Protein Microarrays

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Oriented Immobilization of Farnesylated Proteins by the Thiol-Ene Reaction**

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Protein biochips are of high interest for various fields of biotechnology, such as bioanalytics, proteomics, biocatalysis, and biomaterials.[1–8] For protein-biochip preparation, the oriented (i.e. site-specific) covalent attachment of proteins to surfaces is important because it ensures homogeneous surface coverage and accessibility to the active site of the protein.[9–11] Moreover, the structural sensitivity of proteins calls for chemical transformations that proceed under mild conditions and are compatible with all functional groups present in proteins.[1,2,12–15] The availability of a method for the fast, oriented, and covalent immobilization of expressed proteins from lysates would be of great value. This approach has been demonstrated previously on the basis of protein transsplicing,[16,17] phosphopantetheinyl transferase catalysis,[18] and O^6-alkylguanine-DNA alkyltransferase (SNAP tag).[19,20] We recently described the use of the photochemical thiol-ene reaction for the covalent surface patterning of small biomolecules.[21] We now report that this transformation can be employed for the fast, oriented, and covalent immobilization of proteins under mild conditions, and that it provides a novel means for the direct immobilization of proteins from lysates without any additional chemical derivatization or purification steps.

For the thiol-ene reaction to be applied to proteins, an olefin must be introduced into the protein of interest. In cells, various proteins are posttranslationally S-farnesylated at C-terminal cysteine groups by protein farnesyltransferase (FTase).[22] FTase employs farnesyl pyrophosphate (Fpp) as the farnesyl donor and recognizes a C-terminal "CAAX-box" tetrapeptide sequence (C is cysteine, A is an aliphatic amino acid, X is one of a variety of amino acids). The reaction can also be performed in vitro (Figure 1a).[23] The FTase-catalyzed transfer of synthetic alkyne- or azide-functionalized farnesyl analogues in combination with the Huisgen [3+2] cycloaddition and Staudinger ligation has been explored for the immobilization of proteins.[24,25]

We reasoned that the equipment of proteins with a genetically encodable CAAX tag would enable farnesylation in vitro or in vivo and subsequent photochemical thioether-bond formation between an olefin of the isoprenoid and surface-exposed thiols (Figure 1b).

For a proof-of-principle study, we chose the H-, N-, and K-Ras GTPases, which play major roles in cellular signaling and are among the most important human oncogene products.[26] All Ras isoforms bear the CAAX box and require lipidation for correct function and localization in eukaryotic cells.[27] Full-length H-Ras, N-Ras, and K-Ras were farnesylated in vitro with recombinant FTase, as described previously.[28] Complete farnesylation of H-, N-, and K-RasFar was verified by means of MALDI MS. By using an experimental setup established earlier for small molecules,[21] the farnesylated proteins were drop-cast onto thiol-functionalized SiO_x/Si slides with an intermediate poly(amidoamine) (PAMAM) dendrimer layer (for an illustration of the slide preparation, see Scheme S2 in the Supporting Information). To this end, the protein solution was deposited on the slide surface and covered with a photomask (Figure 1c). A thin liquid-containing chamber was obtained which prevented the drying out and denaturation of the protein. Following exposure to UV light at a wavelength of 365 nm for 10 minutes (6 J cm^{-2}) and subsequent washing to remove unbound material, the slides were incubated with a Cy3-labeled antibody directed against Ras isoforms. Ras-positive microstructures were successfully detected with a fluorescence microarray scanner (Figure 1d; see also Figure S3 in the Supporting Information). The antibody employed recognizes an α helix of the Ras proteins close to the active site and thus indicates that the immobilized Ras proteins are correctly folded.[29,30] However, immobilization efficiencies varied strongly. Whereas K-RasFar immobilization led to highly fluorescent microstructures, the inten-

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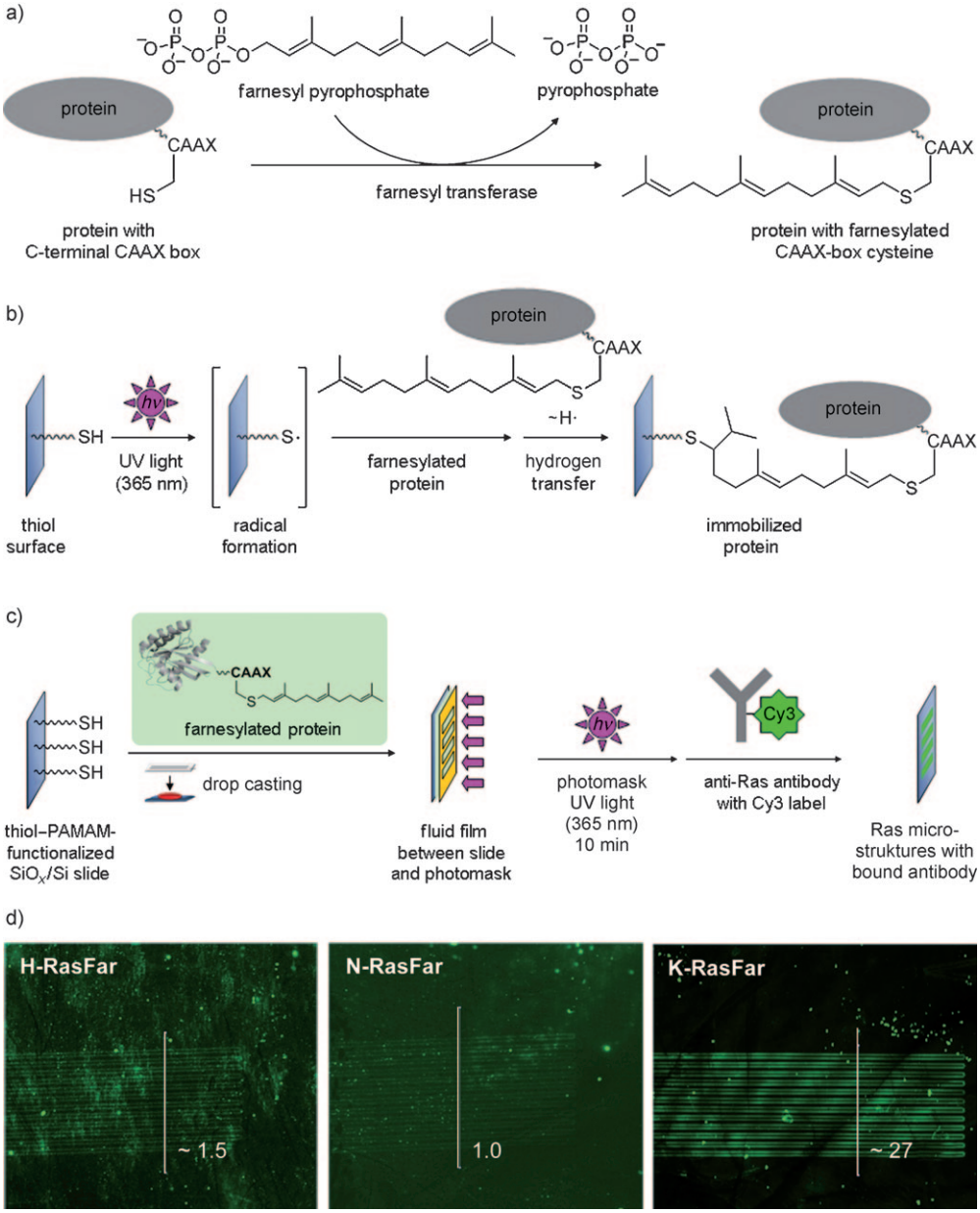

Figure 1. a) Farnesylation of proteins by farnesyltransferase (FTase). FTase recognizes the four amino acid CAAX-box motif at the C terminus of the protein and transfers a farnesyl residue onto the cysteine side chain by using farnesyl pyrophosphate (Fpp) as a lipid donor. b) Proposed mechanism for the thiol-ene photoimmobilization of farnesylated proteins. c) Schematic depiction of the thiol-ene photomicrostructuring of farnesylated Ras isoforms. d) Fluorescence images obtained after the photomicrostructuring of farnesylated Ras isoforms. Average relative fluorescence intensities obtained from cross-sections of the microstructure lines (white lines) are indicated.

sities observed for H-RasFar and N-RasFar were only about 5% of the value for K-RasFar.

The three Ras isoforms share a high sequence identity in the globular part (amino acids 1–171) but differ at the C terminus (Figure 2a). Notably, K-Ras contains a polybasic lysine stretch at the C terminus that is not present in H- and N-Ras. Since the thiol-functionalized SiO_x/Si slides employed were prepared from carboxylic acid functionalized poly(amidoamine) (PAMAM) dendrimers (see Scheme S2 in the Supporting Information),[21] we reasoned that remaining carboxylic acid groups on the surface might have attracted the positively charged K-Ras C terminus (abbreviated as KRT) to increase the concentration of K-RasFar locally and thus promote its more efficient immobilization (Figure 3a). This interaction would also explain the slightly higher photoimmobilization efficiency observed for H-RasFar when compared to N-RasFar, since the C terminus of H-Ras also contains one lysine residue. A control experiment in which C termini with lysine stretches of varying lengths were used confirmed this hypothesis (see Figure S5 in the Supporting Information). Different CAAX-box sequences did not show strong differences in immobilization efficiency (see Figure S6 in the Supporting Information).[31]

We therefore hypothesized that for efficient immobilization on thiol–PAMAM surfaces, proteins of interest should not only be equipped with a CAAX box but also with a polybasic, KRT-like amino acid sequence. To investigate this hypothesis, we generated and farnesylated a variant of the fluorescent mCherry protein with a tetralysine-based KRT-like C terminus in vitro (H_6-mCherry-4KRT; Figure 2b). Protein expression of mCherry modified with the hexalysine-based KRT-like C terminus (abbreviated as 6KRT) failed. However, a control experiment demonstrated that a stretch of four lysine residues in the protein C terminus was sufficient to enable immobilization (see Figure S5 in the Supporting Information).

To enable the comparison of different parameters in parallel, we attempted microarray generation instead of photomicrostructuring. Farnesylated H_6-mCherry-4KRT (H_6-mCherry-4KRTFar) was spotted onto a thiol–PAMAM-functionalized SiO_x/Si slide together with nonfarnesylated H_6-

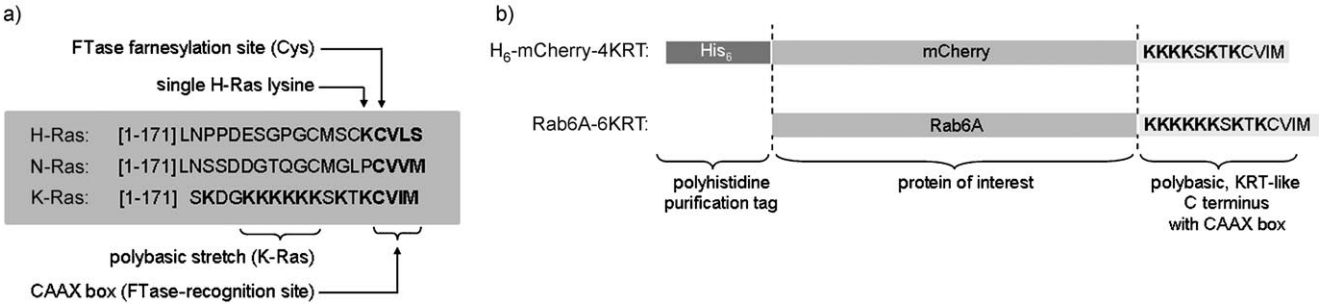

Figure 2. a) C termini of H-Ras, N-Ras, and K-Ras. The CAAX box (the FTase-recognition motif and farnesylation site) and the polybasic stretch of K-Ras, which does not occur in H-Ras and N-Ras, are indicated. The protein core (1–171) is highly similar for all three isoforms. b) Engineered variants of mCherry and Rab6A incorporating polybasic C termini resembling the K-Ras C terminus (KRT) with CAAX-box farnesylation sites.

mCherry-4KRT and H_6-mCherry lacking a prenylatable tag as controls. Following exposure to UV light at 365 nm, immobilized protein was detected by fluorescence scanning (for an illustration of the general work flow see Figure S4 in the Supporting Information). Farnesylated H_6-mCherry-4KRTFar was immobilized efficiently, whereas only trace amounts of the controls were detected (Figure 3 b,c). This result supports the hypothesis that the C-terminal KRT-like sequence strongly enhances thiol-ene photoimmobilization of farnesylated proteins. In contrast, the N-terminal polyhistidine sequence does not seem to influence protein immobilization, since H_6-mCherry, which only bears the polyhistidine tag, was immobilized poorly.

To demonstrate the applicability of this method to other protein classes, we investigated the immobilization of Rab proteins. Rab proteins play decisive roles in vesicular transport,[32,33] whereby Rab6A is involved in vesicular transport originating from the Golgi apparatus.[34] A suitable Rab6A variant with a C-terminal KRT sequence that incorporated a hexalysine stretch (Rab6A-6KRT) was generated and farnesylated in vitro. In this case, the hexalysine-based KRT-like C terminus posed no problems during expression. For immobilization, farnesylated Rab6A-KRT (Rab6A-6KRTFar) was spotted onto a thiol–PAMAM-functionalized SiO_x/Si slide together with Rab6A as a negative control and photoimmobilized as described above. The Rab6A microarray was then incubated with a fusion protein consisting of enhanced green fluorescent protein (eGFP) and the minimal Rab6A-binding domain of bicaudalD2 (eGFP–bicaudalD2);[34,35] this fusion protein could be detected by fluorescence scanning. Figure 3 d shows that Rab6A-6KRTFar was immobilized with high efficiency, whereas the nonspecific immobilization of Rab6A was minimal. Successful recognition by the Rab6A cognate effector bicaudalD2 clearly demonstrated that thiol-ene photoimmobilization yielded correctly folded and functional proteins on the slide surface.

With a view to determine whether this strategy would enable the immobilization of proteins obtained directly from crude cellular lysate, H_6-mCherry-4KRT expression lysate was generated and clarified by ultracentrifugation. H_6-mCherry-4KRT was then farnesylated in the lysate by using FTase and Fpp. The lysate was spotted onto a thiol-functionalized slide without any further purification together with purified H_6-mCherry-4KRTFar as a control. After exposure to UV light at 365 nm, H_6-mCherry-4KRTFar immobilization was confirmed by fluorescence scanning (Figure 4 a). Hence, the photoimmobilization of farnesylated, KRT-bearing proteins is possible directly from expression lysates, thereby obviating laborious purification steps. The nonspecific, covalent immobilization of other lysate proteins can be ruled out owing to the absence of KRT sequences.

To extend the method, we investigated the coexpression of KRT-modified proteins with FTase; this process would directly yield S-farnesylated expressed proteins owing to the presence of the genetically encoded CAAX box and thereby enable thiol-ene photoimmobilization directly from expression lysates without further purification or modification. *E. coli* synthesizes Fpp endogenously but does not have an endogenous protein farnesyltransferase. Therefore, a suitable vector incorporating both FTase subunits was coexpressed in *E. coli* together with the vector encoding H_6-mCherry-4KRT. SDS-PAGE analysis proved the overexpression of the three desired proteins (see Figure S1a in the Supporting Information). To determine whether sufficient *E. coli* Fpp and active FTase were available in the colysate, we generated a microarray with and without the addition of Fpp and FTase. As a modification, a longer thiol linker was used for surface functionalization, since with this linker photoimmobilization efficiency increased. Additionally, the UV-light-exposure time was extended to 20 minutes (12 J cm^{-2}). H_6-mCherry-4KRT lysate which did not contain any FTase was used as a control. The unmodified colysate as well as colysate with additional Fpp or Fpp/FTase showed high immobilization efficiency, whereas an approximately tenfold lower immobilization efficiency was observed for the control (Figure 4 b,c). These results demonstrate that the immobilization of KRT-modified proteins is possible directly from *E. coli* expression lysate after coexpression with FTase and by taking advantage of *E. coli* Fpp. The addition of Fpp and Fpp/FTase to the colysate did not improve immobilization efficiency, which indicates that sufficient amounts of FTase and endogenous Fpp were generated in vivo. The approach was also successfully extended to Ypt1, the yeast analogue of Rab1 (see Figure S7 in the Supporting Information).

In conclusion, the photochemical thiol-ene reaction between S-farnesyl groups attached to a genetically encod-

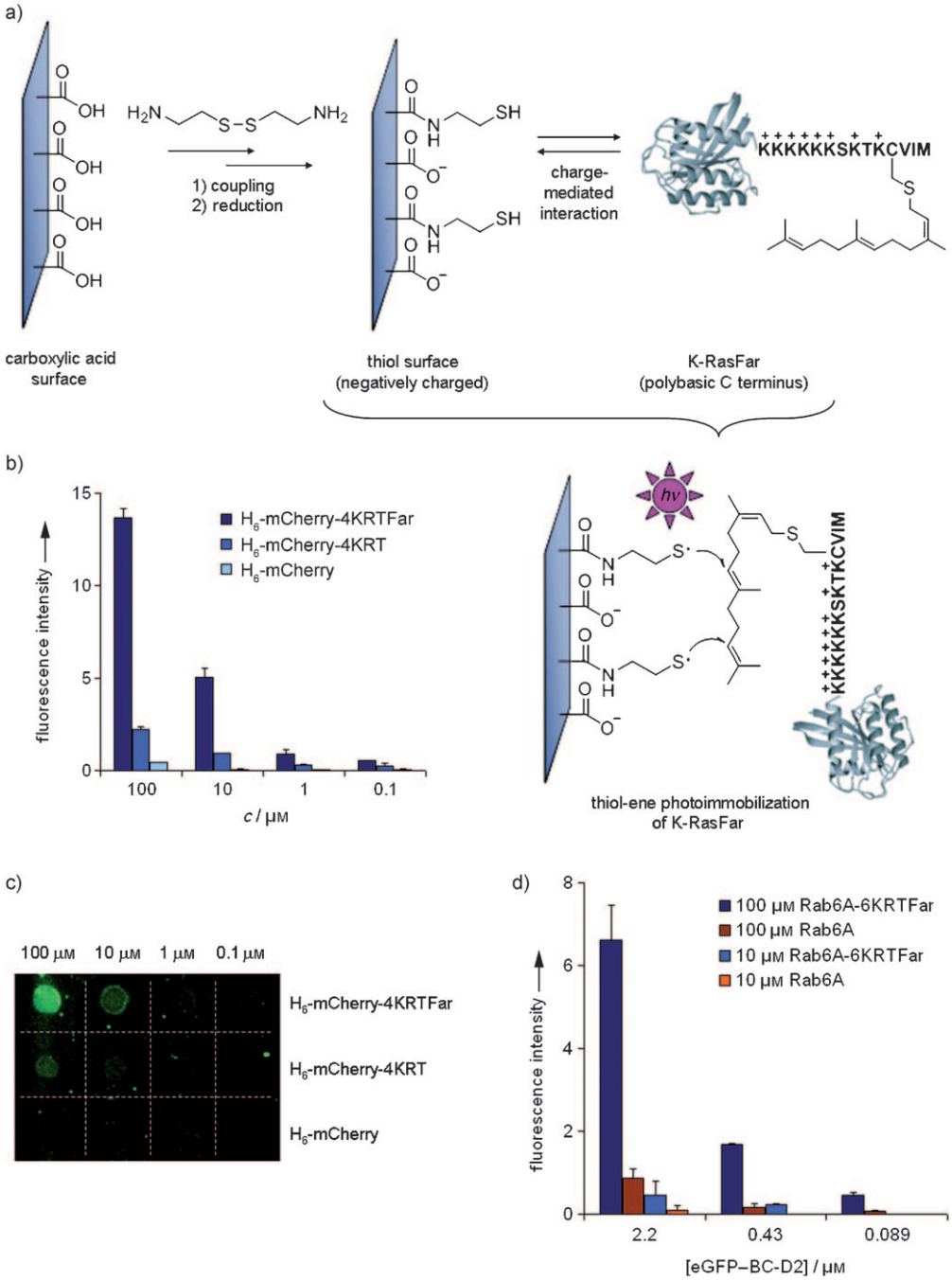

Figure 3. a) Preparation of thiol-functionalized slides from carboxylic acid functionalized slides and charge-mediated interaction with farnesylated K-Ras which could facilitate photoimmobilization. b) Fluorescence intensity observed for an H_6-mCherry-4KRTFar microarray with H_6-mCherry-4KRT and H_6-mCherry as controls. c) Fluorescence image of a subsection of the H_6-mCherry-4KRTFar microarray described in (b). d) Fluorescence intensity observed for a Rab6A-6KRTFar/eGFP–bicaudalD2 microarray with native Rab6A as a control.

able CAAX box at the C terminus of proteins and surface-exposed thiols on PAMAM-functionalized surfaces provides a method for the oriented and covalent immobilization of functional proteins under mild conditions in as little as 10 minutes. The natural farnesyl residue can be introduced into proteins by enzymatic farnesylation in vitro or in vivo by means of coexpression with FTase and by taking advantage of the endogenous *E. coli* Fpp pool. This method opens up the opportunity to immobilize proteins directly from expression lysates without additional isolation, purification, or chemical-derivatization steps otherwise required for oriented and covalent immobilization.

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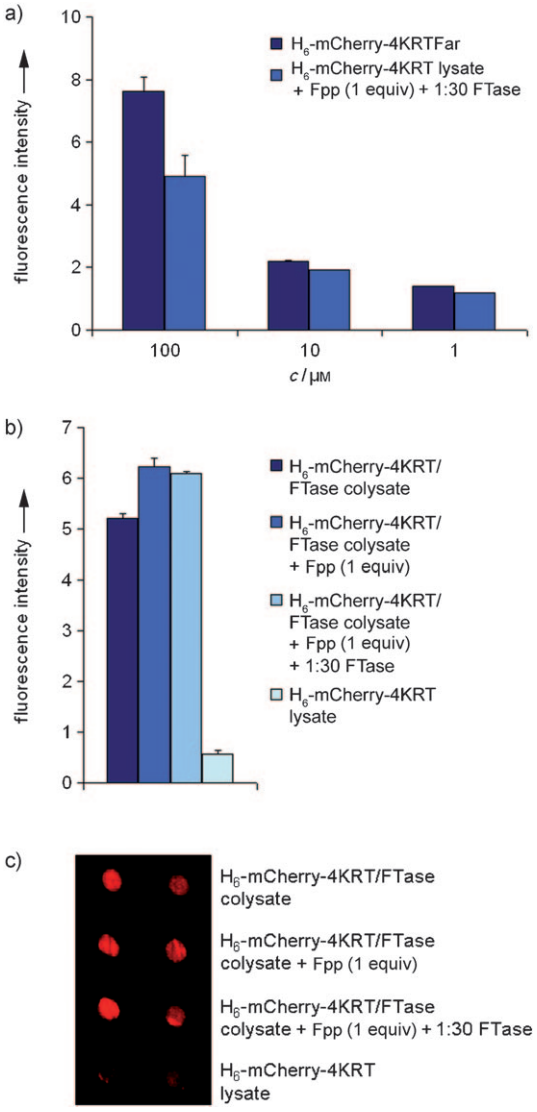

Figure 4. a) Fluorescence intensity observed for an H_6-mCherry4-KRTFar lysate microarray with purified H_6-mCherry-4KRTFar as a control. b) Comparison of the immobilization of H_6-mCherry-4KRT/FTase coexpression lysate (H_6-mCherry-4KRT: 78 μM) and H_6-mCherry-4KRT lysate (75 μM) on a microarray. Additional Fpp and a combination of Fpp and FTase were added to two colysate samples. c) Fluorescence image of a subsection of the colysate microarray described in (b).

Keywords: farnesylation · immobilization · photochemical reactions · protein microarrays · thiol-ene reaction

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