proteins.

Results and Discussion

Optimization of the cGMP–SPR sensor for PKG binding

For our chemical proteomics experiments, we designed a SPR sensor (Figure 1A) with commercially available, chemically modified cGMP (8-(2-aminoethyl)thio-cGMP (8AET-cGMP), see Figure 1C). Unfortunately, direct binding of this modified cGMP did not result in a sensor that was active towards PKG, probably because the distance between the modified cGMP and the sensor surface was too short, which prohibited PKG binding (data not shown). A PKG-active SPR sensor was obtained when this distance was increased using a six-carbon spacer molecule, 6-aminohexanoic acid (Figure 1B) to couple the cGMP to the sensor surface. During the two-step immobilization, an angle shift corresponding to the immobilization of 7.8 ng (18.6 pmol) 8AET-cGMP was obtained. A similar amount of the spacer molecule was immobilized (see the Supporting Information) in the first immobilization step, which shows the high efficiency of the two-step immobilization. In the Experimental Section, the conversion of the angle shift to the corresponding immobilized or bound quantity is more elaborately described.

First we tested the responsiveness of the cGMP–SPR sensor towards PKG by the injection of recombinant PKG. At PKG concentrations between 0.5 and 10 $\mu g\,mL^{-1}$, a linear SPR response was obtained ($y = 10.5x - 1.70$, $R^2 = 0.994$), with a limit of detection of 1 $\mu g\,mL^{-1}$ when 75 µL sample was injected. This corresponds to 6 fmol of PKG_2 being bound to the SPR sensor. When a MALDI-MS identification was performed after the elution of the PKG with sodium hydroxide, the limit of detection increased to 5 $\mu g\,mL^{-1}$, which corresponds to 25 fmol being bound to the sensor surface. As nonspecific binding is the main challenge in affinity extraction, the selectivity of the cGMP–SPR sensor towards PKG was tested through a competi-

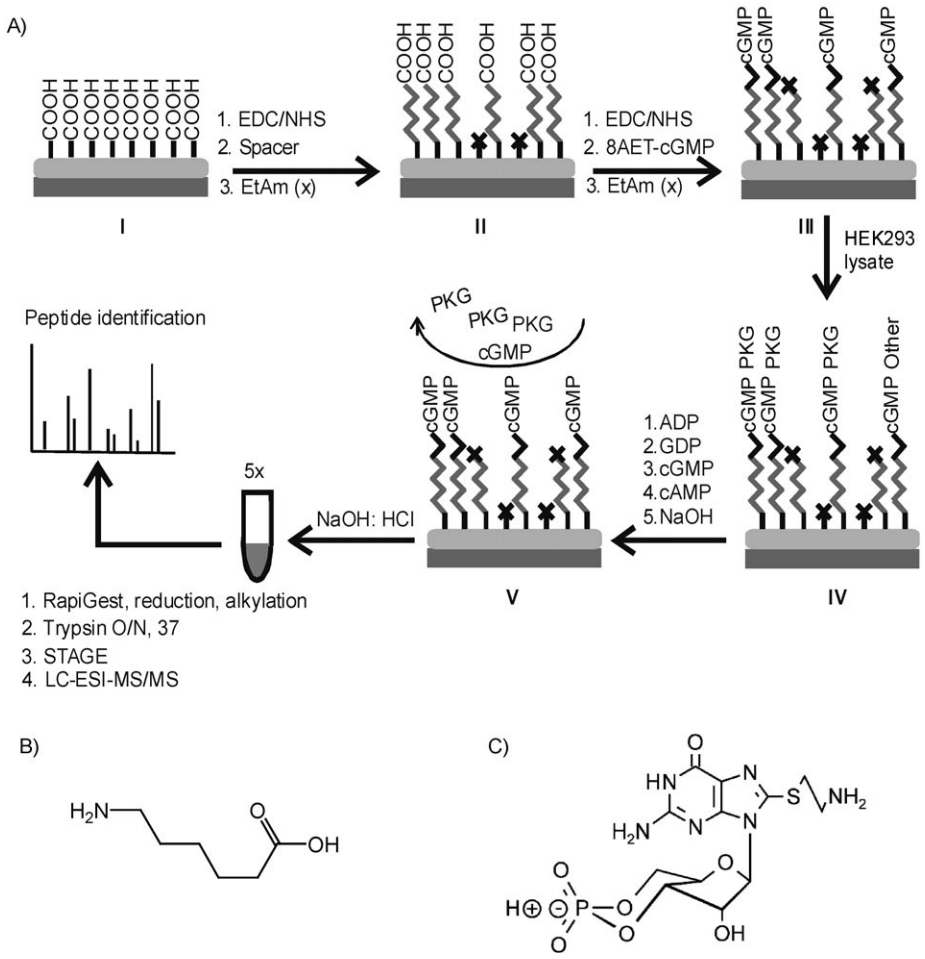

Figure 1. A) Schematic set-up of the SPR–MS approach. This approach can be divided in several steps, I–V. In I, the activated hydrogel is depicted onto which, through a two-step immobilization procedure using a spacer molecule (II), 8AET-cGMP was immobilized (III). After immobilization of the sample, a HEK293 lysate was injected, and cGMP proteins were captured on the sensor surface (IV). By using a five-step sequential elution, proteins were dissociated from the sensor (V). The collected fractions were digested in solution, and were measured by using LC-MS/MS. The structure of the immobilized spacer molecule, 6-aminohexanoic acid, from step II, and the molecular structure of 8AET-cGMP are depicted in (B) and (C), respectively.

tion experiment with free cGMP. The results (see Table 1) indicated that the interaction between the dimeric PKG_2 and the sensor disc was highly selective. At a dimeric PKG_2/cGMP molar ratio of 1:1, merely 8% of the maximal binding remained. Even when the amount of added cGMP was only 20% of the amount of dimeric PKG_2, the binding of the sensor disc was reduced to 20% of the binding of PKG_2 without cGMP. At first this might be an unexpected result. However, the binding of a single cGMP might already prohibit the binding of dimeric PKG_2 to the SPR sensor because the binding of cGMP can result in changes in both the conformation and the surface electronegativity of dimeric PKG_2.[31] Also, the binding affinity of cGMP to the other unoccupied pockets is changed after the binding of the first cGMP. At a tenfold excess of cGMP the interaction between dimeric PKG_2 and the SPR sensor was completely prohibited.

Table 1. Titration of a dimeric PKG_2 sample with cGMP shows the selectivity of the interaction between the 8AET-cGMP immobilized sensor disc and dimeric PKG_2.

Molar ratio of dimeric PKG_2/cGMP	Angle shift [m°]	Angle shift (% of 1:0)
1:0	154.2	100
1:0.2	33.2	21.5
1:1	12.7	8.24
1:2	8.5	5.51
1:10	1.07	0.67
1:20	3.07	1.99

Next, the usability of the developed SPR surface for the selective extraction of PKG from a more complex sample, a cell lysate, was tested. First, a 1 mg mL^{-1} HEK293 cell lysate was spiked with 5 μg mL^{-1} of recombinant PKG, and the sensorgram depicted in Figure 2 was obtained. Then a HEK293 cell lysate was injected without the addition of recombinant PKG, and the second sensorgram of Figure 2 was observed. As expected, significant binding to the SPR sensor disc was observed again, which is indicative of proteins present in the HEK293 lysate binding to the sensor disc. To our surprise, the measured angle shift of the 5 μg mL^{-1} PKG-spiked HEK293 cell lysate was larger than the combined angle shifts of the unspiked 1 mg mL^{-1} HEK293cell lysate and the 5 μg mL^{-1} pure recombinant PKG solution in HBS-N (Figure 2). We hypothesize that PKG is more stable in an environment containing proteins and protease inhibitors than in HBS-N; this results in more of it binding to the surface. It is also possible that PKG binds to the sensor as part of a protein complex, which would also result in the higher SPR signals that were observed.

To identify the proteins other than PKG that are captured on the surface, the sodium hydroxide eluted fraction of the 1 mg mL^{-1} HEK293 cell lysate was digested with trypsin and amended for MALDI-MS identification. Five proteins could be identified: PKG, PKA, actin and nucleoside diphosphate kinases A and B. PKG and PKA are relatively low-abundant proteins in HEK293 cells, but have a high affinity for the 8AET-cGMP sensor. The two diphosphate kinases and actin have a higher abundance in HEK293 cells, and are known to bind primarily to GTP (kinases) and ATP (actin). Both GTP and ATP structures re-

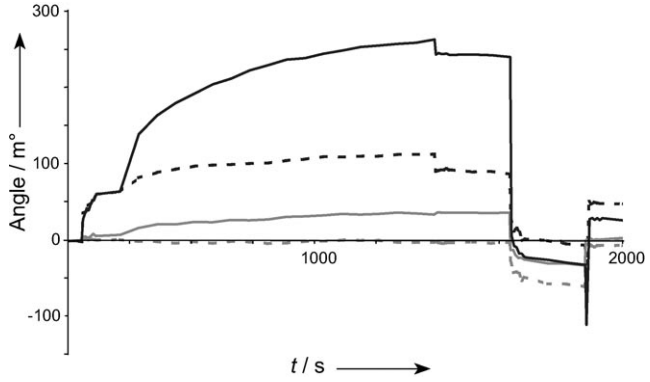

Figure 2. The SPR response gives an indication of the selectivity of the 8AET-cGMP sensor disc towards PKG. The SPR response of the 1 mg mL^{-1} HEK293 lysate (dotted black line) is lower than the SPR response of the lysate that had been spiked to 5 μg mL^{-1} with PKG (black line). The SPR response of a 5 μg mL^{-1} PKG solution in HBS-N (grey line) is depicted for comparison. The grey dotted line reveals that HBS-N does not contribute in any way to the SPR response.

semble cGMP to some extent, which might explain the binding of these high-abundant proteins to the cGMP sensor. It is likely that these proteins can be listed among the earlier mentioned high-abundant, less-specific proteins, which have some affinity for cGMP.

Specific, less-specific and nonspecific binding can be semi-quantitatively monitored and distinguished with sequential elutions

To obtain a better insight into the specificity of the proteins that bind to the sensor disc, five sequential elution steps were introduced by using solutions containing ADP, GDP, cGMP, cAMP and sodium hydroxide, respectively. ADP and GDP were expected to dissociate the more generic nucleotide-binding proteins, (see Figure 3, (black line)) while cGMP and cAMP were expected to dissociate the more specific cyclic-nucleotide-binding proteins from the sensor surface. The protein content of each of these eluates was measured by using LC-MS/MS.

Two consecutive injections were required to expose the SPR chip to a total of 2.89 mg protein from the HEK293 lysate, which should be sufficient for the specific extraction and identification of low-abundant cGMP-binding proteins such as PKG. We determined that after the two injections, 9.2 ng (88.5 m°) of material was bound to the SPR surface (Figure 3B). During the sequential elutions with the different nucleotides and sodium hydroxide, the amount of dissociated material was monitored for each individual step (Figure 3B); this provided a semiquantitative insight as to the amount of nonspecifically, less-specifically and specifically bound proteins. During the first two elution steps using ADP and GDP, 43% (4 ng, 41.5 m°) of the bound material was dissociated from the SPR sensor. Subsequently, 30% (3 ng, 26.9 m°) of the originally bound material dissociated (Figure 3B) in the cGMP fraction, in which we expected the selective displacement of cGMP-binding proteins. The elution profile of the cGMP fraction had a different time-

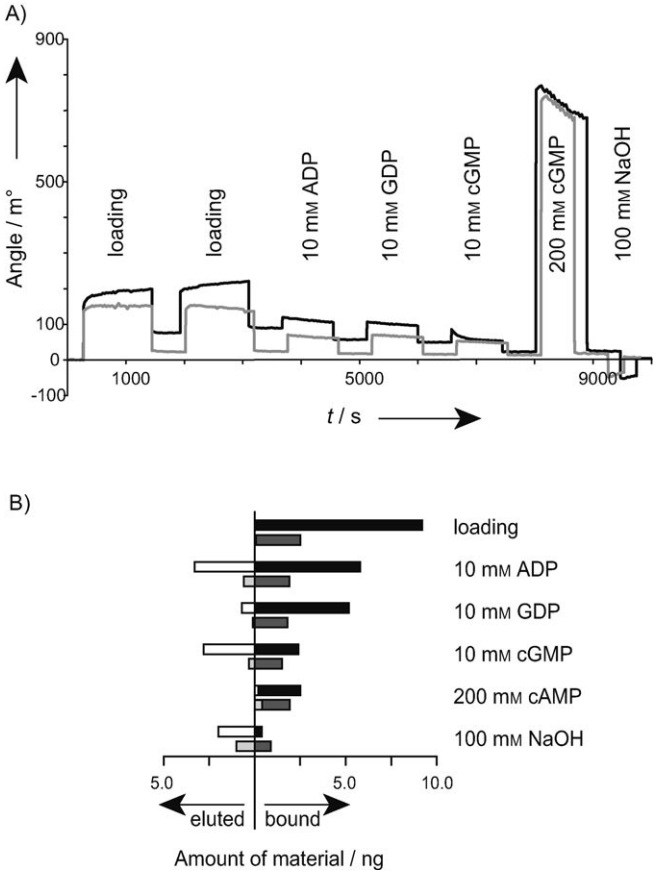

Figure 3. Sequential elution of bound proteins reveals selective dissociation. In A, the black line shows the SPR read-out after injection onto a 8AET-cGMP disc, while the grey line represents the nonspecific binding obtained using a sensor disc on which merely the spacer molecule was immobilized. In the loading step the HEK293 lysate was injected, while sequential elution was performed using ADP, GDP, cGMP, cAMP and NaOH. In B, the amount of material bound to the sensor and the amount of material eluted during each step are depicted for the 8AET-cGMP sensor (black and whites) and for the nonspecific binding (grey scales).

dependent profile than the others (Figure 3A), which might be a characteristic for the dissociation of specifically bound material. Although some decrease in angle shift was also seen during the cAMP elution step, this was probably caused by a bulk shift effect due to the high (200 mM) concentration of cAMP used. In addition, the cAMP dissociation profile was dissimilar to the cGMP dissociation profile. This might indicate that there are differences in selectivity between cGMP and cAMP. After the final regenerative sodium hydroxide elution, a negligible amount of material (0.4 ng, 3.8 m°) remained on the sensor; this showed that the sensor could be used for consecutive measurements.

To further investigate the nonspecific binding, a 6-aminohexanoic acid-immobilized SPR sensor, that is, a SPR chip with the immobilized spacer, but not cGMP, was evaluated. This allowed us to estimate the possible background binding. The HEK293 lysate was injected onto this nonspecific sensor, and the bound material was dissociated during a similar five-step sequential elution procedure (Figure 3A, grey line). After injection, approximately 27% (2.5 ng, 24.1 m°) of the amount of

material attached to the 8AET-cGMP surface was captured on the spacer-immobilized nonspecific sensor. The bound material was mostly removed in the sodium hydroxide step (see Figure 3B), instead of being displaced during the first four elution steps. This shows that electrostatic nonspecific interactions probably contributed the most to the total nonspecific binding.

Identification of the protein composition of each elution fraction results in further insight in specific and nonspecific binding

Further qualitative data about the bound material on the 8AET-cGMP SPR sensor was obtained by LC-MS/MS analysis of the digested eluted fractions, which resulted in a total of 36 identified proteins. By assigning these proteins to specifically eluted fractions, the high-affinity binding partners of cGMP (like PKG and PKA) could be specifically distinguished from the less-specific and the nonspecific binding partners. The last group of proteins could be designated by cross referencing the 36 proteins that were found with the proteins that were identified to bind to the sensor chip that only contained the spacer group (data not shown). In this way, four proteins could be designated as nonspecifically bound to the 8AET-cGMP sensor. As expected, these proteins (14-3-3 protein ζ/δ, α-enolase, heat-shock 70 kDa protein 1 and tubulin β-2 chain) are high-abundant proteins. Another reason for assigning a protein as nonspecific is its presence in more than three different eluted fractions. In this way, another 15 proteins could be assigned to the group of nonspecific binding partners, including the actin that was already detected by MALDI, and nucleoside diphosphate kinase (see the Supporting Information). All of these proteins are highly abundant in the HEK293 cell lysate, and most likely have a low specific affinity for the 8AET-cGMP sensor. Most of the proteins have a natural ligand that shows some structural resemblance with cGMP.

The remaining 17 identified proteins were grouped by their preferential elution fraction (Table 2). The ADP fraction consisted largely of the above mentioned high-abundant nonspecifically bound proteins, which probably caused most of the 36% decrease in the SPR shift (Figure 3B). Besides these high-abundant proteins, a splice isoform of plasminogen activator inhibitor 1 RNA-binding protein was identified as a specific binding partner in the ADP fraction. In the GDP fraction, six specifically bound proteins were identified. Intriguingly, while the highest number of specifically bound proteins was identified in the GDP fraction, the accompanying SPR shift for this fraction was lower than that of the other fractions. Four out of the six identified proteins in the GDP fraction have known affinity towards molecules with structural resemblance to cGMP, such as GMP, GDP, GTP, NAD and DNA/RNA. In the cGMP elution step, the SPR shift decrease of 30% was solely caused by PKG and PKA (Figure 3B). No other proteins were identified in this fraction as specifically bound proteins; this demonstrates the efficiency of the sequential elution procedure, and the resulting selectivity of our approach. Five proteins eluted preferentially in the

Table 2. Identified proteins in the HEK293 cell lysate categorized according to their preferential elution fraction. The number of unique peptides and MASCOT score of the highest scoring fraction are also listed.[a]

	Protein	Swiss acc. no	IPI hum. acc. no.	Binds	M_W [kDa]	Uniq. pept.	Mascot score
ADP fraction							
1	splice isoform 1 of plasminogen activator inhibitor 1 RNA-binding protein		IPI00410693	DNA/RNA	45	4	246
GDP fraction							
2	splice isoform 2 of nucleophosmin		IPI00220740	nucleolin	29	2	90
3	heterogeneous nuclear ribonucleoprotein M isoform A	Q59ES8	IPI00171903	DNA/RNA	78	4	236
4	EEF1A1	P68104	IPI00025447	GMP/GDP/GTP	48	3	185
5	fructose bisphosphate aldolase A	P04075	IPI00465439	other	39	2	117
6	lactate dehydrogenase A	P00338	IPI00217966	adenosine-like	37	3	156
7	eukaryotic initiation factor 4 A-1	P60842	IPI00025491	GMP/GDP/GTP	46	2	141
cGMP fraction							
8	cAMP-dependent protein kinase type I-α regulatory subunit	P10644	IPI00021831	cAMP	43	6	557
9	cGMP-dependent protein kinase 1, alpha isozyme	Q13976	IPI00427589	cGMP	76	4	208
cAMP fraction							
10	vimentin	Q5JVT0	IPI00410474	PKG/Cytoskeleton	54	6	395
11	profilin-1	P07737	IPI00216691	actin	15	2	100
12	replication protein A 70 kDa DNA-binding subunit	P27694	IPI00000811	DNA/RNA	68	3	184
13	single-stranded DNA-binding protein, mitochondrial precursor	Q04837	IPI00029744	DNA/RNA	17	2	167
14	splice isoform B1 of heterogeneous nuclear ribonucleoproteins A2/B1		IPI00396378	DNA/RNA	37	3	234
Sodium hydroxide fraction							
15	60S ribosomal protein L23a	P62750	IPI00021266	DNA/RNA	18	2	125
16	K-ALPHA-1		IPI00328163	cytoskeleton	47	2	401
17	tubulin β 2C-chain	P68371	IPI00007752	cytoskeleton	36	3	356

[a] Nonspecifically bound proteins, that is, proteins that were also identified using the spacer-immobilized sensor, and proteins identified in three or more fractions were not listed here and can be found in Table S1.

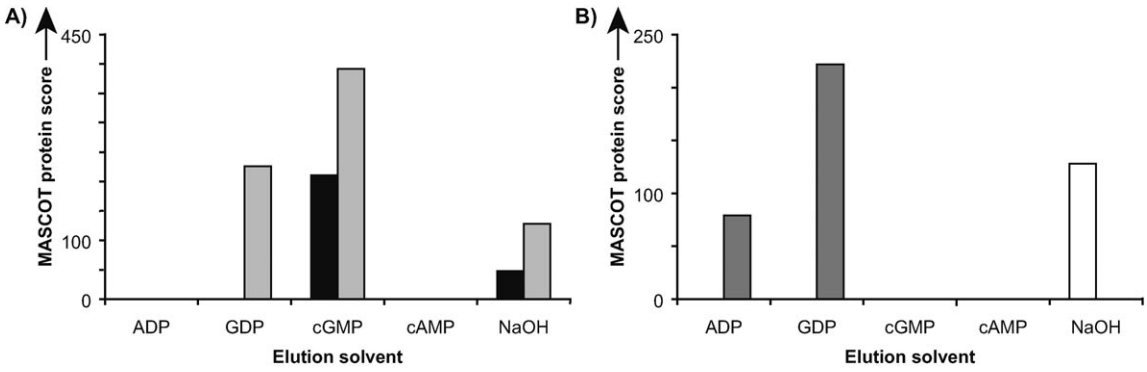

Figure 4. The relative protein abundance as reflected by the MASCOT score versus the elution fraction further emphasizes the selectivity of the used SPR approach. PKG (A, black) dissociated in two fractions, the cGMP and sodium hydroxide (NaOH) fraction respectively, while PKA (A, light grey) also was retrieved in the GDP fraction. Neither PKA nor PKG could be quantitatively displaced, as shown by the recovery of both proteins in the sodium hydroxide regeneration step. For comparison, the elution profiles of two less specifically bound proteins are depicted in B: heterogeneous nuclear ribonucleoprotein M isoform A (dark grey) and 60S ribosomal protein L23a (white).

cAMP fraction: three DNA/RNA binding proteins, vimentin and profilin-1. The last two were probably retained via secondary interactions with actin, a high-abundant nonspecifically bound protein. Interestingly, vimentin is also a putative GKAP.[32] In the sodium hydroxide fraction, three proteins were identified: two cytoskeleton proteins and one DNA/RNA binding protein. Although DNA/RNA has some structural similarity with cGMP, 60S ribosomal protein L23a probably interacted via electrostatic interactions with the SPR sensor, which was a similar result compared to the two cytoskeleton proteins. This hypothesis was supported by the identification of these proteins in the sodium hydroxide elution fraction that was obtained from the nonspecific-spacer-immobilized sensor (data not shown).

To further demonstrate that the use of a sequential elution procedure resulted in improved selectivity, the measured MASCOT protein scores for the PKA and PKG, which are both low-abundant cyclic-nucleotide binding proteins, were further examined (Figure 4A). MASCOT scores can be used to estimate protein abundances.[33] PKG eluted primarily in the cGMP and in the sodium hydroxide elution steps. Surprisingly PKA was also found in the GDP elution step. PKA therefore seemed to have more promiscuous binding characteristics, which may be explained by the fact that cGMP is not PKA's native ligand. On the other hand PKA, like PKG was also retrieved in the sodium hydroxide fraction; this indicates strong binding to the SPR sensor. For comparison, the elution profiles of two less specifically bound proteins are depicted in Figure 4B. Heterogeneous nuclear ribonucleoprotein M, isoform A was less specifically bound to the sensor surface, and likely had a lower affinity towards cGMP, as it could be eluted with ADP and GDP. 60S Ribosomal protein L23a was only retrieved in the regeneration step with sodium hydroxide; this is indicative of a very strong interaction with the sensor disc. However, this interaction was likely to have been caused by electrostatic interactions, as discussed above. Accordingly, proteins with different degrees of affinity towards the immobilized cGMP were separated by using the sequential elution approach. Only the cGMP-binding proteins PKG and PKA were retrieved in the cGMP elution fraction; this shows the improved selectivity of our approach.

When the identified proteins are classified according to their known cofactor dependence or nucleotide-binding characteristics (Table 2), it is clear that most proteins are known DNA/RNA-binding proteins, and proteins that have an affinity towards small molecules with a structural resemblance to cGMP, such as GMP, GDP, GTP and adenosine-like compounds. Therefore, most of the identified proteins after the sequential elution steps were indeed specifically, or less-specifically, bound to the 8AET-cGMP sensor.

Conclusions

In this work an alternative chemical proteomics approach is presented that is based on SPR combined with sequential elution and identification by using LC-MS/MS. We analyzed the affinity-enriched cGMP-binding proteins from a HEK293 cell lysate using an 8AET-cGMP immobilized sensor surface. With this methodology we could distinguish between proteins that bind nonspecifically, less-specifically and specifically to cGMP. Sequential elution steps with ADP and GDP resulted in the dissociation of nonspecifically and less-specifically binding, high-abundant proteins. As a consequence, the bias towards these proteins could be reduced in the remaining elution steps; this resulted in a higher selectivity of specifically bound low-abundant proteins. In the cGMP elution step, only PKG and PKA dissociated; this clearly demonstrates the improved selectivity of our approach.

Our sensor-based methodology has several advantages over the conventional bead-based chemical-proteomics approach. First, the extraction of proteins can be quantitatively monitored, and each dissociation step provides a unique insight in the extraction procedure and the moment of equilibrium. Second, the sensor disc can be reused, and therefore, the procedure can be automated; this leads to better reproducibility from sample to sample. In addition, sensor-based and bead-based chemical proteomics yield largely complementary data. Scholten et al[6] identified 42 proteins by using the 2AH–cGMP affinity chromatography beads and a HEK293 cell lysate, of which approximately eight overlapped with the SPR proce-

dure. We identified relatively more DNA/RNA-binding proteins and less structural/actin-binding proteins compared to the pull-down experiments. Thus, the selectivities of the two modified cGMP-immobilized surfaces is somewhat different and, therefore, yield complementary data.

In conclusion, the data described here reveal that the coupling of SPR to LC-MS/MS and the use of sequential elution steps can be successfully applied to the efficient and specific extraction and semiquantitative identification of small-molecule-binding proteins from complex cellular mixtures. The approach therefore offers an alternative to conventional bead-based chemical proteomics.

Experimental Section

Materials: All chemicals were purchased from commercial sources and were of analysis grade unless stated otherwise. HEPES, 6-aminohexanoic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), ethanolamine, dithiothreitol (DTT) and ammonium hydrogen carbonate were purchased from Fluka. Sodium chloride, hydrochloric acid, boric acid and formic acid (FA) came from Merck. Sodium hydroxide was obtained from BDH Aristar (VWR, Amsterdam, the Netherlands) and Rapigest was purchased from Waters (Etten-Leur, the Netherlands). cGMP, cAMP and 8AET-cGMP (8-(2-aminoethyl)-thio-cGMP) were purchased from Biolog (Bremen, Germany), ADP and GDP were obtained from Sigma–Aldrich, as were iodoacetamide (IAA) and *N*-hydroxysuccinimide (NHS). Protease inhibitor cocktail and trypsin were purchased from Roche. HPLC-S gradient grade acetonitrile (AcN) was purchased from Biosolve (Valkenswaard, The Netherlands) and high purity water was obtained from a Milli-Q system (Millipore, Bedford, MA USA) and was used for all experiments. Bovine PKG Iα was recombinantly expressed in Sf9-insect cells according to Feil et al.[34] and was purified according to the method described by Dostmann et al.[35] Subsequently, it was stored at −30 °C in 50% glycerol.

Samples: Human embryonic kidney (HEK293) cells were cultured in suspension, harvested and lysed as described by Scholten et al,[6] by using HBS-N (10 mM HEPES, 150 mM sodium chloride, pH 7.4), to which a protease inhibitor cocktail was added as a lysis buffer for the 50×10^6 cells. The protein content of the supernatant was determined to be 7.23 mg mL^{-1} by using the Bradford assay.[36] The purified PKG solution (2 mg mL^{-1}, 50% glycerol) was dialyzed against HBS-N before use.

System set-up: A schematic representation of the extraction procedure for cGMP-binding proteins using a cGMP immobilized SPR sensor followed by LC-MS/MS identification is depicted in Figure 1. A SPR sensor (G-COOH sp, IBIS technologies, Hengelo, the Netherlands) was placed inside an IBIS iSPR equipped with a 4 mm i.d. cuvette system (IBIS technologies). All solvents were added by using the built-in autosampler, and were mixed continuously during the reaction, or interaction measurements. The SPR was kept at 24 °C during all measurements. Before immobilization, the hydrogel bed was activated by three washes with sodium hydroxide (100 mM), with in between washes with HBS-N, which was used as the running buffer.

A 1:1 mixture (200 μL) of EDC (400 mM) and NHS (100 mM) in water was used to activate the SPR sensor (Figure 1 A step I) for the immobilization of 6-aminohexanoic acid (10 mg mL^{-1}, 100 μL; Figure 1 B) in borate pH 8.5 (50 mM). After 30 min the sensor was washed with HBS-N followed by the deactivation of the remaining activated carboxylic functions using 1 M ethanolamine in borate pH 8.5 (50 mM; Figure 1 A step II). The second immobilization step (Figure 1 A step III) was performed in the same manner, except for the injection of the hexanoic acid solution, which was replaced by a solution of 8AET-cGMP (0.63 mg mL^{-1}, 1.5 mM; Figure 1 C) in borate pH 8.5 (50 mM).

After immobilization, HEK293 cell lysate (2×200 μL) was injected, with in-between and afterwards a 10-min wash to remove the non-specifically bound material (Figure 1 A, step IV). Next, the bound material was dissociated by using the sequential elution procedure (Figure 1 A step V): first ADP (100 μL, 10 mM) was added to the cuvette, mixed for ten minutes, and then the eluate was transported to a 96-well plate; this was followed by a HBS-N wash of the sensor. The second, third and fourth elution step were conducted in a similar manner using GDP (10 mM), cGMP (10 mM) and cAMP (200 mM), respectively. Sodium hydroxide (100 mM) was used in the last dissociation step to regenerate the sensor. All experiments were performed at least in duplicate.

Proteolytic cleavage: The dissociated material was transported to separate Eppendorf tubes (Hamburg, Germany) for further sample handling. Hydrochloric acid (1 μL, 1 M) was added to each sodium hydroxide fraction (100 mM) in order to reduce the pH to a value suitable for proteolytic digestion. Before digestion, RapiGest (0.1% *w/v*) was added to the samples to denature the proteins. Reduction and alkylation were achieved by using dithiothreitol (DTT, 2 mM) at 56 °C for 15 min, and iodoacetamide (IAA, 4 mM) at room temperature for 30 min in the dark, respectively. The samples were digested overnight at 37 °C by using trypsin/sample 1:100 (*w/w*). To quench the digest activity and to degrade the RapiGest, all of the samples were acidified with 5% formic acid (*v/v*), and left for 4 h at 37 °C. The insoluble degradation product of RapiGest was spun down at 14000 rpm for 10 min. The supernatant was used for LC-MS/MS analysis.

LC-MS/MS: 40 μL of the digest was desalinated using a C_{18} STAGE-tip.[37] The trapped peptides were eluted in a 96-well plate using 1 μL of 5% formic acid (*v/v*) in 50% acetonitrile (*v/v*) and 39 μL of 5% formic acid (*v/v*). NanoLC-ESI-MS/MS was performed as described by Meiring et al[38] and Pinkse et al[39] by using an Agilent 1100 HPLC system (Amstelveen, the Netherlands) that was connected to a Thermo Finnigan LTQ-MS (Bremen, Germany) that was equipped with a 20 mm × 100 μm i.d. Reprosil C18-RP trap column (Dr. Maisch, Ammerbuch-Entringen, Germany) and a 200 mm × 50 μm i.d. Reprosil C18-RP analytical column. 30 μL of peptide mixture was separated by using a 60-minute 100 nL/min linear gradient from 0% to 40% solvent B (0.1 M acetic acid in 80% acetonitrile (*v/v*)), in which solvent A was 0.1 M acetic acid. The MS was operated in the positive-ion mode, and parent ions were selected for fragmentation in data-dependent mode.

After MS, the data was analyzed with MASCOT (http://www.matrixscience.com) and Scaffold (http://www.proteomesoftware.com) software by using the IPI human FASTA 3.16 database with trypsin as an enzyme. The software allowed for three miscleavages, and searched in a MS window of 0.5 Da and a MS/MS window of 0.9 Da. The identified proteins were further analyzed for their nucleotide-binding properties by using the online SWISS-PROT protein database (http://www.expasy.org), or by literature searching.

Validation experiments for PKG and the cGMP-sensor: To test the 8AET-cGMP sensor, a titration was conducted by using a constant amount of recombinant dimeric PKG_2 (9.2 pmol, 75 μL of 9.4 μg mL^{-1} in HBS-N). cGMP was titrated-in using the molar ratios: 1:20. 1:10, 1:2, 1:1, 1:0.2 and 1:0 (PKG_2/cGMP). The SPR procedure

was as described above, except that the final dissociation of the bound material was conducted by using 100 μL 100 mM sodium hydroxide.

The linearity and the limits of detection (LOD) were determined by using a calibration curve of dimeric PKG_2 in HBS-N at a concentration of 0, 0.5, 1.0, 5.0 and 10 $\mu g\,mL^{-1}$. SPR read-outs were measured twice at each PKG concentration by using sodium hydroxide (100 mM) as the elution solvent.

Reference sensor to determine nonspecific interactions: A 6-aminohexanoic acid-immobilized sensor disc was used to determine nonspecific binding. The sensor disc was made as described above, except that after the first ethanolamine deactivation, the sensor was washed with HBS-N after which it was ready for use. Injection, interaction, elution, digestion and LC-MS/MS analysis of the HEK293 cell lysate was conducted in the same manner as described above for the 8AET-cGMP sensor disc.

Semiquantification of material bound to the SPR sensor: The angle shift has a linear correlation with the amount of material bound to the surface: 120 m° angle shift corresponded to 1 $ng\,mm^{-2}$ material bound. As the used cuvette had an inner diameter of 4 mm, an angle shift of 120 m° corresponded to 12.5 ng of material bound to the total sensor surface.

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