Reversible, site-specific immobilization of polyarginine-tagged fusion proteins on mica surfaces

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Abstract A large variety of genes is expressed as fusion proteins for the purpose of characterization and purification in molecular biology. We have used this strategy to append polyarginine peptides in order to achieve specific binding of the Arg-tag to atomically flat, negatively charged mica surfaces. We show that the model protein, hexaarginine-tagged green fluorescent protein (GFP), binds to mica via its Arg-tag based on ion exchange of naturally occurring potassium cations. Only non-specific binding was observed with the control protein that is free of the Arg-tag. This novel technology will be widely applicable to orient functional proteins on flat surfaces.

Key words: Mica; GFP; Polyarginine; XPS

1. Introduction

The immobilization of functional proteins on flat surfaces is of crucial importance for studying their interaction with ligands and examining their structure by means of electron and scanning probe microscopy and other biophysical techniques requiring a solid interface.

Targeting proteins at specific sites and anisotropically immobilizing them on a surface while preserving their functionality is a major precondition to facilitate biochemical recognition and interaction, to present selected sites for structural investigation, to induce two-dimensional crystallization, and to develop new biosensors and supramolecular assemblies.

Several efforts have been made to immobilize proteins with controlled orientation either covalently utilizing single reactive thiol groups of cysteine residues [1], or non-covalently, but specifically via immobilized antibodies [2,3], the biotin/streptavidin system [4], or metal-chelating Langmuir-Blodgett films [5–8] and metal-chelating self-assembled monolayers [9,10] for binding of polyhistidine fusion proteins.

Mica, with the ideal structure $KAl_2[AlSi_3O_{10}](OH,F)_2$, refers to a group of layered aluminosilicate minerals whose crystals exhibit a large degree of basal cleavage, allowing them to be split into very thin atomically flat sheets.

Due to its flatness and hydrophilic surface, mica has been established as a standard substrate for electron and scanning probe microscopy applications (see for example: [11–14]). Therefore, chemical modification of and site-specific immobilization on mica would extend its field of applications towards more sophisticated molecular architectures.

The complex multilayered structure of mica with its surface-exposed negatively charged honeycomb arrangements of $Si(Al)O_4$ tetrahedra has been used as substrate for monolayer formation of amphiphilic organic molecules, such as alkylphosphonic acids [15] and organosilanes [16–20]. Unfortunately, the former are not robust under aqueous conditions and the latter are often isotropically rough with monolayer formation characterized by low reproducibility, especially if terminated with a nucleophilic group in the ω-position.

As an alternative to the attachment of a two-dimensional siloxane network onto the mica surface, efforts have been made to alter the surface chemistry by exchanging the surface cations (mostly potassium) at the basal (001) cleavage plane with other inorganic and organic ions [21,22]. Surfactant adsorption of long-chain alkylammonium salts, such as cetyltrimethylammonium bromide (CTAB) [23,24] and N-dodecylpyridinium chloride (NDP) [21] are known to hydrophobize negatively charged minerals. Exchange with bivalent cations has been used to mediate binding of DNA for SPM studies [25].

Sheldon and coworkers have shown that the amidine group of 2,2′-azobisisobutyramidine hydrochloride (AIBA) binds as a bication without deprotonation and can act as an azo initiator for the polymerization of styrene directly on the mica surface [21,26].

We have extended this strategy to the guanidino group of arginine residues and in this study we report our results on the immobilization of proteins bound via a polyarginine fusion peptide.

In analogy to the polyhistidine fusion strategy, used to facilitate purification of recombinant proteins by metal affinity chromatography, we have appended a polyarginine sequence to two different model proteins to achieve specific docking of the positively charged guanidino groups of the Arg-tag to the negatively charged mica surface by ion exchange (as illustrated in Fig. 1).

As a model protein we have chosen green fluorescent protein equipped with a hexaarginine sequence at either its N- or C-terminus. The reversible, Arg-tag-specific binding was investigated by fluorescence and X-ray photoelectron spectroscopy.

This strategy, based on site-directed mutagenesis, could facilitate the uniform and specific orientation of immobilized proteins on a standard substrate used for many surface-related applications.

2. Materials and methods

2.1. Materials

Muscovite mica was obtained from Provac (Liechtenstein). The plasmid pGFPuv was from Clontech (Palo Alto, CA) and the vector pET28a$^+$ was from Novagen (Madison, WI). All other reagents were from Sigma Chemical (St. Louis, MO) and of highest available grade. Ultrapure water with a resistance of 18 MΩcm was used for all aque-

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ous buffers (purified by passage through a Milli-Q purification system).

2.2. Preparation of $GFPH_6$, $GFPH_6R_6$, $GFPR_6$

For the addition of six histidine residues to the N-terminus of GFP, two oligodeoxyribonucleotides were designed: one corresponding to the N-terminal part of the GFP gene (5′-GGAATTCCATATGAGT AAAGGAGAAGAACTTTTC-3′, (#1)) and a second corresponding to the C-terminal part (5′-GACCGGCGCTCAGTTGGAATTC-3′, (#2)). These oligodeoxyribonucleotides were used for the PCR with 20 ng of linearized pGFPuv as template. The amplified fragments, digested with *Nde*I and *Bam*HI, were ligated with the linearized expression vector pET28a$^+$. The resulting plasmid $pGFPH_6$ was used for transformation of *E. coli* BL21(DE3). Standard protocols were followed for DNA handling and bacterial transformation [27].

To introduce a tag of six arginine residues on either the N- or C-terminal part of GFP, the same procedure was used with the following oligodeoxyribonucleotides: (#2) and 5′-GGAATTCCATATGCGCC-GTCGCCGTCGCCGTATGAGTAAAGGAGAAGAACTTTTC-3′ for $GFPH_6R_6$, (#1) and 5′-TTGGAATTCATTAGCGACGGCGAC-GGCGACGCGCGGTGCCTTTGTAGAGCTCATCCATG-3′ for $GFPR_6$. The PCR and cloning procedure was performed as described above. The resulting plasmids $pGFPH_6R_6$ and $pGFPR_6$ were used to transform *E. coli* BL21(DE3).

2.3. Expression and purification of the recombinant proteins

All proteins carry a vector-encoded tag of a hexahistidine sequence for purification by metal chelate affinity chromatography on a Ni^{2+}/NTA matrix (Qiagen, Santa Clarita, CA). The cells were grown at 37°C by shaking in LB-medium containing 25 μg/ml Kanamycin. At an OD_{600} of 0.8 the cells were induced with 1 mM IPTG, and 5 h later, they were harvested by centrifugation at $6000\times g$ for 10 min. The cells were lysed by addition of lysozyme at a concentration of 100 μg/ml and 10% (v/v) of 1% Triton X-100 in 50 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM EDTA. After incubation for 30 min on ice, $MgCl_2$ was added to a final concentration of 40 mM. The liberated DNA was digested by adding 0.2 μg DNaseI per ml lysate. The lysate was incubated for 15 min on ice and then centrifuged at $30\,000\times g$ for 40 min. The clear supernatant was dialysed against buffer containing 10 mM HEPES/NaOH pH 7.4, 50 mM NaCl, and then applied to a Ni^{2+}/NTA column. Weakly bound proteins were eluted with 10 mM imidazole pH 8.0. The His-tagged proteins were eluted with 500 mM imidazole in the case of the $GFPH_6$ and with 500 mM imidazole, 500 mM NaCl for all the other variants (the Arg-tag caused a strong ionic interaction with the Ni^{2+}/NTA matrix). The eluted proteins were dialyzed against buffer containing 10 mM HEPES/NaOH pH 7.4, 50 mM NaCl, 50% glycerol and stored at −20°C. The purity of the recombinant proteins was estimated by SDS-polyacrylamide gel electrophoresis and found to be greater than 95%.

2.4. Protein adsorption to mica

Mica sheets were cut into pieces of 5×5 cm^2 and freshly cleaved immediately before use. Droplets of protein solutions ($GFPH_6$, $GFPR_6$, $GFPH_6R_6$) at a concentration of 10 μg/ml were applied onto the previously unexposed, hydrophilic surfaces resulting in aqueous films of approximately 4 cm^2 in size. After incubation for 5 min, the mica sheets were washed with 10 ml of water. The central parts, 1 cm^2 in size, were then cut out to ensure that no contaminants from the edges could falsify the subsequent analyses. For each data point four surfaces were analyzed and the readings were averaged.

These surfaces, stored separately in Eppendorf tubes, were then subjected to consecutive 1 min washing steps with 400 μl 10 mM HEPES/NaOH buffer pH 7.4 containing increasing concentrations of salt with different mono- and bivalent cations (50, 125, 250 mM, Na^+, K^+, Mg^{2+}). For quantitation of active, adsorbed GFP, the eluates were collected separately and analyzed by fluorescence measurement at 509 nm (excitation at 395 nm) using an SLM8000 spectrophotometer (Aminco, Silver Spring, MD) and GFP of known concentration as standard.

Qualitative determination of immobilized GFP was carried out with X-ray photoelectron spectroscopy (XPS) using the N1s narrow scans normalized against the corresponding Si2s peaks, an element that does not occur in proteins. For this purpose the adsorbed proteins were washed with the same salt-containing solutions as mentioned above (without buffer) and finally rinsed with ultrapure water and dried under a stream of nitrogen. This ensured that the XPS spectra were not dominated by crystallized salts.

2.5. Amino acid adsorption to mica

To adsorb the amino acids arginine, lysine and histidine on mica, the sheets were cut and freshly cleaved as described in Section 2.4. Droplets of amino acid solutions at a concentration of 25 mM were applied onto the mica. The incubation times, the elution of the adsorbed amino acids with increasing concentrations of NaCl (1 and 10 mM in water), and the detection of the amount of bound amino acids by XPS were performed as described above.

2.6. X-ray photoelectron spectroscopy (XPS)

XPS was carried out on a Surface Science Model 150 XPS spectrometer with an AlKα source (1486 eV), a quartz monochromator, hemispherical analyzer, and a multichannel detector. A nickel grid, directly positioned above the samples, and a charge neutralizer were used to prevent artifacts due to charging effects. The spectra were accumulated at a take-off angle of 35° and an angular acceptance of 30°, with a 250×1000 μm spot size at a pressure of less than 1×10^{-8} Torr. The N1s peaks shown in this study are normalized against Si2s and corrected for the number of scans and the atomic sensitivity factors.

3. Results and discussion

The strategy presented here for the site-specific immobilization of proteins is based on the ion exchange capacity of naturally occurring cations on the negatively charged cleavage plane of atomically flat mica. Positively charged polypeptide tags with high affinity to mica were genetically fused to either the N- or C-terminus of GFP. In order to design this tag, the interaction of positively charged amino acids to the mica surface was investigated in a preliminary experiment by XPS measurement.

Table 1 shows the extent of release of arginine, lysine and histidine from the mica surface and its dependence on the salt concentration in the wash buffer. The emitted photoelectrons of the nitrogen atoms of the adsorbed molecules were used to monitor the residual amount of the amino acids on the mica after consecutive washing with increasing concentrations of salt. Although we have rationed the N1s peak signals to the silicon Si1s peak intensities of the underlying silica structure, we did not attempt to calculate the absolute amounts of adsorbates. However, the extent of desorption of the amino acids could be estimated based on the relative photoelectron

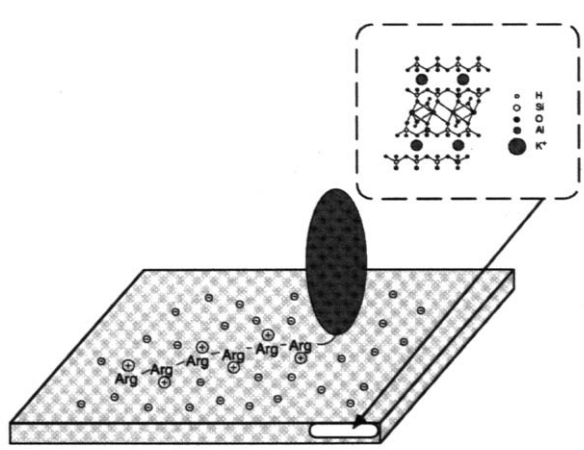

Fig. 1. Schematic illustration of a protein immobilized to the mica surface via its Arg-tag (not drawn to scale). The muscovite mica structure is shown in the inset.

Fig. 2. Schematic presentation of the three different GFP variants. The N- and C-terminally added sequences are shown in the one letter amino acid code. The hexaarginine tag is marked in bold letters and the GFP is shown as a gray bar.

counts. In this experiment, the amount of bound amino acids after thorough washing with ultrapure water was set to 100%. After a first rinsing step with 1 mM NaCl, 78% of the adsorbed arginine, but only 54% of lysine and 53% of histidine remained on the surface. Further treatment with a 10 mM NaCl solution decreased the amount of bound amino acids to 58, 36 and 30% for Arg, Lys and His, respectively. These data show that arginine is more tightly associated with the mica than the other positively charged amino acids. The extent of ion exchange on the mica surface is associated to the enthalpy of hydration of the cations that are involved. We chose NaCl instead of KCl in the salt buffer, because K^+ and the positively charged nitrogens of the bulky amino acid have a lower enthalpy of hydration than Na^+.

This result and the previously reported binding of 2,2′-Azobisisobutyramidine hydrochloride [21,26], a compound with a positively charged amidine group (resembling the guanidino group of the arginine) led to the decision to add a polyarginine tag to a protein.

Because of its intrinsic fluorescence, we chose the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* as the model protein in this study. GFP has been expressed in a variety of species including bacteria [28]. The chromophoric group emits efficiently in the green spectral region if the protein is in its native conformation. This allows the quantitation of the amount of native protein that was released from the surface after consecutive elution steps by measuring fluorescence. Fig. 2 gives an overview of the three different GFP constructs that were designed for this experiment. $GFPH_6$ carries an N-terminal His-tag alone in order to facilitate the

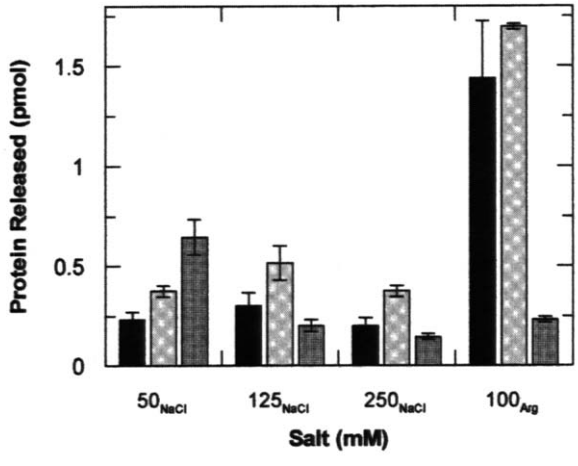

Fig. 3. Stepwise elution of immobilized protein as a function of consecutive washes of the same surface with increasing NaCl concentration in the wash buffer followed by a 100 mM Arg wash. The values for $GFPR_6$, $GFPH_6R_6$ and $GFPH_6$ are shown in black, light gray and dark gray, respectively.

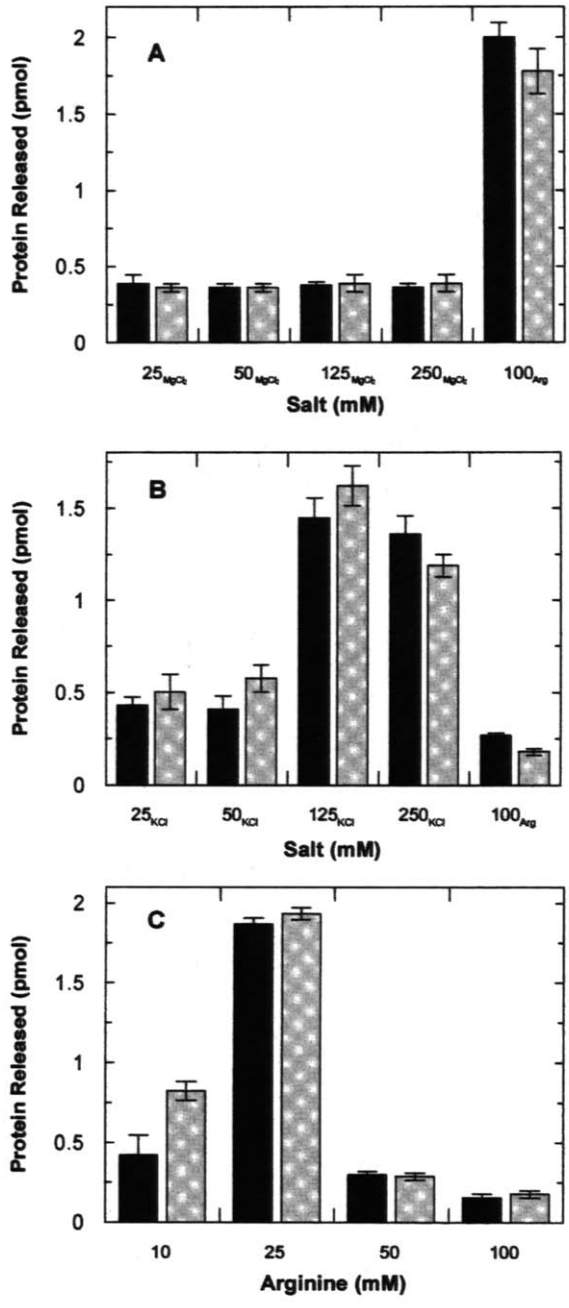

Fig. 4. Dependence of the elution of immobilized protein on the $MgCl_2$ (A), KCl (B) and Arg (C) concentration. $GFPR_6$ is shown in black and $GFPH_6R_6$ is shown in light gray.

purification of the protein by Ni^{2+}/NTA affinity chromatography. $GFPR_6H_6$ carries in addition a stretch of six arginine residues at the N-terminal region, whereas $GFPR_6$ has the same Arg-tag at the C-terminal region and the His-tag at the N-terminus.

In a first experiment the release from mica of all three prebound GFP constructs was tested using consecutive washes with increasing concentrations of NaCl, followed by a final wash with 100 mM Arg. Only about 1 pmol of the Arg-tag free $GFPH_6$ remained bound after extensive washing with 10 mM HEPES/NaOH, pH 7.4. In contrast about 3 pmol of both Arg-tagged species remained bound after this wash. Es-

Table 1
Release of arginine, lysine and histidine from mica under different salt conditions as determined by XPS (for details see Section 2)

Wash	Bound amino acid (%)		
	Arg	Lys	His
Water	100	100	100
1 mM NaCl	78	54	53
10 mM NaCl	58	36	30

The data shown correspond to the amount of bound amino acid as a percentage of the value after washing with water (set to 100%).

sentially all of the Arg-tag free $GFPH_6$ was removed from the surface by consecutive washing steps with increasing concentrations of NaCl (see columns in Fig. 3 from left to right). In contrast, only about 50% of the two GFP variants comprising hexaarginine-tags ($GFPR_6H_6$ and $GFPR_6$) came off with NaCl. The complete release could be achieved by elution with arginine-containing wash buffer (final column in Fig. 3). It is rather likely that this arginine-releasable protein was exclusively bound via its Arg-tag, whereas that released in the NaCl washing steps stemmed primarily from protein electrostatically bound to the surface via other charged groups in the protein.

For numerous applications it is important to immobilize proteins under physiological conditions. Enzymes, for example, often need the presence of Mg^{2+} ions and K^+ ions for maximum catalytic activity. The compatibility of this immobilization strategy with these ions was tested and the result is shown in Fig. 4. Multiple washing steps with increasing concentrations of $MgCl_2$, followed by a final arginine wash revealed the same release characteristics caused by NaCl (Fig. 3). The location of the Arg-tag on either the C-terminus or the N-terminus of the protein had no influence on the interaction with the mica. In addition, the slightly hidden position of the

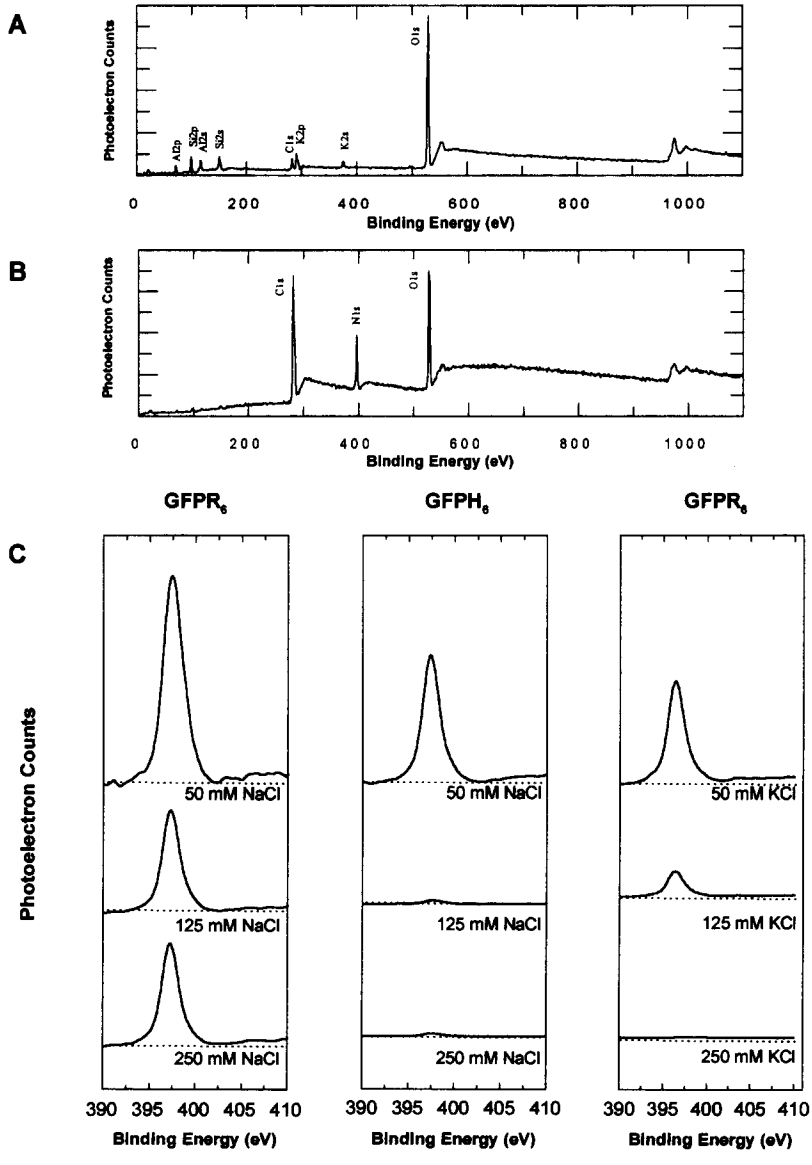

Fig. 5. XPS-survey spectra of (A) freshly cleaved mica and (B) $GFPR_6$ immobilized to mica. The binding strength of bound $GFPR_6$ and $GFPH_6$ to mica after washing with increasing amounts of NaCl and KCl is shown in C as monitored by the XPS N1s narrow scans (arbitrary units and normalized to the Si1s signal). The dashed line means no photoelectron counts.

Arg-tag of $GFPH_6R_6$ did not decrease its binding efficiency compared to $GFPR_6$.

The monovalent cation K^+ is similar to arginine in its ability to release the $GFPH_6R_6$ and the $GFPR_6$ from the mica substrate, although higher concentrations are necessary. Potassium is the naturally occurring cation in muscovite mica and has a lower enthalpy of hydration than sodium, explaining its 'power' for inducing Arg-tagged GFP desorption from the mica.

These experiments demonstrate that GFP with an Arg-tag on one of its termini can be reversibly and specifically bound via this sequence onto the mica surface.

In order to investigate the complete release of protein from the mica surface after these washing steps, we have examined the mica with XPS. The XPS spectrum of freshly cleaved mica as shown in Fig. 5A exhibits the corresponding peaks for aluminum (Al2p (73.1 eV), Al2s (118.2 eV)), silicon (Si2p (101.4 eV), Si2s (152.2 eV)), potassium (K2p (292.9 eV), K2s (377.8 eV)) and oxygen (O1s (532.0 eV)). All these elements are constituent components of the mica silica structure (the very small carbon peak (C1s) at 284.6 eV is due to contaminations from the environment). After adsorption of $GFPR_6$ (see Fig. 5B), the XPS spectrum changes considerably towards increased carbon (C1s (284.6 eV)), oxygen (531.8 eV), and nitrogen (N1s (399.6 eV)) peaks. Due to the thickness of the protein film, the signals for the silicon and aluminum peaks disappear into the noise level. The survey spectra of $GFPH_6R_6$ and $GFPH_6$ are nearly identical, although the latter shows less amounts of bound protein after rinsing with water (data not shown). The detection of nitrogen on the surface and its removal upon protein desorption under different buffer conditions is shown in Fig. 5C with N1s narrow scans. Whereas the Arg-tagged $GFPR_6$ showed a similar decrease by a factor of two, as shown before by fluorescence (Fig. 3), $GFPH_6$ lacking the Arg-tag was washed off completely at 125 mM NaCl.

In order to rule out irreversible attachment of $GFPR_6$, we have carried out a similar experiment with KCl mediated desorption as shown in the right panel of Fig. 5C, demonstrating that at very high KCl concentrations (250 mM) no protein was detectable. These results are consistent with the fluorescence measurement (Fig. 3) and clearly indicate that $GFPR_6$ binds reversibly via its Arg-tag and without denaturation.

Considering that the GFP is a cylinder with a height of 4–5 nm and a diameter of 3–4 nm [29], a 1 cm^2 area of mica could theoretically bind 6–8 pmol GFP in a densely packed monolayer. The added amounts of desorbed Arg-tagged protein after consecutive washing steps with increasing salt concentrations and finally arginine correspond to 3–4 pmol (as estimated from the sum of the bars in Figs. 3 and 4). As described above, XPS showed that the consecutive washes shown in Figs. 3 and 4 led to the complete release of all protein bound (Fig. 5). This suggests that GFP forms a non-crystalline, but densely packed, protein monolayer on the mica surface. The fact that the desorbed protein was still fluorescent demonstrates that adsorption and desorption did not disrupt the native structure of the protein.

We have also carried out the analogue set of experiments with glutathione-S-transferase and obtained the identical Arg-tag mediated binding behavior (data not shown). This strongly indicates that Arg-tag fusion proteins could be of general applicability, even for larger proteins. In each case, the maximum ionic strength must be determined and adjusted in order to minimize random, non-specific electrostatic interactions of the target protein and to achieve an attachment situation where the protein is only bound via its Arg-tag. It is likely that proteins immobilized in this way exhibit uniform orientation. The fact that mica is atomically flat could help to investigate the structure of uniformly oriented biomolecules by electron and scanning probe microscopy and other surface-related biophysical assays. It should be noted, however, that the charge distribution on the surface of a protein of interest could influence its adsorption properties. Patches of arg-rich areas could act as additional adsorption sites and jeopardize any attempts to achieve uniform orientation.

The stability of immobilized Arg-tagged proteins allows functional studies under physiological conditions and even at high ionic strength. In many cases, proteins lacking the polyarginine sequence should not bind at such high salt concentrations, which could also facilitate in situ purification directly on the mica substrate.

This concept should be widely applicable to a large number of proteins and represents a powerful strategy to design anisotropic protein surfaces for applications in structural biology, biosensing and biophysics.

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