Acta Biochimica Polonica

Vol. 50 No. 4/2003

1155–1163

QUARTERLY

Application of electrophoretic methods for detection of protein–porphyrin complexes✪

Agnieszka Kowalska[1]✉, Piotr Kwiek[2], Ewa Miłosz[1], Grzegorz Gondek[2], Anna Romiszewska[3], Alfreda Graczyk[3] and Anna J. Podhajska[1]

[1]*Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Gdańsk, Poland;* [2]*Department of Acousto-optics and Laser Physics, Institute of Experimental Physics, Faculty of Mathematics, Physics and Informatics, University of Gdańsk, Gdańsk, Poland;* [3]*Institute of Optoelectronics, Military University of Technology, Warszawa, Poland*

Received: 13 October, 2003; revised: 26 November, 2003; accepted: 09 December, 2003

Key words: PDT, photodynamic therapy, electrophoresis, porphyrins

Simple methods for detection and isolation of protein–porphyrin complexes were elaborated in our laboratory. They are based on the separation of protein–porphyrin complexes in native polyacrylamide gel and measurement of their fluorescence, with the use of two detection systems: the commercially available Gel Doc™ 2000 system, and a system specially designed for the purpose of these investigations, concerning protein–porphyrin interactions. The fluorescent complexes can be electro-transferred from the gel onto PVDF membrane, eluted and analyzed in order to identify the protein interacting with porphyrins.

Photodynamic diagnosis (PDD) and therapy (PDT) are very promising methods in the therapy of cancer and some other diseases. PDT is based on the dye-dependent photo-oxidation of biological targets in the treated tissues (Henderson & Dougherty, 1992; Vrouenraets *at al.*, 2003). The first step is the intravenous, topical or oral administration of a photosensitizer followed by irradiation at the appropriate wavelength (in the red), which initiates a number of oxidation reactions, leading to target destruction.

Although many different substances have been investigated in order to improve the effi-

✪This work was supported by the State Committee for Scientific Research (KBN, Poland) grants: PB/0010-1-2601-9 and DS/B000-4-0010-1.

✉Corresponding author: tel./fax: (48 58) 301 2807; e-mail: agak@biotech.univ.gda.pl

Abbreviations: HDL, high density lipoprotein; HS, human serum; HSA, human serum albumin; PAG, polyacrylamide gel; PDD, photodynamic diagnosis; PDT, photodynamic therapy; $PP(AA)_2Arg_2$; L-arginine di(amino acid) protoporphyrinate; $PP(Thr)_2Arg_2$, L-arginine di(*N*-threonyl) protoporphyrinate; $PPArg_2$, L-arginine protoporphyrinate; PVDF, polyvinylidene difluoride.

ciency and applicability of PDT (Gomer, 1991; Woodburn *at al.*, 1992; Sternberg & Dolphin, 1998; Kelty *at al.*, 2002), the most important role in PDT is played by porphyrins and their derivatives. Photofrin®, a commercial photosensitizing agent approved in several countries (including the U.S.A., Japan and Canada) for the treatment of various types of cancer, is a mixture of porphyrin derivatives (Dougherty *et al.*, 1987).

New derivatives of protoporphyrin IX are being investigated in our laboratory (Fig. 1). These photosensitizers were obtained by attaching two amino-acid residues to the porphyrin ring to improve the anti-tumor efficacy of such derivatives. The substances were also additionally modified with two arginines to

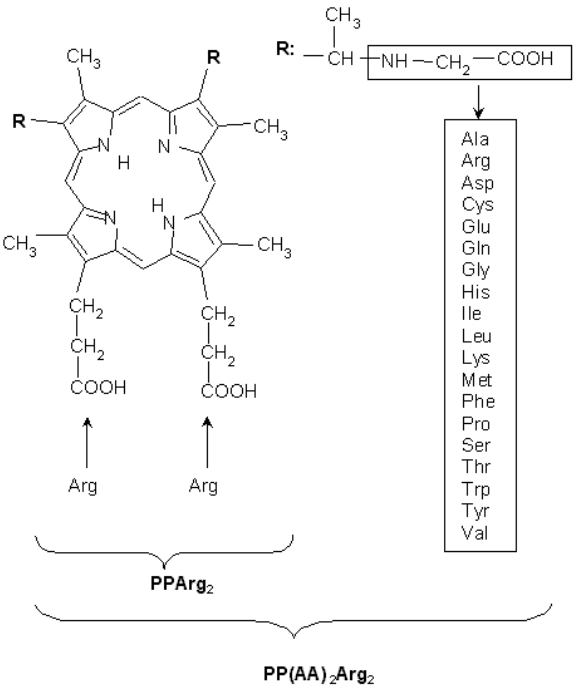


Figure 1. Structure of the amino-acid protoporphyrin derivatives: $PPArg_2$ and $PP(AA)_2Arg_2$ (AA indicates an amino acid substituted at the R positions in the protoporphyrin molecule).

improve their solubility in physiological solutions. The preliminary studies performed on both cell lines and living organisms proved them to be very promising and effective photosensitizers (Konarski *et al.*, 1987; Graczyk & Konarski, 1995; Żołądek *et al.*, 1997).

Despite a significant progress in the research and application of PDT in recent years, the phenomena occurring at the molecular level in PDT-treated tissues and cells are still poorly understood. Therefore, in this paper we present relatively simple methods that were devised to detect *in vitro* and identify proteins (e.g. those present in body fluids such as serum) able to bind porphyrins exemplified by the new protoporphyrin IX derivatives. Detection of the protein–porphyrin complexes is possible by measuring their fluorescence directly in polyacrylamide gels.

MATERIALS AND METHODS

Reagents. L-arginine protoporphyrinate IX ($PPArg_2$) and its diamino acid derivative, having an additional threonine substituents at the protoporphyrin ring ($PP(Thr)_2Arg_2$), were prepared in the Military University of Technology (MUT, Warsaw, Poland) (Graczyk & Konarski, 1995). All other chemicals, human serum and human serum proteins were purchased from Sigma-Aldrich Co.

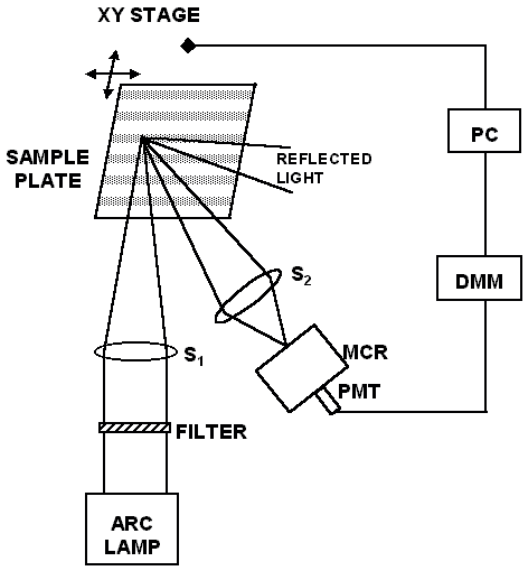
Detection of protein–porphyrin complexes in polyacrylamide gels. To detect protein–porphyrin complexes, mixtures of those compounds were separated in native polyacrylamide gels and the fluorescence of the complexes formed was measured using one of the following detection systems: (1) SYS I – the commercially available Gel Doc™ 2000 gel documentation system (Bio-Rad Laboratories Inc., U.S.A.) additionally equipped with a red filter, and (2) SYS II – an instrumentation set specially designed for the purpose of our investigations. Finally, gels were stained with Coomassie Brilliant Blue and documented using the Gel Doc™ 2000 system or were used for other purposes.

Electrophoresis. Samples containing serum albumin, other serum proteins or whole serum were incubated in 5 mM Hepes, pH

7.4, with or without a given porphyrin derivative, for 2 h at 4°C in the dark. Then, the samples were separated by native polyacrylamide gel electrophoresis (PAGE). The gels (size 11 × 13 cm, 1 mm thick) were prepared according to standard procedures, without the addition of SDS or β-mercaptoethanol. Gradient in the gels was non-linear. Electrophoresis was conducted for about three hours in a vertical apparatus at room temperature, at 120 V, in the dark. Then, the fluorescence was measured using the following detection systems:

System I. In SYS I the porphyrins in the gel were excited using UV light (302 nm) from a transilluminator (Gel Doc™ 2000). Having passed through an amber filter (Gel Doc™ 2000) and a red absorption filter (high-pass filter $\lambda > 600$ nm, MUT, Warsaw, Poland), fluorescence was measured by means of a CCD camera (Gel Doc™ 2000). With very weak signals longer exposures assured more reproducible results. Images were saved using the Quantity One® software.

System II. The diagram of SYS II is presented in Scheme 1. Glass-plates with the gel were placed in an XY – scanning system (line tables, Isel Automation, Eiterfeld, Germany). The porphyrins in the gel were excited at 405 nm from a high-pressure mercury ARC LAMP (HBO 200 Watt, Osram GmbH, Berlin, Germany), projected through an interference FILTER (405 nm ± 11 nm) and focused on the gel by the lens S_1. The gel was illuminated at an angle of about 45°. The fluorescent light from the sample was directed onto the entrance slit of the monochromator MCR (SPM2, Carl Zeiss Jena, Jena, Germany) by the S_2 lens. The geometry of the setup ensured a high signal-to-noise ratio. To register the fluorescence at 630 nm (resolution ± 2 nm), a photomultiplier PMT (M12FCC51, Carl Zeiss Jena, Jena, Germany) was used. The photomultiplier was located at the exit of the monochromator with a glass prism (G60). The exit slit had a size of 0.1 × 6 mm. The signal from the photomultiplier was measured with a multimeter DMM (PM2525, Philips, Eindhoven, Netherlands) with a standard communication interface (GPIB, General Purpose Interface Bus) that transmitted data to a computer PC. The computer kept additional control of the XY-scanning system, so that shifting of the gel and the fluorescence measurement were coordinated. Automatic fluorescence measurement in gel lanes with a spatial resolution of 0.1 mm was possible. The scanning step was 0.5 mm.



Scheme 1.

Blotting and elution of complexes. Electro-transfer of proteins and protein–porphyrin complexes from the gel to a PVDF membrane was carried out under non-denaturing conditions, according to standard procedures for wet blotting, using Tris/glycine buffer, pH 8.3, without SDS. After blotting the membrane was examined under UV light of the SYS I system and the fluorescent bands were marked. Proteins were eluted from the PVDF membrane with 2% SDS and 1% Triton X-100. The eluted proteins were analyzed by 0.1% SDS – 10% polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Detection of protein–porphyrin complexes with the use of system I (SYS I) for fluorescence measurement

Samples containing human serum at a final concentration of 4% incubated with or without $PPArg_2$ were separated using non-denaturing gradient PAGE. The fluorescence of the protein–porphyrin complexes was detected in UV light of SYS I (Fig. 2A). Some distinct fluorescence signals were visible in lane 2 while no fluorescence signals were detected in the case of serum proteins without porphyrin (lane 1). The fluorescence signals in Fig. 2A correspond to some of the protein bands stained with Coomassie Brilliant Blue (Fig. 2B).

In order to prove specificity of the detection of protein–porphyrin complexes, several purified serum proteins were used. Some of them are known to interact with porphyrins, e.g. albumin (HSA) and high density lipoprotein (HDL) (Kongshaug, 1992; Rosenberger & Margalit, 1993; Kongshaug & Moan, 1995; Maziere *et al.*, 1990, 1991; Polo *et al.*, 2002) whereas interaction with haptoglobin or fibrinogen has not been described.

Samples containing a given protein were incubated with or without $PPArg_2$. After electrophoresis the fluorescence measurement was carried out using SYS I (Fig. 2C) and then the gel was stained with Coomassie Brilliant Blue (Fig. 2D).

The results presented in Fig. 2C show that fluorescent bands of complexes were detected only in the case of the samples containing $PPArg_2$ and albumin (lane 2) or $PPArg_2$ and lipoprotein (lane 4), while no fluorescence was observed in the porphyrin mixtures with haptoglobin (lane 6) or fibrinogen (lane 8).

The results obtained indicate that it is possible to visualize protein–porphyrin complexes in samples containing purified proteins as well as in multi-component mixtures (e.g. serum) using a simple, commercially available detection system. However, since the excitation beam of the UV lamp (302 nm) in this system does not match the maximum of

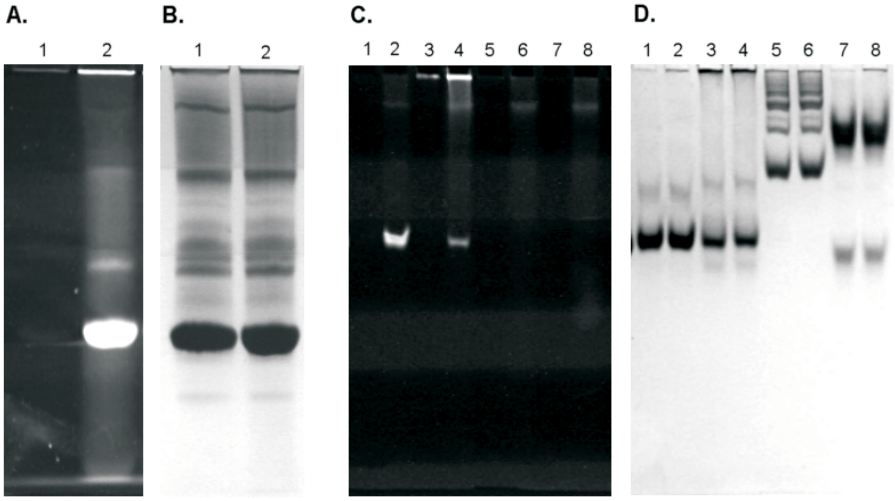


Figure 2. Detection of serum protein–porphyrin complexes in PAG using SYS I detection system.

A. Fluorescence image of non-denaturing PAG (6, 8, 10 and 12%). Lane 1, human serum (4% HS in 50 μl sample.); lane 2, HS + $PPArg_2$ (0.1 mg/ ml). **B**. The same gel after Coomassie Brilliant Blue staining. **C**. Fluorescence image of non-denaturing PAG (6 and 10%). Samples (40 μl) contained about 0.25 mg/ml of a given protein and 0.025 mg/ml of $PPArg_2$ or no porphyrin. Lane 1, HSA; lane 2, HSA + $PPArg_2$; lane 3, high density lipoprotein (HDL); lane 4, HDL + $PPArg_2$; lane 5, haptoglobin; lane 6, haptoglobin + $PPArg_2$; lane 7, fibrinogen; lane 8, fibrinogen + $PPArg_2$. **D**. The same gel after Coomassie Brilliant Blue staining.

porphyrin absorption (about 400 nm), but lies in the range of absorption of some other substances, in some cases additional unspecific fluorescence may appear (not shown).

Detection of protein–porphyrin complexes with the use of system II (SYS II) for fluorescence measurement

Samples containing human serum with or without $PP(Thr)_2Arg_2$ were separated by non-denaturing 10% PAGE. After electrophoresis, the gel, together with the glass gel plates, was placed in a gel scanner of SYS II and the fluorescence was excited and measured, followed by staining with Coomassie Brilliant Blue.

The fluorescence peaks (Fig. 3A) corresponding to protein bands in the gel (Fig. 3B) indicate the presence of albumin–porphyrin (an intensive band at 40 mm of the migration distance) and other complexes.

Porphyrin interactions with various albumin forms (monomers, dimers and oligomers) could also be visualized using the method described above (Fig. 4). The multimeric forms of this protein occur naturally and are present in commercial preparations (Radivic *et al.*, 1979; Scorza & Minetti, 1998). Since albumin is the major serum protein engaged in bio-distribution of various drugs, there are also many publications concerning interactions of porphyrin photosensitizers with this protein (e.g. Ding *et al.*, 2001;

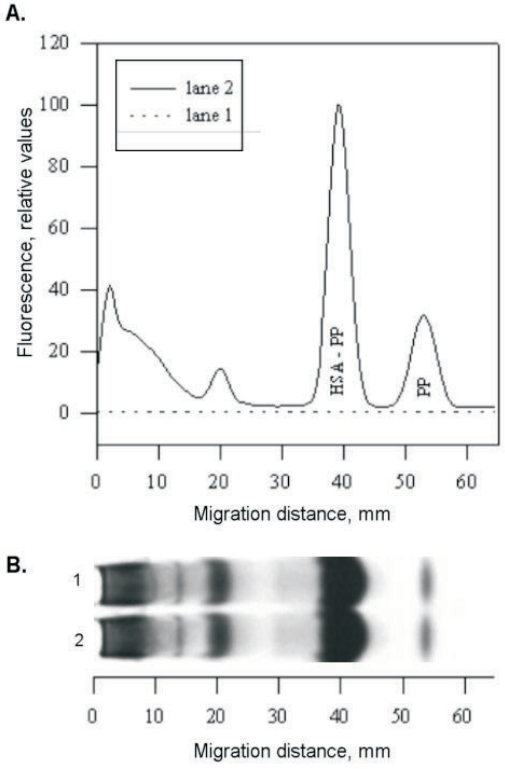


Figure 3. Detection of serum protein–porphyrin complexes in PAG using SYS II detection system.

A. Fluorescence intensity measured in lanes 1 and 2 of the gel below. **B**. Non-denaturing PAG (10%) stained with Coomassie Brilliant Blue. Lane 1, human serum (4% HS in 50 μl sample); lane 2, HS + $PP(Thr)_2Arg_2$ (0.1 mg/ml).

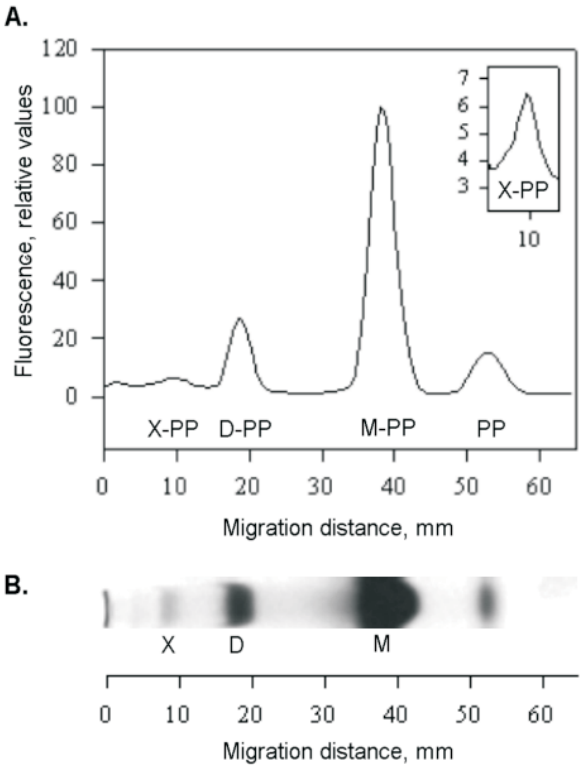


Figure 4. Detection of porphyrin (PP) complexes with albumin (HSA) monomers (M), dimers (D) and multimers (X) in PAG using SYS II.

A. Fluorescence intensity measured in the lane of the gel below. B. Non-denaturing PAG (10%) stained with Coomassie Brilliant Blue. Lane, HSA (3.3 mg/ml in 50 μl sample) + $PP(Thr)_2Arg_2$ (0.5 mg/ml).

Bartosova *et al.*, 1994). However, we found no data concerning the interaction of oligomeric albumin forms with porphyrins. Here we present a simple method of separating and visualizing porphyrin complexes with different forms of a protein (e.g. albumin).

Since in the SYS II system both excitation and emission wavelengths lay exactly within the porphyrin absorption and emission spectra, no unspecific signals were detected.

Post-electrophoretic formation of protein–porphyrin complexes in the gel

In the case of weak protein–porphyrin interactions, complexes tend to decay during electrophoretic separation. To improve the detection of such weak interactions we propose post-electrophoretic staining of the gel in a porphyrin solution as an additional step of the method.

Samples containing HSA with or without $PPArg_2$ were separated by non-denaturing PAGE. After electrophoresis, the gel was inspected under UV in SYS I (Fig. 5A). Then, the gel was immersed for 30 and 60 min in a water solution of $PPArg_2$ and the fluorescence was documented (Fig. 5B and C). Finally, the gel was stained with Coomassie Brilliant Blue (Fig. 5D).

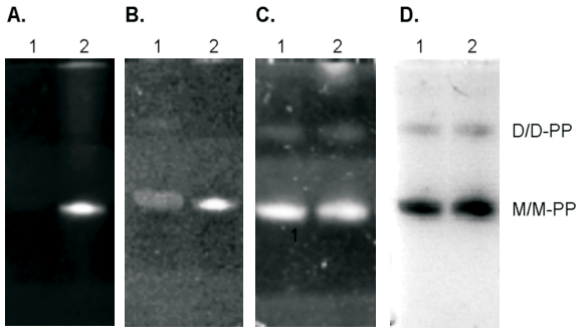


Figure 5. Post-electrophoretic formation of albumin–porphyrin complexes in PAG.

A. Fluorescence image of the gel (10%) directly after non-denaturing PAGE. Lane 1, HSA (0.6 mg/ml in 25 μl sample); lane 2, HSA + $PPArg_2$ (0.5 mg/ml). **B**. and **C**. Fluorescence images of the gel incubated for 30 and 60 min, respectively, in the $PPArg_2$ solution (5 μg/ml). **D**. The gel stained with Coomassie Brilliant Blue. M/M-PP, migration pattern of albumin monomers and their complexes with porphyrins; D/D-PP, migration pattern of albumin dimers and their complexes with porphyrins.

After one-hour gel staining in the porphyrin solution, no significant difference in fluorescence intensities of the protein–porphyrin complexes in particular lanes of the gel was observed. This indicates that the staining of the gel in the porphyrin solution allowed the formation of albumin–porphyrin complexes.

Electro-transfer of protein–porphyrin complexes to the PVDF membrane

Samples containing HSA alone or HSA with different amounts of $PPArg_2$ were separated by non-denaturing 10% PAGE. Following electrophoresis, fluorescence of the albumin–porphyrin complexes was documented using SYS I (Fig. 6A). Next, the proteins and protein–porphyrin complexes were electro-transferred to the PVDF membrane as described in Methods. After blotting, the membrane was inspected under UV light of SYS I (Fig. 6B). The fluorescent band containing protein-porphyrin complexes was cut out and eluted from the membrane. Next it was separated by SDS/PAGE together with protein molecular mass marker (Fig. 6D). In order to visualize the protein components of the complexes present in the separated samples, a duplicate membrane was stained with Coomassie Brilliant Blue (Fig. 6C).

The experiment proved that complexes of porphyrins with albumin or other proteins (not shown) remained stable during electro-transfer to the PVDF membrane and could be visualized using the described detection systems. This gives further advantages, such as elution of complexes from the membrane, determination of molecular mass and complexity of the porphyrin binding proteins by SDS/PAGE and other methods aiming to

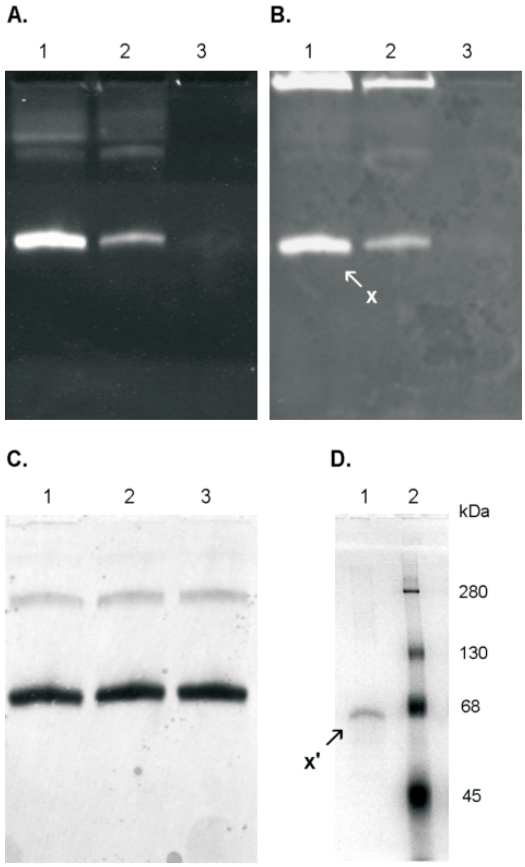


Figure 6. Electrotransfer of albumin–porphyrin complexes to PVDF membrane.

A. Fluorescence (SYS I) of albumin–porphyrin complexes in non-denaturing PAG (10%). Lane 1, HSA (1 mg/ml in 12 μl sample) + $PPArg_2$ (40 μg/ml); lane 2, HSA (as above) + $PPArg_2$ (10 μg/ml); lane 3, HSA (as above). **B**. Fluorescence of the same (panel A) albumin–porphyrin complexes transferred to the PVDF membrane (SYS I). **C**. The PVDF membrane (lanes as above) stained with Coomassie Brilliant Blue. **D**. SDS-denaturing PAGE. Lane 1, the protein component (**x'**) of the albumin–porphyrin complex (**x**), eluted from the PVDF membrane; lane 2, molecular mass marker.

identify the protein component of the complex (e.g. by sequencing).

Comparison of the fluorescence detection capability of the two measurement systems, SYS I and SYS II

Samples containing HSA and different amounts of $PP(Thr)_2Arg_2$ (serial diluted from 5 mg/ml to 0.04 mg/ml) were separated by non-denaturing 10% PAGE. The fluorescence of the complexes was measured first with the use of SYS II and then with SYS I. The data obtained were compared (Fig. 7).

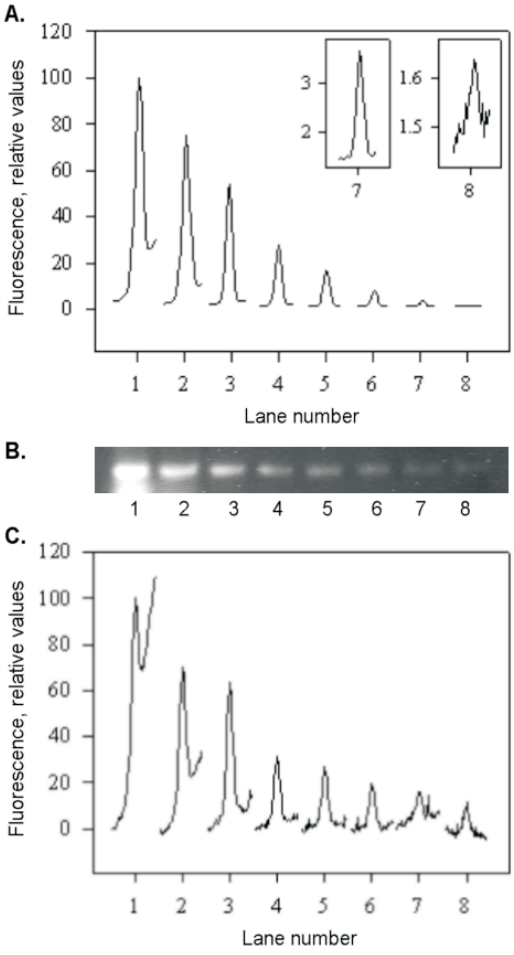


Figure 7. Comparison of fluorescence detection of protein–porphyrin complexes, separated by PAGE, with the use of two systems: SYS I and SYS II.

A. Fluorescence intensity of HSA–$PP(Thr)_2Arg_2$ complexes in the lanes 1–8 of the gel below, measured with SYS II. **B**. Fluorescence of HSA–$PP(Thr)_2Arg_2$ complexes in non-denaturing PAG (10%) observed in UV light of SYS I. Samples separated in the gel contained a constant amount of HSA (0.5 mg/ml in 20 μl) and decreasing amounts of $PP(Thr)_2Arg_2$: lane 1, 5 mg/ml; lane 2, 2.5 mg/ml; lane 3, 1.25 mg/ml; lane 4, 0.62 mg/ml; lane 5, 0.31 mg/ml; lane 6, 0.15 mg/ml; lane 7, 0.078 mg/ml, and lane 8, 0.039 mg/ml in the sample. **C**. Fluorescence intensities of HSA–$PP(Thr)_2Arg_2$ complexes in the lanes 1–8 of the gel above determined with SYS I.

There was no significant difference as regards the level of fluorescence detection by the two detection systems – in both cases even the very low fluorescence signal in gel lane 8 was detected. The protein level at which the complexes were still detectable was about 1 μg or even less.

Owing to the selective narrow-band excitation, close to the absorption maximum of the porphyrin derivatives examined, the emission plots obtained with SYS II were not distorted by any undesired fluorescence related to the wide emission band of the exciting lamp (as in SYS I). This fact essentially improved the quality of the data recorded using SYS II, as compared to analogous profiles obtained with SYS I.

Measurement of fluorescence by SYS I is easier and faster, another advantage of this system being the possibility of taking a picture of the fluorescence as well as of stained gel. This enables easier and more accurate matching of fluorescence to corresponding protein bands. The limitations are the optical parameters that do not correspond exactly to the porphyrin absorption and emission spectra, while matching those of some other substances.

CONCLUSIONS

We elaborated a method of detection of fluorescent porphyrin complexes with soluble proteins, purified or present in multi-component mixtures, using native PAGE. The range of detected interactions can be enhanced by post-electrophoretic staining of the gel in porphyrin solution. And, in order to identify components of the complex, electrotransfer onto PVDF with subsequent elution and additional methods, can be used. The methods described in this paper are currently applied in our studies leading to understanding mechanisms responsible for the uptake and accumulation of new porphyrin photosensitizers by human cells.

We would like to thank Drs. Diana Wojtkowiak, Bogdan Falkiewicz and Krzysztof Bielawski for suggestions and help during the experimental work. Special thanks are addressed to Professor Waclaw Szybalski for reading the manuscript and helpful comments.

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