

Immobilisation of engineered molecules on electrodes and optical surfaces

J.Maly¹, E.Illiano, M.Sabato, M.De Francesco, V.Pinto, A.Masci, D.Masci, J.Masojidek², M.Sugiura³,
R.Franconi and R.Pilloton

ENEA-CR-Casaccia Via Anguillarese 301, 00060 S.Maria di Galeria Rome, Italy

¹Department of Biology, University of J.E.Purkyne, 40001 Usti nad Labem, Czech Republic

²Inst. of Microbiology, Academy of Sciences, 379 81 Trebon, Czech Republic

³Osaka Prefecture University, Osaka, Japan

ABSTRACT

Monolayers of genetically modified proteins with an hexahistidine tag, (His)₆, were obtained by using a Ni-NTA chelator synthesized on gold sputtered surfaces (via sulphide bonds), or on gold and graphite (via siliating 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;
- b) a photosystem II (PSII) core complex isolated from the mutant strain CP43-H of the thermophilic cyanobacterium *Synechococcus elongatus*.

An scFv, previously isolated from a synthetic 'phage display' library, was further engineered with an alkaline phosphatase activity genetically added between the carboxi-terminal of the scFvs and the (His)₆ to allow direct measurement of immobilisation.

Renewable specific binding of (His)₆-proteins to gold and graphite surfaces and fast and sensitive electrochemical or optical detection of analytes were obtained. Additionally, "on chip" protein pre-concentration was conveniently achieved for biosensing purposes, starting from crude unpurified extracts and avoiding protein purification steps.

Keywords: biosensors, gold, self assembled monolayers, recombinant antibodies, histidine tag, photosystem II

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' (Bird *et al.*, 1988; Huston *et al.*, 1988), 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 (Smith & Scott, 1993; Winter *et al.* 1994).

In this paper an original procedure for synthesizing functional groups (Ni-NTA) suitable for immobilisation of engineered (His)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 (Desiderio *et al.*, 2001) 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)₆ tag, obtaining the fusion protein scFv-AP-(His)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 biosensors. 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 (Koblizek et al, 1999; Koblizek et al,2001).

MATERIALS AND METHODS

a) Preparation of the scFv-AP-(His)₆

-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) (Desiderio *et al.*, 2001). The scFv gene sequence was digested from the original pDN332 phagemid and subcloned as a *SfiI-NotI* fragment in the pDAP2 expression vector (CODON genetic system GmbH), for the production of a bifunctional protein, scFv-AP-(His)₆, with both antigen binding activity as well as AP activity, and an (His)₆ (Kerschbaumer *et al.*, 1996).

-Expression of the scFv-AP-(His)₆ fusion protein: Competent TG1 *E. coli* cells were transformed with the ligation product and cultured at 37°C for 16h on 2xYT agar (1.5%) medium containing 100 µg/ml of ampicillin and 2% glucose (2xYT-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 OD₆₀₀=0.05 in 1L of 2xYT-AG and grown at 30°C with vigorous shaking (250rpm). At OD₆₀₀ of about 0.8-0.9, the cells were collected by centrifugation and resuspended in 1L of 2xYT containing 100µg/ml ampicillin and 1mmol/L IPTG (isopropyl-1-thio-β-D-galactopiranoside) to induce protein expression. The cells were then cultured for 16h at 30°C. After centrifugation at 3000g for 20min, the bacterial pellet was resuspended in ice-cold TES medium (0.2mol/L Tris-HCl pH 8.0, 0.5mmol/L EDTA, 0.5mol/L sucrose, 1ml TES for 100ml initial culture), containing protease inhibitors (Roche). After adding water-diluted TES (1:5, 1.5ml for 100ml initial culture), the cells were incubated on ice for 30min and then centrifuged at 4000g for 25min. The supernatant (Periplasmic Extract fraction 1, EP1) was collected. Pellets were resuspended in 15ml 5mmol/L MgSO₄, containing protease inhibitors, and shaken for additional 10min before further extraction. The supernatant EP2 was collected after centrifugation at 20000g for 20min.

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, dialized against PBS and quantified by reading the absorbance at 280nm. Protein purity was checked by SDS-PAGE (12%w/v) followed by Coomassie staining. Approximately 360 µg of purified protein was obtained from 1L of bacterial culture.

b) Purification of PSII-(His)₆ core complex

Thermophilic cyanobacterial *Synechococcus elongatus* 43H cells expressing psbC with an (His)₆ extension were used for purification of PSII-(His)₆ core complexes (Sugiura *et al.* 1999). The eluate from a Ni²⁺-affinity column was diluted in 40mmol/L MES/NaOH (pH 6.5), 100mmol/L NaCl, 15mmol/L CaCl₂, 15mmol/L MgCl₂, 0.03% DM and 10% glycerol for reducing amount of imidazole, and then they were

concentrated with a Centriprep-100 (Amicon, Inc., MA) to a chlorophyll concentration of 9.2mg/mL (imidazole content was finally 12.5mmol/L).

c) Immobilisation procedures

-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: 1h 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 non-polar solutions (chloroform, 12h). In the case of plastic support, gold was sputtered on both sides of the small circles (o.d.=6mm) 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.

-Synthesis of the Ni-NTA chelator and Ni²⁺ 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 (1h at room temperature) with 10% 3-aminopropyltriethoxysilane (3-APTES) in PBS for obtaining -NH₂ groups exposed out the surface. Glass or plastic slides with sputtered gold were treated with 20mmol/L cysteamine dissolved in ethanol for at least 12h and resulted in a self assembled monolayer (SAM) with the -NH₂ 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% N α -N α -bis(carboxymethyl)-L-Lysine Hydrate, e) 1% NiSO₄; the chelator without spacer was obtained by omitting steps (b) and (c); each step of synthesis lasted 2h. At the end of each step samples were washed in PBS.

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

-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 16h. 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 1ng/ μ L. AP activity immobilised on the samples was determined at 405nm using p-nitrophenylphosphate as a substrate. Electrochemical AP activity measurement was performed with screen printed electrodes (Krejci Engineering, Ltd) in a flow μ -cell as described in fig.1.

-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 μ gChl/mL at 4°C in complete darkness for 16h. 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.

RESULTS

The two procedures for chelator synthesis were compared in terms of quantity of Ni-NTA groups and in terms of specific and non-specific 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 (tab.1). Moreover, surface density of the NTA groups was higher compared to commercial Ni-NTA treated μ -titre-plates: threefold on graphite printed on PVC, tenfold on sputtered gold (glass or plastic).

Table 1: Ni²⁺ surface density (ng/cm²) on synthesized and commercial surfaces determined by AdCSV

Substrate	chemical treatment	Ni ²⁺ surface density (ng/cm ²) _{±s.d.} (n=3)*	C.V.
sputtered gold on plastic	blank	4.3 _{±0.1}	2,3%
	spacer	122 _{±3.0}	2.6%
	blank	4.4 _{±0.2}	4.5%
	no spacer	114 _{±2.0}	1.7%
printed graphite ink on PVC	no spacer	27	-
commercial μ -titre-plates	-	10	-

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

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 200mmol/L imidazole and assuming the residual AP activity as a measure of non-specific binding (Tab.2). Again, no significant differences of the surface binding capacity were observed for both chelators,

Table 2: AP activity of immobilised scFv-AP- (His)₆ before and after washing with imidazole

Substrate	Treatments	surface area	OD ₄₀₅ _{±s.d.} (n=3)	C.V.
sputtered gold on plastic with spacer	scFv loading with purified protein	0.6cm ²	0.563 _{±0.030}	5.3%
	washing with 200mmol/L imidazole	0.6cm ²	0.153 _{±0.010}	6.5%
sputtered gold on plastic without spacer	scFv loading with purified protein	0.6cm ²	0.679 _{±0.010}	6.8%
	washing with 200mmol/L imidazole	0.6cm ²	0.230 _{±0.030}	13.0%
sputtered gold on plastic with spacer	scFv loading with bacterial extracts (" on chip" pre-concentration)	0.6cm ²	0.061 _{±0.012}	19.7%
sputtered gold on plastic without spacer		0.6cm ²	0.004 _{±0.002}	50.0%
commercial Ni-NTA coated μ -titre-plates		1.0cm ²	0.014 _{±0.003}	21.4%

After immobilisation and elution with imidazole, the scFv-fusion protein retained binding activity toward the antigen as confirmed by ELISA on CMV-coated μ -titre-plates.

The possibility of direct pre-concentration of (His)₆-proteins on sensor surface ("on chip" pre-concentration) using rough bacterial extracts was evaluated. "On chip" pre-concentration was obtained after 16h of substrate treatment and better results (lower non-specific binding) were obtained with the substrate with chelator having a lysine spacer (Tab.2) which showed an higher binding capacity (about fourfold) than commercial (His)₆-proteins sorbing μ -titre-plates (even higher, sevenfold, if the unfavourable surface area ratio of about 0.6:1, tab.2, 3rd column, is taken into account). Milk (5%) BSA (5%) and cytochrome c (5%) solutions were used with the aim of blocking non-specific 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 200mmol/L imidazole were determined, by using ascorbate-2-phosphate as a substrate for AP (fig.1). "On chip" pre-concentration, 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, 20mmol/L) at 620 nm after illumination of the samples for 45min 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, no matter whether the samples with or without spacer were used. After washing of the PSII-(His)₆ loaded samples with 200mmol/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 non-specific binding was significantly lower. Experiments were well reproducible and PSII-(His)₆ core complex was not denaturated during immobilisation since it did not lose oxygen evolving activity (tab.3). The amount of PSII-(His)₆ bound to the substrate without the spacer was higher compared to the substrate with the lysine spacer.

Table 3: PSII-(His)₆ core complex activity measured by photoreduction of DCPIP before and after washing with imidazole

Substrate	Treatment	OD ₆₂₀ *+s.d. (n=3)
sputtered gold on plastic with spacer	PSII-(His) ₆ core omplex loading	0.032 _± 0.013
	after washing with imidazole 200mmol/L	0.006 _± 0.007
sputtered gold on plastic without spacer	PSII-(His) ₆ core omplex loading	0.047 _± 0.006
	after washing with imidazole 200mmol/L	0.007 _± 0.006

*absolute value of OD₆₂₀ decrease with respect to the blank sample

The presence of PSII-(His)₆ was also confirmed by measuring fluorescence in the region between 600 and 700nm. 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 curve of the PSII-(His)₆ core complex was observed (fig.2) with a maximum at about 685nm and a longer-wavelength (725nm), 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.

Also amperometric detection of PSII-(His)₆ activity with a screen printed gold electrode polarised at +600mV vs Ag/AgCl and in presence of duroquinone as artificial electron acceptor was achieved (data not shown).

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 a good proof 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 non-specific binding and in length of the optimal chelator arm can be expected using different (His)₆-proteins. Finally, "on chip" protein pre-concentration was conveniently achieved for biosensing purposes, starting from crude unpurified extracts and avoiding time consuming, and expensive protein purification steps.

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Legends of figures

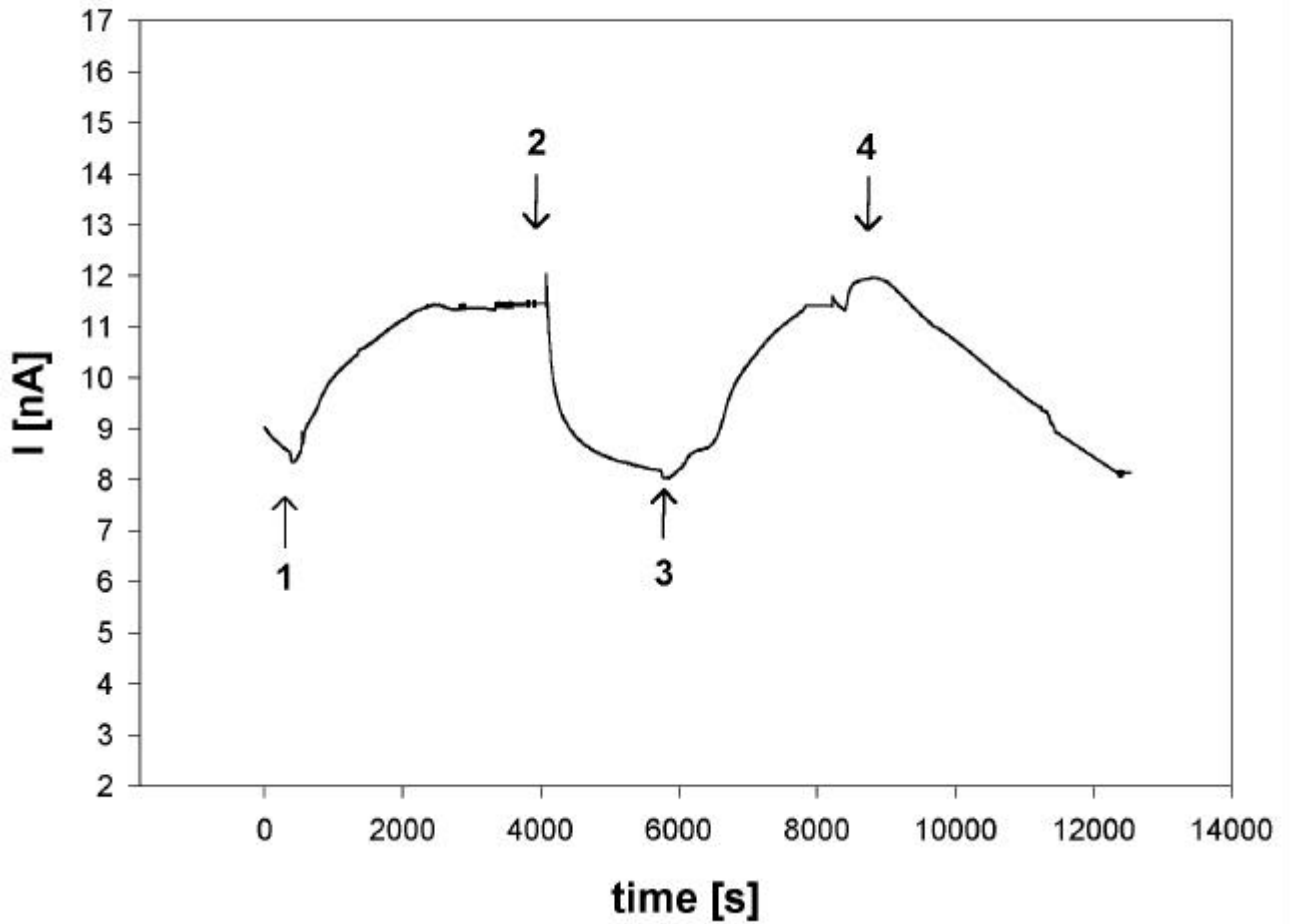


Figure 1: Immobilised AP activity checked electrochemically with a screen printed gold electrode at +400mV vs Ag/AgCl in a flow μ -cell. (1) and (3) Current increased with substrate (2mM ascorbate-2-phosphate) and recovered the baseline as soon as the buffer was injected (2). At the end of the recording also quick removal of the (His)₆-protein was observed when imidazole was used as feeding solution together with the substrate (4).

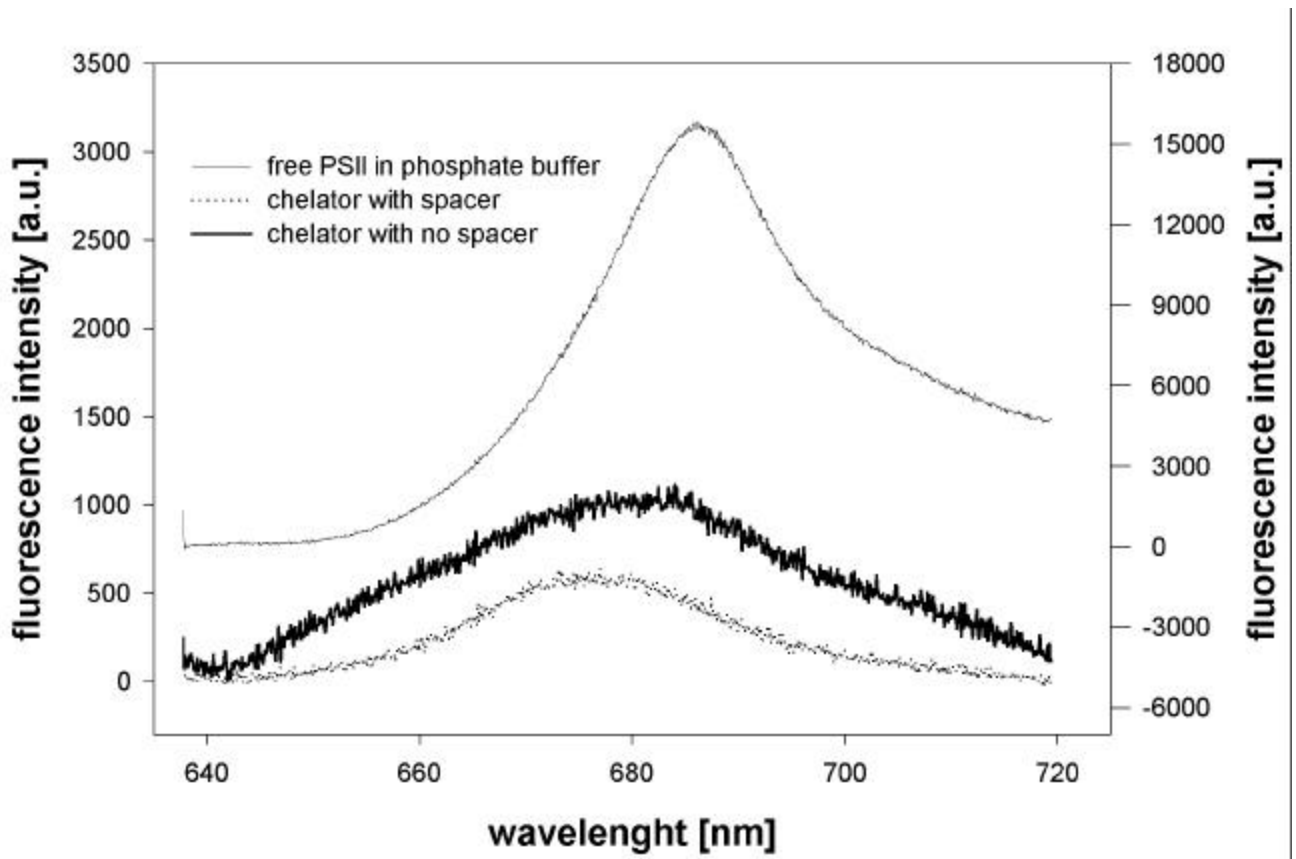


Figure 2: Fluorescence spectra of PSII-(His)₆ core complex immobilised on plastic sheets by using Ni²⁺ chelator (40 scans in four independent repetitions).