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Spectroscopic properties of a chlorophyll-based photosensitive dye entrapped in sol-gel fibre-optic applicators

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One of the promising modalities of modern medical treatment is interstitial laser therapy, where a special fibre-optic applicator is used to ensure a proper curing light distribution in the pathological lesion. Such an applicator can act as a light diffuser, and simultaneously it can serve as a carrier of the therapeutic medium, e.g. a photosensitive dye for photodynamic therapy in situ. For applicator construction, silica based sol-gels coatings are proposed in this paper. The sol-gel applicators were prepared from the silicate precursor TEOS (tetraethylorthosilicate) mixed with ethyl alcohol in acid-catalysed hydrolysis. A suitable amount of surfactant (Triton X-100) was used. The carrier matrices were produced with a solvent to precursor molar ratio of 20. In these studies, optical fibres from Laser Components were used (core diameter 400 nm, HCS, low OH). The external jacket was mechanically removed at a distance of 25 mm. The modified dip-coating method was exploited to cover the bare fibres with sol-gel material. Two types of applicators were produced, silica sol-gels with an addition of chlorophyll-derived sensibilisator (Photolon) in two various concentrations. It was proved that the immobilization of Photolon in a silica sol-gel does not destroy its chemical activity and does not disturb contact with the external environment.

Key words: *sol-gel coatings; fibre optic applicator; chlorophyll-based photosensitiser*

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1. Introduction

Photodynamic therapy (PDT) of malignant tumours is a treatment method based on selective accumulation of light-absorbing agents (photosensitisers) in pathologic tissue, capable of stimulating photoreactions after irradiation by light of a certain wavelength, which destroy the targeted cells.

Various dyes have already been used in photodynamic therapy. This paper is focused on chlorin derivatives. A chlorin is a large heterocyclic aromatic molecule, consisting at the core of 3 pyrrole rings and one reduced pyrrole ring, all coupled by methine linkages. Magnesium-containing chlorins are called chlorophylls, and are the main photosensitive pigments in chloroplasts. A related compound, with 2 reduced pyrroles, is called bacteriochlorin. Due to their strong photosensitivity, chlorins are used as photosensitising agents in experimental PDT therapies.

Water-soluble derivatives of chlorophyll were first introduced as potential drugs by Snyder (USA) in 1942 [1]. The next important step was done by Allen [2]. He revealed that the major chemical compound of native chlorin mixtures is chlorin e_6, which under oral and intravenous administration possess low toxicity and demonstrates hypotensive, antisclerotic, spasmolytic, anaesthetic, and antirheumathoid activity. The first PDT usage of a chlorin concerns pheophorbide α-derivatives. Some of them were patented as prospective photosensitizers for PDT in Japan in 1984 by Sakata et al. [3]. In 1986, an American group reported a photosensitizer meeting crucial PDT requirements: good tumour affinity and intensive absorption in the middle red part of the visible spectrum [4]. Their choice was mono-L-aspartyl-chlorin e_6 (MACE). At present, this compound is at stage III of clinical trial studies in Japan. This group has even patented other functionally advanced chlorin and bacteriopheophorbide α-derivatives as photosensitizers for PDT [5]. In the 1990s, a Byelorussian group at first headed by G. Gurinovich reported research on water-soluble chlorin-type photosensitizers derived from nettle [6]. Recently, their work was focused on the spectral-luminescent characteristics of chlorine e_6 and the newly developed photosensitizer – Photolon – in whole blood [7].

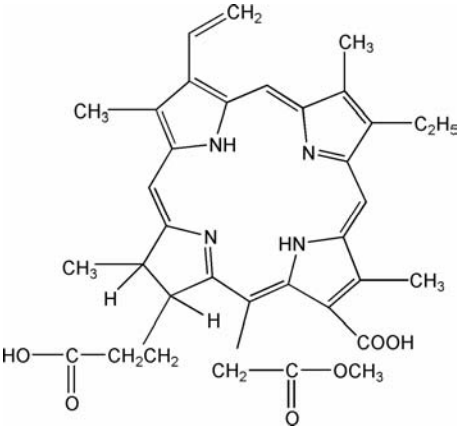

Fig. 1. Chemical structure of the Photolon according to [8]

Photolon – a chlorin derivative – belongs to the class of one-photon-based photosensitizers [8]. The dye is approved for cancer treatment in some countries [9]. Photolon is referred to a group of new sensitizers for treating cancer by means of photodynamic therapy. It can be administrated intravenously or topically. Recently, some non-oncologic applications have been reported as well [10]. The formula is shown in Fig. 1.

Interstitial laser thermotherapy is also a new method designed for minimally invasive therapy of pathologic lesions in environments difficult to access (e.g. the brain, liver). Fibre-optics laser applicators are used to perform interstitial therapy with laser light. The applicator is inserted into the pathologic lesion and curing laser light is guided through the fibre [11, 12]. We have already demonstrated that sol-gel coatings may improve the characteristics of applicators. Depending on the amount of compounds used for producing sol-gels, different optical and structural properties could be achieved [13–15].

Our idea was to combine interstitial therapy with photodynamic therapy. In this paper, we report on the development of a modified light delivery system – a sol-gel fibre-optic applicator doped with Photolon. The sol-gel was deposited on the optical fibre core and the influence of immobilized chlorophyll-derived dye on the applicator’s spectral properties was investigated.

2. Preparation of sol-gel matrices

2.1. Materials

Solvents and reagents were obtained from commercial sources and used without further purification: a) solvents, methanol (Merck, UV-grade), ethanol (Merck), pyridine (Aldrich), b) chemicals, zinc acetate (Merck), Triton X-100 (Aldrich), tetraethylorthosilane (TEOS) (Fluka), hydrochloric acid (Merck), phosphate buffered saline (PBS) pH 7.2 (Biochrom). Photolon (Belmedpreparaty in cooperation with Haemato Ltd., Germany) was used as the active photosensitizer. Aqueous solutions were prepared with Millipore water (Milli-Q/USA). Phosphate buffered saline (PBS) pH 7.2 (Biochrom) was used to mimic physiological conditions, otherwise aqueous solutions were prepared with Millipore water.

The sol-gel films were produced from sol-gel hydrolyzate prepared from the silicate precursor TEOS mixed with solvent (ethanol, 96 % v/v). The sol-gel material was prepared with a solvent to precursor molar ratio R of 20. Hydrochloric acid (37%, w/w) was added as a catalyst in a proportion that ensured acidic hydrolysis (pH $\approx$ 2). The mixture was stirred at room temperature for 4 hours with a magnetic stirrer (speed 400 rev./min).

2.2. Preparation of sol-gel coatings on optical fibres

Hard Clad Silica (HCS) fibres of low OH (CFO 1493-12), originating from Laser Components, were used (fused silica core $\phi_1 = 400$ μm; cladding $\phi_2 = 430$ μm; ETFE buffer diameter 730 μm). They feature high transmission values from UV to IR (200–2400 nm). The fibres were cut into 200 cm sections. The external jacket was mechanically removed up to 2.5 cm from one end, then the cladding was treated with a hot torch. The residuals were removed with linen cloth and cleaned with ethanol. A modified dip-coating method was applied to cover the bare fibres with sol-gels. The fibres were placed vertically in a plastic container with a specially designed bottom, possessing an opening with a controlled diameter to ensure the required speed (1 cm/h) of hydrolyzate outflow. The container was filled with hydrolyzate up to the non-removed cladding. Just before the deposition procedure, the surfactant Triton X-100 (Aldrich) was added to the liquid hydrolyzate (20 μl of Triton per 1 ml of hydrolyzate).

2.3. Photolon-doped coatings

Aqueous stock solutions (8.2×10^{-4} M and 1.6×10^{-4} M Photolon) were prepared by dissolving the photosensitizer in water (Millipore grade). Aliquots of 50 μl stock solution and 20 μl of Triton X-100 were added to 1 ml of the freshly prepared hydrolyzate to get final Photolon concentrations of 3.8×10^{-5} M and 7.6×10^{-6} M, respectively. The newly prepared hydrolyzate was used to produce doped fibre-optic coatings according to the procedure described above. The stock solutions and the sol-gel modified fibres were stored at room temperature in darkness.

For metal insertion, zinc acetate was dissolved at room temperature in methanol (20 mg/ml) and the fibre was placed in the solution. The dopant sol-gel fibre was left there for 72 h. Then the fibre was removed, rinsed with ethanol, dried in air, and its luminescence and absorption spectra were recorded.

3. Spectroscopic measurements

3.1. Apparatus

Fluorescence spectra were obtained by means of a fluorescence spectrometer LS-50B (Perkin Elmer/UK). The spectra were recorded in the wavelength range from 350 to 750 nm (100 nm/min, excitation slit 3 nm, emission slit 5 nm) by using quartz micro-cuvettes (optical length 0.5 cm, Hellma, Germany). 10 scans were accumulated.

The absorption spectra of Photolon solutions were obtained by means of a Lambda 2 (Perkin Elmer, UK) spectrophotometer (optical length 1 cm, wavelength range

300–750 nm, 250 nm/min). A hand-held UV-lamp (λ_{exc} = 365 nm) was used for predetecting and controlling the luminescent sol-gel coating.

3.2. Spectroscopic examinations of Photolon in solvents

The Photolon molecule shows a partially reduced porphyrine moiety (Fig. 1). Its molecular structure is similar to the structure of chlorine e_6 molecule, which can be isolated after hydrolysing the 5-membered exocyclic β-ketoester moiety of pheophorbide A. It is well known that the absorption and fluorescence of tetrapyrrolic macrocycles are sensitive to changes in their molecular environment.

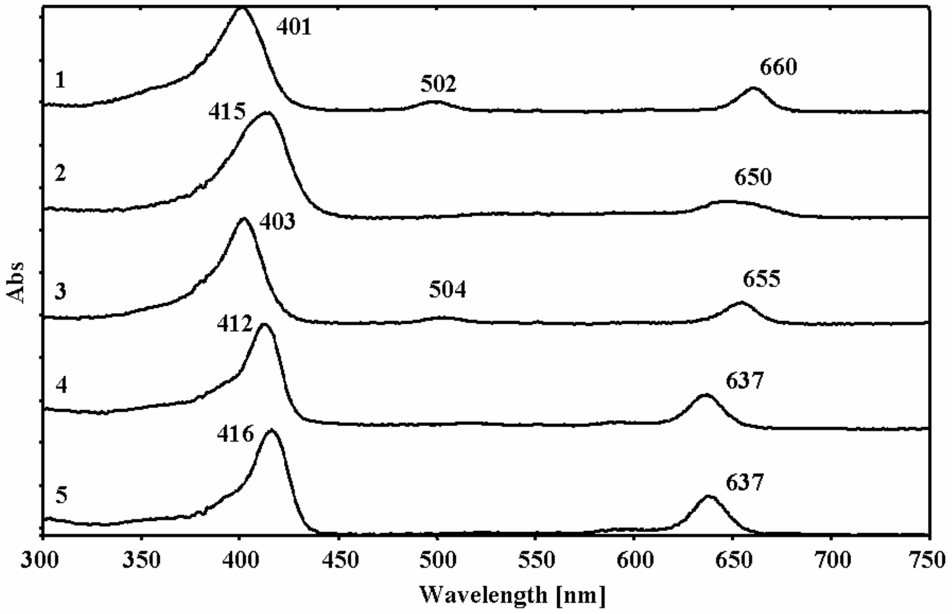

Fig. 2. Absorption spectra of Photolon in various solvents (from the top to the bottom): 1 – in methanol; 2 – in methanol acidified with hydrochloric acid; 3 – in phosphate-buffered saline (pH 7.2); 4 – after addition of zinc acetate in methanol; 5 – after addition of pyridine to 4

The absorption and fluorescence spectra of Photolon were recorded in various solvents. A stock solution of Photolon dissolved in methanol (1×10^{-6} M) was prepared and visible spectra were recorded (Fig. 2). The Soret band and additional absorption bands in the wavelength range 500–700 nm (Q-bands) were observed. Two prominent absorptions, at 401 nm (Soret band) and at 660 nm (chlorine-type band), are visible. The addition of 0.1 ml of the concentrated hydrochloric acid to 2.5 ml of the methanolic dye solution leads to a bathochromic shift (λ_{Soret} = 415 nm). A second strong absorption band appears at 650 nm. The absorption intensity decreases and the bands become broader. In phosphate-buffered saline (PBS), the absorption of Photolon remains essentially the same as in methanol (403 nm and 655 nm). After adding zinc acetate, a spectral change due to the insertion of zinc into the macrocycle is detected within minutes. The Soret band shifts towards 412 nm and the chlorine-type absorption band undergoes a hypsochromic shift to 637 nm. When pyridine is added to the zinc complex, a further shift of the Soret band (λ_{max} = 416 nm) is recorded.

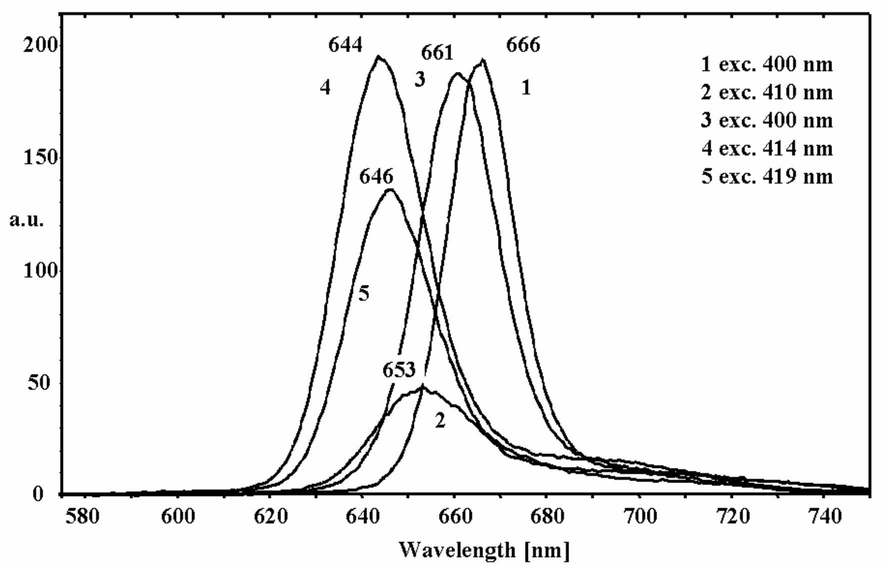

Fig. 3. Fluorescence spectra of Photolon in solutions: 1 – in methanol; 2 – in methanol acidified with hydrochloric acid; 3 – in PBS (pH 7.2); 4 – after addition of zinc acetate in methanol; 5 – after addition of pyridine to 4

The fluorescence spectra recorded in the wavelength range of 575–750 nm are shown in Fig. 3. In methanol, the fluorescence maximum of Photolon is observed at 666 nm (λ_{exc} = 400 nm). In acidified methanol, the maximum is shifted towards 653 nm upon excitation at 410 nm. At the same time, the emission intensity decreasesd by a factor of approx. 4. In aqueous solution (PBS, pH = 7.2), the emission maximum is shifted to 661 nm (λ_{exc} = 400 nm). Compared to the fluorescence spectrum of Photolon in methanol, the zinc complex in methanol shows strong fluorescence at 644 nm (λ_{exc}= 414 nm). The coordination of pyridine to this metal complex, however, leads to the fluorescence signal at 646 nm.

3.3. Spectroscopic examinations of Photolon in sol-gel applicators

For fluorescence measurements, the sol-gel coated fibres were placed diagonally inside a cuvette. Spectra were recorded in air, in solvents, and after chemical altering. In order to avoid artifacts, the spectra were recorded at various positions on the same fibre.

First, the unmodified quartz fibre and sol-gel coated fibres without dopant were investigated in order to check their spectroscopic properties. Excitations from 395 up to 420 nm ($\Delta\lambda$ = 5 nm) using the pure core fibre as a target gave only a very weak fluorescence emissions in the range of 525–575 nm. A negligible fluorescence is also observed in air for the undoped sol-gel coated fibres. The addition of solvents leads to a further decrease in the fluorescence intensity.

Fibres with a sol-gel Photolon-doped coating were investigated in air and in the presence of solvent. Spectra were recorded for fibres doped with two concentrations of Photolon (3.8×10^{-5} M and 7.7×10^{-6} M) (Fig. 4). Fluorescence and fluorescence excitation spectra were recorded in the wavelength range 550–775 nm and 350–625 nm, respectively.

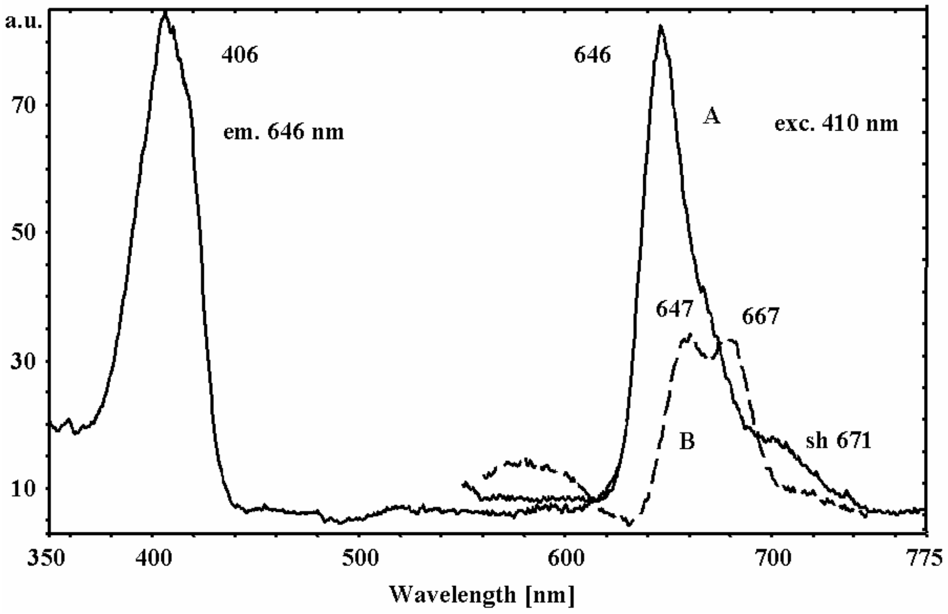

Fig. 4. Excitation and emission spectra in air, sol-gel applicator
(A: 3.8×10^{-5} M; B: 7.7×10^{-6} M Photolon)

The fluorescence intensity of the sol-gel applicator was found to depend on the Photolon concentration (Fig. 4). A similar dependence (not shown here) was observed in absorption spectra. The fluorescence was determined in the range of 620–750 nm. The excitation spectra were recorded in a step-by-step mode ($\Delta\lambda$ = 5 nm) with λ_{exc} in the range of 395–420 nm. In Fig. 4, the fluorescence spectra (λ_{exc} = 410 nm) are shown for fibres measured in air just after the coating procedure. For the fibre referred to as A (3.8×10^{-5} M Photolon) in Fig. 4, the emission maximum at 646 nm and a shoulder at 671 nm were recorded. For the fibre with a lower dye concentration (7.7×10^{-6} M Photolon), assigned to B, maxima at 647 nm and 667 nm were detected. The excitation spectrum shows a strong absorption at 406 nm (λ_{em} = 646 nm).

Photolon dissolved in methanol shows the absorption maximum at 401 nm and a strong emission at 666 nm. The wavelengths of absorption and emission maxima in PBS (pH 7.2) are similar – 403 nm and 646 nm, respectively (Figs. 2 and 3). Both in methanol and PBS only the free base of Photolon is present. Adding hydrochloric acid to the methanolic solution, however, leads to a protonated form of the dye (emission, λ_{max} = 646 nm). The addition of acid is crucial for sol-gel preparation, in order to initiate polymerisation, hence this will also lead to a protonation of the chromophore. Therefore, as indicated by the emissions at 647 and 667 nm, two different species – the neutral and a protonated form – seem to be present in the sol-gel matrix.

3.4. Spectroscopic examinations of Photolon-doped sol-gel applicators in various environments

Due to a high solubility of Photolon in organic solvents, fluorescence measurements were done in order to check whether the dopant leaks from the coating and to detect changes in the spectral properties of the applicator. The release of Photolon from the sol-gel matrix was also checked for aqueous solutions of PBS and Millipore water as described above, but no fluorescence could be detected. Therefore, after the measurement was completed, the modified fibre was removed and the remaining solvent was examined again.

Figure 5 shows the spectra for a doped fibre (R = 20, 3.8×10^{-5} M Photolon) placed in methanol. Compared to the measurement in air, the overall intensities are slightly lower. The fluorescence is recorded in the wavelength range of 620–750 nm. The emission spectra (λ_{exc} = 395, 407, and 420 nm) do not differ from the spectra in air (Fig. 4). Emission maxima appear at 647 and 668 nm. Excitation at 395 nm leads to strong fluorescence at 668 nm. Upon excitation at 420 nm, the intensity decreases by up to 50%. Emission at 647 nm leads to a Soret band at 408 nm, whereas emission at 667 nm leads to a Soret band at 404 nm. A minor absorption in the Q-band range at 500 nm (λ_{em} = 667 nm) is also observed.

An example of the protonation of a Photolon-doped fibre is shown in Fig. 6. In order to determine the protonated species of Photolon encapsulated in the sol-gel matrix, 100 μl conc. hydrochloric acid was added. After 10 minutes, the spectra were measured (Fig. 6), then the solvent was replaced by a PSB-buffer. The emission spectra were immediately recorded and the process of neutralization was monitored. An excitation spectrum was recorded before and after protonation.

The emission spectrum of Photolon cations shows strong fluorescence at λ_{max} = 649 nm (λ_{exc} = 410 nm) and a Soret band at 407 nm (λ_{em} = 50 nm). In PBS, the fluorescence is shifted toward 667 nm (λ_{exc} = 405 nm) and the Soret band is determined to be at 405 nm (λ_{em} = 666 nm). In accordance to the absorption spectrum of Photolon in PBS (Fig. 2), the excitation spectrum in PBS shows an additional absorbance at approximately 506 nm.

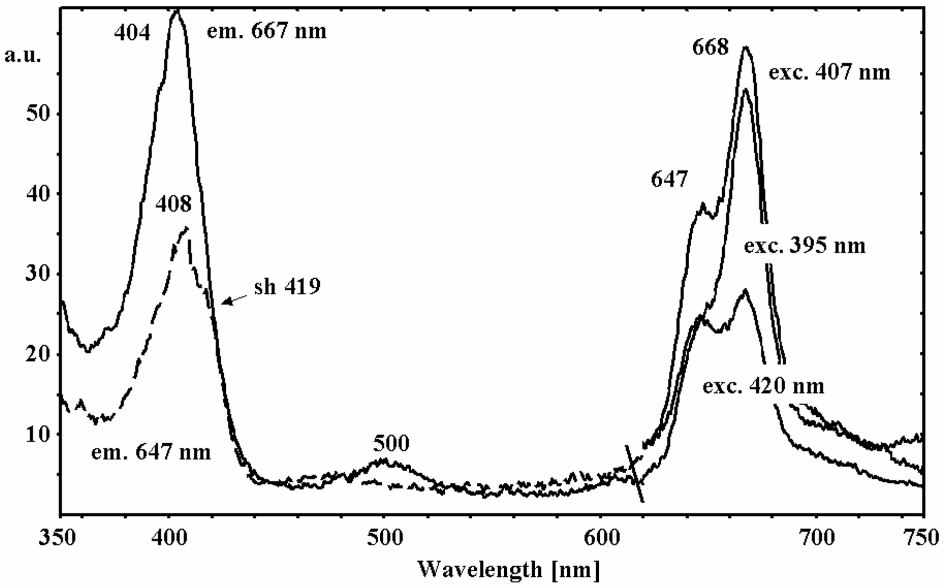

Fig. 5. Fluorescence excitation and emission spectra of a fibre $R = 20$ (3.8×10^{-5} M Photolon) placed in methanol

To monitor the chemical reactivity of the embedded Photolon, the fibre was left in a solution of zinc acetate (20 mg/ml) in methanol for 12 h at room temperature. Prior to this, it was placed in PBS to ensure the presence of the neutral species. After 12 h the fibre was removed and the solution, as well as the fibre in the dry state, were examined. No fluorescence was detected in the organic solution.

In result, there is another strong fluorescence at 667 nm ($\lambda_{\mathrm{exc}} = 395$ nm), which is assigned to the free base of Photolon. Another emission band is observed at 646 nm ($\lambda_{\mathrm{exc}} = 415$ nm) – this may be caused by the partial protonation of Photolon. The emission at 646 nm, however, leads to an excitation spectrum with a broad Soret-band at 407 nm and a shoulder at 416 nm (not shown).

We conclude that no metal insertion takes place. Neither a longer reaction time, gentle heating of the reaction solution, nor high concentrations of zinc acetate enforced metal insertion.

Due to the initial preparation conditions and to sol-gel polymerisation, the neutral and the cationic species of Photolon are present in different amounts. The ratio can be changed with protonation and by using different solvents. While the insertion of zinc into the dye is observed within minutes when Photolon is dissolved in methanol, the doped fibre remains unchanged. It is well known [16] that porphyrin monomers exist only at very low dye concentrations or in a strong acidic medium. Usually, at normal conditions, porphyrins as well as chlorin will dimerise. Therefore, we conclude that besides the limitation due to the pore diameter of the sol-gel, the embedded Photolon molecules (preferred species: dimer) cannot be reached by the zinc cations in the sol-gel matrix. Also, no phenomenon of dye aggregation (which happens through a selfenforced process that builds up supramolecular structures, namely H- (stacked) or J- (side by side) complexes [17], was not observed during spectroscopic examinations.

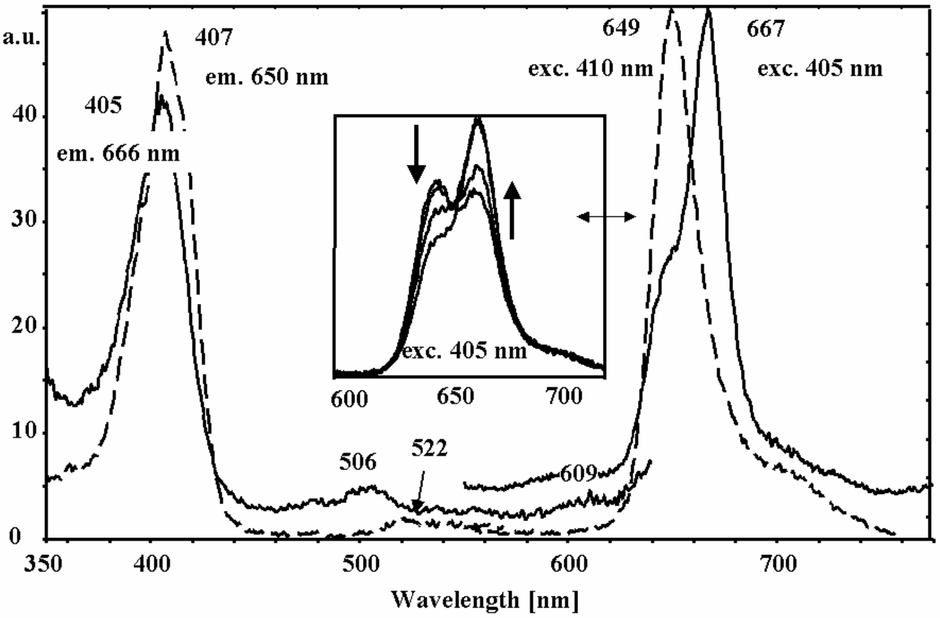

Fig. 6. Excitation and emission spectra from applicator (3.8×10^{-5} M Photolon) placed in 0.5 M HCl (dashed line) and PBS-buffer (pH 7.2). Inset: fluorescence spectral evolution by time (λ_{exc} = 405 nm) during reaction in PBS

4. Conclusions

Our work was focused on the examination of the optical properties of doped silica sol-gel coatings, which may be used for photodynamic therapy in situ. It has already been proved [18] that Protoporphyrine IX-doped sol-gel coatings deposited on the fibre core changes the light propagation conditions, depending on the sol-gel *R* factor (solvent to precursor molar ratio) and on the coating technology as well. These observations should be considered in the construction of sol-gel based applicators.

In this paper, we have shown that Photolon entrapped in sol-gel preserves its chemical activity and may contact with the external environment. Chemical reactions like protonation occurred quite fast. This indicates that the interconnected porous network could be easily penetrated by relatively small molecules. On the other hand, reactions with zinc cations cannot be clearly observed by spectroscopic examination. The silica sol-gel structure was modified by adding Photolon in such a way that pores are too small or there is a kinetic effect. The insertion of zinc ions into the sol-gel matrix might take more time than the duration of our experiments, 72 h at room temperature, and 6 h at 50 °C. The diameter of the zinc ion is about 83 pm, and a solvation sphere has to be taken into account. Solvation phenomena make necessary preparation of the silica sol-gel matrix with a diameter of pores much larger than that of the introduced molecule.

The absorption peak of zinc-Protoporphyrin is 12 times higher and shifted as compared the absorption peak of Protoporphyrin IX [18]. We did not observe a similar phenomenon in the case of Photolon with and without Zn ions – the intensity of absorption is approximately the same. This could explain lack of a clean signature of metallation of the chlorin derivative. The Zn-Photolon complex is not so easy distinguishable in absorption spectrum.

Photolon molecules did not leave the pores of the silica sol-gel coating and no aggregation was observed. The protonation of Photolon occurred slowly when the concentration of H^+ ions was low. The higher the concentration of H^+ ions, the faster appeared the protonated form of Photolon. The deprotonation of the dye, conducted in a PBS environment (pH~7.2), occurred after 15 min.

Structural changes caused by the addition of Photolon to the sol-gel matrix influence not only the optical properties, but also the durability of the coatings. During the examinations described in this paper, lasting a few days in various conditions (various solvents, drying in air), no changes in the stability of coatings were observed. We did not notice any alterations in the Photolon-doped silica sol-gel coatings.

Further investigations will involve studies on dye metallation (the insertion of Zn ions) and oxidation (under O_3 and H_2O_2 conditions). We are going to prepare Photolon-doped silica sol-gel coatings with various values of R, not only $R = 20$ (chosen because of the quality of the coating, stability, and optical properties).

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