Immobilisation of engineered molecules on electrodes and optical surfaces

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Abstract

Monolayers of genetically modified proteins with an hexahistidine tag, (His)\textsubscript{6}, were obtained by using a Ni–NTA chelator synthesized on gold-sputtered surfaces (via sulphide bonds), or on gold and graphite (via sililating agents) working electrodes of screen-printed devices.

Two kinds of proteins were produced and purified for this study:

(a) a recombinant antibody, derived from the ‘single-chain Fv’ (scFv) format, and
(b) a photosystem II (PSII) core complex isolated from the mutant strain CP43-H of the thermophilic cyanobacterium \textit{Synechococcus elongatus}.

An scFv previously isolated from a synthetic ‘phage display’ library was further engineered with an alkaline phosphatase activity genetically added between the carboxy-terminal of the scFvs and the (His)\textsubscript{6} to allow direct measurement of immobilisation.

Renewable specific binding of (His)\textsubscript{6} proteins to gold and graphite surfaces and fast and sensitive electrochemical or optical detection of analytes were obtained. Additionally, “on chip” protein preconcentration was conveniently achieved for biosensing purposes, starting from crude unpurified extracts and avoiding protein purification steps.

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1. Introduction

Biomolecules modified by genetic engineering represent a new and powerful approach for obtaining simpler artificial structures with new or improved properties (i.e. specificity, stability, sensitivity) useful for biosensors development. For instance, insertion of specific molecular tags can be used for oriented and reversible immobilization, preconcentration and purification, whereas, easier and sensitive analytical detection can be achieved by gene fusion with selected enzyme activities. Moreover, antibody-derived fragments, like the single-chain Fv antibody fragment ‘scFv’ [1,3], offer the necessary sensitivity required for a sensing element, and new desired specificities can be isolated from ‘phage display’ antibody libraries through the ‘panning’ procedure [7,9].

In this paper, an original procedure for synthesizing functional groups (Ni–NTA) suitable for immobilisation of engineered (His)\textsubscript{6} proteins on different sensor materials (gold, plastic, graphite, glass, quartz) are presented for gold and graphite surfaces, and evaluated in terms of specific binding and activity of two kinds of different engineered proteins: an scFv-derived antibody and a PSII core complex. An scFv already isolated from a synthetic phage display library [2] and characterized for binding activity (Villani et al., unpublished) was further engineered at the C-terminus in order to add an alkaline phosphatase (AP) activity before the (His)\textsubscript{6} tag, obtaining the fusion protein scFv–AP–(His)\textsubscript{6}.

The fusion protein was produced in the bacterial periplasm and used, rough or purified, for optimal (oriented) immobilization through the synthesized Ni–NTA chains on gold and graphite surfaces. No loss of functional properties (ability to bind the antigen) was observed, indicating suitability of recombinant antibodies as sensing molecular tools for bio-
sensors. The engineered PSII core complex, containing an (His)$_{6}$ tag, was also purified and immobilised by using the same Ni–NTA chelator on gold surfaces. The engineered PSII–(His)$_{6}$-derived molecule represent a further step for the development of new PSII-based sensing devices [5,6].

2. Materials and methods

2.1. Preparation of the scFv–AP–(His)$_{6}$

2.1.1. Preparation of the scFv–AP–(His)$_{6}$ expression plasmid

The scFv (CMV-G4) bacterial clone, expressing soluble scFv, was picked out from selection against a plant virus (cucumber mosaic virus, CMV) [2]. The scFv gene sequence was digested from the original pDN332 phagemid and subcloned as a Sfi–Not fragment in the pDAP2 expression vector (CODON Genetic System) for the production of a bifunctional protein, scFv–AP–(His)$_{6}$, with both antigen-binding activity, as well as AP activity and (His)$_{6}$ [4].

2.1.2. Expression of the scFv–AP–(His)$_{6}$ fusion protein

Competent TG1 *Escherichia coli* cells were transformed with the ligation product and cultured at 37 °C for 16 h on 2 × YT agar (1.5%) medium containing 100 μg/ml of ampicillin and 2% glucose (2 × YT-AG). Single colonies were subjected to PCR screening. After a mini-induction experiment (in order to assess the ability of the fusion protein to bind the antigen in ELISA and the presence of AP activity), a positive clone was picked and cultured overnight at 30 °C for maxi-induction. The culture was diluted to OD$_{600}$ = 0.05 in 1 l of 2 × YT-AG and grown at 30 °C with vigorous shaking (250 rpm). At OD$_{600}$ of about 0.8–0.9, the cells were collected by centrifugation and resuspended in 1 l of 2 × YT containing 100 μg/ml ampicillin and 1 mmol/l IPTG (isopropyl-1-thio-β-D-galactopyranoside) to induce protein expression. The cells were then cultured for 16 h at 30 °C. After centrifugation at 3000g for 20 min, the bacterial pellet was resuspended in ice-cold TES medium (0.2 mol/l Tris–HCl, pH 8.0, 0.5 mmol/l EDTA, 0.5 mol/l sucrose, 1 ml TES for 100 ml initial culture) containing protease inhibitors (Roche). After adding water-diluted TES (1:5, 1.5 ml for 100 ml initial culture), the cells were incubated on ice for 30 min and then centrifuged at 4000g for 25 min. The supernatant (Periplasmic Extract fraction 1, EP1) was collected. Pellets were resuspended in 15 ml 5 mmol/l MgSO$_{4}$ containing protease inhibitors and shaken for additional 10 min before further extraction. The supernatant EP2 was collected after centrifugation at 20,000g for 20 min.

2.1.3. Purification of the scFv–AP–(His)$_{6}$ fusion protein

Fractions EP1 and EP2 were independently concentrated by ultrafiltration on a Diaflo YM10 membrane (Amicon) and subjected to immobilised-metal affinity chromatography (IMAC) using Ni–NTA (QIagen), following the procedure recommended by the manufacturer. Eluted fractions were collected, dialyzed against PBS and quantified by reading the absorbance at 280 nm. Protein purity was checked by SDS-PAGE (12% w/v) followed by Coomassie staining. Approximately 360 μg of purified protein was obtained from 1 l of bacterial culture.

2.2. Purification of PSII–(His)$_{6}$ core complex

Thermophilic cyanobacterial *S. elongatus* 43 H cells expressing psbC with an (His)$_{6}$ extension were used for purification of PSII–(His)$_{6}$ core complexes [8]. The eluate from a Ni$^{2+}$-affinity column was diluted in 40 mmol/l MES/NaOH (pH 6.5), 100 mmol/l NaCl, 15 mmol/l CaCl$_{2}$, 15 mmol/l MgCl$_{2}$, 0.03% DM and 10% glycerol for reducing amount of imidazole, and then they were concentrated with a Centriprep-100 (Amicon, MA) to a chlorophyll concentration of 9.2 mg/ml (imidazole content was finally 12.5 mmol/l).

2.3. Immobilisation procedures

2.3.1. Deposition of thin gold layers on glass and plastic surfaces

In all experiments, thin transparent layers of gold were deposited by commercial sputter coaters. The best deposition procedure on glass was achieved as follows: 1 h washing of glass slides in cold ‘piranha’ solution (sulphuric acid/hydrogen peroxide 1:2), drying at 80 °C directly before the deposition. The gold layers deposited in this way were stable during the chelator synthesis and resistant even to nonpolar solutions (chloroform, 12 h). In the case of plastic support, gold was sputtered on both sides of the small circles (OD = 6 mm) cut from a laser printer transparent foil. The conditions for deposition were the same as in the case of glass slides, but they were not washed or chemically treated before deposition.

2.3.2. Synthesis of the Ni–NTA chelator and Ni$^{2+}$ assay

Two types of chelators were synthesized for (His)$_{6}$ protein immobilisation. The one with the long-arm spacer created by inserting lysine molecule into the chain and the other without spacer. Graphite thick films deposited by screen printing on PVC sheets were treated (1 h at room temperature) with 10% 3-aminopropyltriethoxysilane (3-APTES) in PBS for obtaining–NH$_{2}$ groups exposed out the surface. Glass or plastic slides with sputtered gold were treated with 20 mmol/l cysteamine dissolved in ethanol for at least 12 h and resulted in a self-assembled monolayer (SAM) with the –NH$_{2}$ groups exposed out the surface. These functional groups were used for further synthesis of both chelators. The synthesis followed the common scheme: (a) 10% glutaraldehyde in PBS, (b) 5% lysine, (c) 10% glutaraldehyde in PBS, (d) 5% Na$_{2}$-Na-bis (carboxymethyl)-l-lysine hydrate, (e) 1% NiSO$_{4}$. The chelator without spacer
was obtained by omitting steps (b) and (c). Each step of synthesis lasted 2 h. At the end of each step, samples were washed in PBS.

Then, Ni was eluted from the samples with 25% HNO₃ for determination by adsorptive cathodic stripping voltammetry (AdCSV) according to the DIN 38406E16 Standard Method for trace metals analysis. In a polarographic vessel, 0.5 ml of sample were added to 8 ml of water, 0.25 ml of DMG solution, 0.5 ml of ammonium chloride buffer, pH = 9.5 ± 0.2, and bubbled with nitrogen for 5 min. Ni content determination was carried out with three standard additions.

2.3.3. Immobilisation of scFv–AP–(His)₆ protein

The amount of fusion protein bound to the plastic and glass support was assayed after loading at 4 °C in the dark for 1 or 16 h. Then, the calibration curve of fusion enzyme activity was obtained and the maximum capacity of chelator groups determined. The saturating concentration of protein in the loading buffer was found to be 1 ng/µl. AP activity immobilised on the samples was determined at 405 nm using p-nitrophenylphosphate as a substrate. Electrochemical AP activity measurement was performed with screen-printed electrodes (Krejci Engineering) in a flow microcell as described in Fig. 1.

2.3.4. Immobilisation of PSII–(His)₆ core complex

The highest quantity of immobilised PSII–(His)₆ core complex on the surface of plastic and glass substrates was obtained by incubation in MES buffer solution (pH = 6.5) containing PSII–(His)₆ equivalent to 300 µg Chl/ml at 4 °C in complete darkness for 16 h. Chlorophyll fluorescence measurements were done on a spectrofluorometer (TRIAX 180, Jobin Yvon-Spex) at laboratory temperature. Fourier transform infrared (FTIR) spectra were also obtained (Perkin Elmer). In both cases, 40 scans were done for each measurement in at least four independent repetitions.

3. Results

The two procedures for chelator synthesis were compared in terms of quantity of Ni–NTA groups and in terms of specific and nonspecific binding capacity of (His)₆ proteins. Only data on plastic are reported because results were comparable in all experiments.

Ni²⁺ determination by AdCSV on the substrates revealed high amount of NTA groups with no significant differences between the chelator with or without lysine spacer (Table 1). Moreover, surface density of the NTA groups was higher compared to commercial Ni–NTA treated microtitre plates: 3-fold on graphite printed on PVC, 10-fold on sputtered gold (glass or plastic).

In order to perform direct measurements of immobilisation, an scFv fused to AP was used. Specific immobilisation of scFv–AP–(His)₆ through the (His)₆ was confirmed by the possibility of eluting the protein with 200 mmol/l imidazole and assuming the residual AP activity as a measure of nonspecific binding (Table 2). Again, no sig-

![Fig. 1. Immobilised AP activity checked electrochemically with a screen-printed gold electrode at +400 mV vs. Ag/AgCl in a flow microcell. (1 and 3) Current increased with substrate (2 mM ascorbate-2-phosphate) and recovered the baseline as soon as the buffer was injected (2). At the end of the recording, quick removal of the (His)₆ protein was also observed when imidazole was used as feeding solution together with the substrate (4).](image-url)
significant differences of the surface binding capacity were observed for both chelators. After immobilisation and elution with imidazole, the scFv fusion protein retained binding activity toward the antigen as confirmed by ELISA on CMV-coated microtitre plates.

The possibility of direct preconcentration of (His)$_6$ proteins on sensor surface (“on chip” preconcentration) using rough bacterial extracts was evaluated. “On chip” preconcentration was obtained after 16 h of substrate treatment and better results (lower nonspecific binding) were obtained with the substrate with chelator having a lysine spacer (Table 2) which showed an higher binding capacity (about fourfold) than commercial (His)$_6$ proteins sorbing microtitre plates [even higher, sevenfold, if the unfavourable surface area ratio of about 0.6:1 (Table 2, third column) is taken into account]. Milk (5%), BSA (5%) and cytochrome c (5%) solutions were used with the aim of blocking nonspecific binding of proteins, but in all cases, specific binding was also reduced.

AP activity of the scFv fusion protein was electrochemically determined after binding to a printed gold electrode with the chelator without spacer. The calibration curve of enzyme activity as well as washing out the protein with 200 mmol/l imidazole were determined, by using ascorbate-2-phosphate as a substrate for AP (Fig. 1). “On chip” preconcentration, starting from the crude bacterial extracts containing the engineered scFv fragment, was also possible with screen-printed gold electrodes (data not shown).

The presence and activity of PSII–(His)$_6$ was tested by photoreduction of 2,6-dichlorophenolindophenol (DCPIP, 20 mmol/l) at 620 nm after illumination of the samples for 45 min with white light at the laboratory temperature. In all cases, the decrease in absorption of oxidised form of DCPIP due to the activity of PSII–(His)$_6$ was observed, whether the samples used were with or without spacer. After washing of the PSII–(His)$_6$ loaded samples with 200 mmol/l imidazole, photoreduction of DCPIP was measured once again. In this way, results already obtained with scFv–AP protein were confirmed. A significant decrease of activity (nearly to zero) was observed. Contrary to the antibody fragment, the engineered PSII–(His)$_6$ core complex was nearly completely washed out and nonspecific binding was significantly lower. Experiments were well reproducible and PSII–(His)$_6$ core complex was not denatured during immobilisation since it did not lose oxygen evolving activity (Table 3). The amount of PSII–(His)$_6$ bound to the substrate without the spacer was higher compared to the substrate with the lysine spacer.

The presence of PSII–(His)$_6$ was also confirmed by measuring fluorescence in the region between 600 and 700 nm. The sample (PSII–(His)$_6$ on plastic or glass) was illuminated with blue light (400–530 nm) and fluorescence detected against the blank (plastic, glass with chelator without PSII–(His)$_6$). In all cases, the typical fluorescence

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Chemical treatment</th>
<th>Ni$^{2+}$ surface density (ng/cm$^2$) ± S.D. (n = 3)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputtered gold on plastic</td>
<td>blank</td>
<td>4.3 ± 0.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Sputtered gold on plastic</td>
<td>spacer</td>
<td>122 ± 3.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Printed graphite</td>
<td>no spacer</td>
<td>27</td>
<td>–</td>
</tr>
<tr>
<td>Commercial microtitre plates</td>
<td>–</td>
<td>10</td>
<td>–</td>
</tr>
</tbody>
</table>

DP-voltammogram at the HMDE. Deposition potential = – 0.8 V; deposition time = 90 s, start potential = – 0.80 V; final potential = – 0.45 V; DP amplitude = – 50 mV. The Ni peak potential is at – 0.97 V vs. Ag/AgCl.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>AP activity of immobilised scFv–AP–(His)$_6$ before and after washing with imidazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputtered gold on plastic with spacer</td>
<td>scFv loading with purified protein</td>
<td>0.6</td>
</tr>
<tr>
<td>Sputtered gold on plastic without spacer</td>
<td>scFv loading with purified protein</td>
<td>0.6</td>
</tr>
<tr>
<td>Sputtered gold on plastic with spacer</td>
<td>washing with 200 mmol/l imidazole</td>
<td>0.6</td>
</tr>
<tr>
<td>Sputtered gold on plastic without spacer</td>
<td>washing with 200 mmol/l imidazole</td>
<td>0.6</td>
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Table 1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Chemical treatment</th>
<th>Ni$^{2+}$ surface density (ng/cm$^2$) ± S.D. (n = 3)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputtered gold on plastic</td>
<td>spacer</td>
<td>122 ± 3.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Sputtered gold on plastic</td>
<td>no spacer</td>
<td>114 ± 2.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Printed graphite</td>
<td>no spacer</td>
<td>27</td>
<td>–</td>
</tr>
<tr>
<td>Commercial microtitre plates</td>
<td>–</td>
<td>10</td>
<td>–</td>
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Table 2

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<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>OD$_{620}$ ± S.D. (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputtered gold on plastic</td>
<td>complex loading</td>
<td>0.032 ± 0.013</td>
</tr>
<tr>
<td>Sputtered gold on plastic</td>
<td>after washing with imidazole 200 mmol/l</td>
<td>0.006 ± 0.007</td>
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Table 3

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<th>Substrate</th>
<th>Treatment</th>
<th>OD$_{405}$ ± S.D. (n = 3)</th>
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<tbody>
<tr>
<td>Sputtered gold on plastic</td>
<td>complex loading</td>
<td>0.047 ± 0.006</td>
</tr>
<tr>
<td>Sputtered gold on plastic</td>
<td>after washing with imidazole 200 mmol/l</td>
<td>0.007 ± 0.006</td>
</tr>
</tbody>
</table>

For the substrate without spacer after washing with imidazole 200 mmol/l.

* Absolute value of OD$_{280}$ decreases with respect to the blank sample.
curve of the PSII–(His)$_6$ core complex was observed (Fig. 2) with a maximum at about 685 nm and a longer-wavelength (725 nm), broad shoulder (the shape of curve was compared to the one obtained with the PSII–(His)$_6$ core complex in solution). A decrease of fluorescence was observed after washing the sample with the imidazole solution. Good reproducibility and high signal reached in this experiment confirmed the applicability of this procedure for immobilisation of PSII and preparation of optical biosensing devices (for example, using fiber optics).

PSII–(His)$_6$ immobilisation was observed with FTIR absorption in case of glass samples in reflectance mode. Sample was let to dry before measurement at laboratory temperature and difference spectra were recorded before and after PSII–(His)$_6$ loading. Typical absorption bands (amid I and amid II bands) in the 1000–2000 cm$^{-1}$ regions were obtained.

In addition, amperometric detection of PSII–(His)$_6$ activity with a screen-printed gold electrode polarised at $+600$ mV vs. Ag/AgCl and in presence of duroquinone as artificial electron acceptor was achieved (data not shown).

4. Conclusions

Higher Ni–NTA loading was obtained on both graphite and gold surfaces compared to commercial products. Similar results with both (His)$_6$ proteins are good proofs of possible universal use of this procedure for sensing purposes; it can be applied to several technological materials by changing the first step of chemical treatment. Imidazole can be specifically used for washing out the sensing molecule, for renewing it or replacing the biomediator depending on the analysis requirements. Slight difference in nonspecific binding and in length of the optimal chelator arm can be expected using different (His)$_6$ proteins. Finally, “on chip” protein preconcentration was conveniently achieved for biosensing purposes, starting from crude unpurified extracts and avoiding time-consuming and expensive protein purification steps.

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