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Sensitization of *Salmonella enterica* with 5-aminolevulinic acid-induced endogenous porphyrins: a spectroscopic study†

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Photodynamic therapy (PDT) of bacterial strains presents an attractive potential alternative to antibiotic therapies in search of the solution for the chemoresistance problem. The efficacy of the treatment is dependent on the interaction of photochemically active substances called photosensitizers (PSs) with the bacterial cell wall or their intracellular accumulation. In addition to exogenous PSs, other molecules such as 5-aminolevulinic acid (5-ALA), a natural precursor of heme, are gaining interest. When provided exogenously to cells, 5-ALA uptake results in the overproduction of various photoactive porphyrins. The pattern of their intracellular accumulation and release to the surroundings depends on incubation conditions such as the applied 5-ALA concentration, cell density and incubation duration. The detection of endogenously synthesized porphyrins in samples of *Salmonella enterica* cells and supernatants was accomplished after 4 h and 20 h incubation periods by means of fluorescence spectroscopy. The relative proportions of different types of porphyrins were assessed by modeling the registered spectra with the fluorescence spectra of standard porphyrins. After the shorter incubation period, the dominant porphyrins in the supernatant medium were coproporphyrins. The longer incubation period shifted the relative proportion of intracellular porphyrins from protoporphyrin IX towards water-soluble porphyrins such as uroporphyrin I, which interfered with additional by-products. The time-dependent changes in compositions of both intracellular and extracellular porphyrins imply that 5-ALA-induced sensitization might have triggered a complex protective mechanism of bacterial cells. Thus, identification and evaluation of the relative amounts of porphyrins, which accumulate in bacterial cells and are extruded outside after different time periods, could provide access to valuable information, working towards more efficient applications of 5-ALA-based antibacterial PDT.

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1. Introduction

Salmonella is one of the most frequently isolated foodborne pathogens and a major worldwide public health concern, accounting for 93.8 million foodborne illnesses every year.[1] 22 million invasive systemic infections are incited by the obligate human pathogens *Salmonella typhi* or *paratyphi*, and result in 200 000 deaths, mainly in the developing world.[2] The growing concern about the enhanced risk of infectious diseases and the efficacy of the treatment is prompted by the increasing resistance of bacterial pathogens to commonly used antibiotics and antibacterial agents.[3] The combined application of photosensitizers (PSs) and appropriate light radiation, known as photodynamic therapy (PDT), to induce photochemical damage that could eradicate sensitized bacterial cells is gaining increased attention as a potent alternative means in search of the solution for the chemoresistance problem.[4–6] The effectiveness of the treatment depends, among other factors, on the accumulation of the photosensitizing agents near the vital cellular targets. Due to essential structural differences in the cell envelope between Gram-positive and Gram-negative bacteria,[7] the photosensitizing properties of different types of PSs have been tested for seeking the highest photo-induced bactericidal efficacy.[4,8]

The intracellular production of tetrapyrrolic compounds, which are involved in many vital metabolic processes,[9] is a common feature of all living cells including prokaryotes.[10] The application of natural precursors such as 5-aminolevulinic acid (5-ALA) or its derivatives for the recruitment of metabolic reactions in the sensitization process could be a plausible choice, especially in the case of Gram-negative bacteria, when

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intracellular penetration of extrinsic agents is hindered.[6,11] Thus, the excess amounts of endogenous porphyrins acting as PSs can lead to the photoinduced destruction of bacteria.[12] Typically, irradiation of bacterial cells with light in a blue spectral region results in the excitation of bacterial porphyrins, formation of reactive oxygen species due to energy transfer from the excited triplet state and subsequent destruction due to photoinduced oxidative stress.[13–15]

However, the complex pathway of the synthesis of endogenous porphyrins and the differences in their metabolic activity do not allow the creation of a universal treatment procedure. The time-dependent changes in intracellular accumulation and the type of produced porphyrin, which can directly affect the outcome of the treatment,[16] also depend on a calculated choice of the external factors, such as the applied concentration of the precursor and the time of exposure. On the other hand, the extrusion of exogenous molecules, such as antibiotics, toxic compounds and various dyes, through membrane channels and efflux pumps to the exterior of a cell is an intrinsic feature of bacteria,[17] which is essential in the case of resistance to both externally applied PSs and internal ones.[18,19] Efflux pumps play a significant role in the survival capacity of *Salmonella* cells as protective means to reduce intracellular oxidative stress[20] and can be related to the virulence as well.[21]

The studies of 5-ALA-induced sensitization in various strains of bacteria revealed different combinations of accumulated water-soluble porphyrins and hydrophobic ones, such as protoporphyrin IX.[22] Conventional assessment methods include fluorescence spectroscopy and high performance liquid chromatography (HPLC). In many cases, the fluorescence spectra registered under excitation at a certain single wavelength cannot ensure reliable quantitative or qualitative assessment of the samples containing different types of porphyrins. The application of several excitation wavelengths for the fluorescence registration along with spectral modeling using the spectra of standard porphyrins allows a more accurate analysis of the registered fluorescence spectra through the evaluation of the relative amounts of porphyrins in the samples.[23] The application of organic solvents as a usual step during the chromatographic analysis of the formed porphyrins has its drawbacks too. As porphyrins are taken out from their surrounding environment, solvent molecules might distort the natural dynamic state of intracellular porphyrins by interacting with biomolecules or metal cations.

To the best of our knowledge, to date, there is only one article reporting about the sensitizing capacity of *Salmonella* cells after the application of exogenous 5-ALA. The properties of fluorescence spectra measured in bacterial cell suspensions indicated the production of different types of porphyrins related to the biosynthesis of heme.[16]

Since both the amount and the type of endogenous PSs can affect the degree of photodamage to *Salmonella* cells, the data on the production and accumulation of porphyrins in the bacterial culture can give valuable information for seeking higher efficacy of the treatment. The aim of the present study was to perform the evaluation of the relative amounts of porphyrins being synthesized and accumulated in *Salmonella enterica* cells and those being excreted into the incubation medium by applying the fluorescence spectroscopy method. It is relevant for designing improved modalities of the 5-ALA-based photodestruction of the bacterial cells with regard to both the incubation duration and the sensitization pattern.

2. Materials and methods

2.1. Chemicals

5-aminolevulinic acid (5-ALA) and hydrochloric acid (HCl, 36.5–38%) were obtained from Sigma-Aldrich. The stock solution of 5-ALA (0.1 M) was freshly prepared before use by dissolving 5-ALA in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) and the pH was adjusted to 7.4. As standards for fluorescence spectroscopy measurements, the following porphyrins were used: uroporphyrin I dihydrochloride, coproporphyrin I dihydrochloride and protoporphyrin IX disodium salt, purchased from Porphyrin Products, USA.

2.2. Growth conditions of bacterial cells, incubation with 5-ALA and preparation of bacterial samples

This study was conducted with Gram-negative bacteria, *Salmonella enterica* serovar Typhimurium strain DS88 [SL5676 SmR (pLM32)] resistant to tetracycline, kindly provided by Prof. D. H. Bamford (University of Helsinki, Finland).

The bacterial culture was grown aerobically at 37 °C in Luria–Bertani (LB) medium overnight. This culture was diluted with fresh LB medium that had an initial optical density of 0.10 ± 0.01 (Eppendorf, BioPhotometer) at 600 nm. The culture was grown until it reached an optical density of 1.0 ± 0.1 at 600 nm, corresponding to $\sim 5 \times 10^8$ colony forming units (CFU) per ml. Then the pellets were harvested by centrifugation (at 7500*g* for 15 min, Beckman) and washed twice with sterile PBS. The pellets were resuspended and diluted with PBS to densities of 5×10^8 CFU ml^{-1} (fraction I) and 3×10^7 CFU ml^{-1} (fraction II) before the experiments. Samples of equal volumes (15 ml) were taken and then 5-ALA was added to obtain the concentrations of 10^{-3} M (fraction I) and 5×10^{-4} M (fraction II). The samples were incubated in the dark for 4 h and 20 h at 37 °C in a shaking incubator (150 rpm, Environmental Shaker–Incubator ES-20). At the end of these periods, 5 ml aliquots of bacterial cell suspensions were withdrawn, centrifuged (at 8000*g* for 15 min, Eppendorf 5810R), and then the supernatants were sterilized by filtration through a 0.22 μm filter, while bacterial pellets were resuspended in PBS and collected for fluorescence spectroscopy measurements. All samples of fraction II were additionally treated with HCl (its final concentration in all samples was 1.5 M) and incubated in the dark for 30 min at 37 °C in a thermomixer (Eppendorf Thermomixer comfort). The samples of the bacterial culture, which was grown under the same conditions and prepared in the same way as in the cases of fractions I and II, but without the addition of 5-ALA, served as the corresponding controls for the detection of the autofluorescence background.

2.3. Incubation of bacterial samples with standard porphyrins

The stock solutions of uroporphyrin I (UP I, 2×10^{-4} M) and coproporphyrin I (CP I, 2×10^{-3} M) were prepared in PBS and rectified ethyl alcohol (96%), respectively. A stock solution of protoporphyrin IX (PP IX, 2×10^{-3} M) was prepared by firstly dissolving the powder in a small volume of dimethyl sulfoxide (DMSO, 10%) and NaOH (2×10^{-4} M) and then diluting with PBS. All stock solutions were kept in the dark at 4 °C.

The stock solutions of UP I, CP I and PP IX were added into bacterial cell suspensions and supernatants to reach the final concentration of porphyrins, 2×10^{-6} M. A part of these samples was prepared by diluting with HCl (a final concentration of 1.5 M), and the other parts were diluted with PBS (to keep the bacterial cell density and the volume of supernatants the same). Then the samples were incubated in the dark for 30 min at 37 °C in a thermomixer (Eppendorf Thermomixer comfort). The bacterial cell density in all samples, which were prepared for the fluorescence spectroscopy measurements, was 3×10^7 CFU ml^{-1}. The measurements were triplicated for each standard porphyrin. Since there had been no significant differences observed in the main fluorescence features such as the peak position and general shape of the registered spectra, the averaged spectra were used in the spectroscopic analysis of the fluorescence spectra observed in the samples of bacterial cells after incubation with 5-ALA.

2.4. Fluorescence spectroscopy measurements

All samples containing the bacterial culture treated with 5-ALA were finally diluted with PBS (fraction I) or PBS with HCl (fraction II) to maintain the bacterial cell densities mentioned in Section 2.2 before the fluorescence spectroscopy measurements. Fluorescence excitation and emission spectra were recorded by means of a LS55 spectrofluorimeter (PerkinElmer, USA). The wavelengths used for excitation were 390 nm, 395 nm, 405 nm, 410 nm and 420 nm, and slits of 5 nm bandwidth were set for both excitation and emission paths. The emission spectra were recorded in the spectral region from 550 nm to 750 nm, while excitation spectra were recorded from 350 nm to 450 nm. The registered fluorescence spectra were analyzed using OriginPro 9.1 software.

3. Results

5-ALA-induced formation of porphyrins in the samples of *Salmonella enterica* cells was followed *in vitro* by using a direct and sensitive non-invasive method – fluorescence spectroscopy. The presence of porphyrins was demonstrated by the appearance of characteristic bands in the registered fluorescence excitation and emission spectra. The excitation at different wavelengths in the absorption region of the Soret band was used to distinguish the fluorescence properties of porphyrins and to perform their spectral separation.

3.1. Spectroscopic evaluation of endogenously synthesized porphyrins in fraction I

Fig. 1 presents the spectra obtained after the incubation of the bacterial cells (fraction I) with 5-ALA for 4 h and 20 h and the subsequent preparation of the samples in PBS. The main fluorescence features such as the peak position and general shape of the spectra differed in the cells and in the supernatants. After the incubation period of 4 h, the fluorescence emission spectrum with a maximum at 633 nm and a smaller band at 703 nm was observed in the cells, as shown in Fig. 1A, which is characteristic of PP IX.[22] The prominent Soret band was located at 410 nm in the fluorescence excitation spectrum and also indicated the presence of PP IX. The peak near 585 nm was clearly visible in the fluorescence emission spectrum under excitation at 410 nm only, and might be assigned to several metalloporphyrins, including Zn-PP IX and complexes of water-soluble porphyrins,[24–26] which possess a red-shifted Soret band with respect to that of free-base porphyrins. Excitation at 395 nm yielded broad fluorescence bands at 615 nm and at about 675 nm, which appeared to be the result of combined emission by different water-soluble porphyrins, similar to that previously reported in ref. 22. After the incubation period of 20 h, an emission spectrum of bacterial cells had a distinguished peak of PP IX at 633 nm, when excited at 410 nm, and a less expressed one when excitation was at 395 nm (Fig. 1C). Two major emission bands were located at 615 nm and at about 676 nm in the spectrum with excitation at 395 nm, and the peak near 396 nm in the corresponding excitation spectrum, allowing us to ascribe a 615/676 nm component to water-soluble porphyrins. However, in this case, the fluorescence band at 615 nm was narrower in comparison with that detected after a 4 h incubation period.

The spectroscopic analysis of the supernatants prepared after 4 h and 20 h incubation periods with 5-ALA, which had been performed by comparing the recorded spectra with the spectral properties of the standard porphyrins, showed that the most abundant porphyrins in the supernatants were coproporphyrins with the Soret band detected at 391 nm or at 392 nm (Fig. 1B and D). After the incubation period of 4 h, the fluorescence bands underwent a slight bathochromic shift (from 612/671 nm to 614/673 nm) when excitation at 395 nm was changed to 410 nm (Fig. 1B, see curves 3 and 4, respectively), thus implying the presence of different states of CP I in the supernatant medium. One possible interpretation is that a fraction of CP I showing a stronger absorption at the blue spectral side of the Soret band was a free monomer in aqueous medium, while another fraction might be associated with cellular proteins present in the supernatant medium, as reported in ref. 27. However, the presence of another coproporphyrin isomer cannot be ruled out (Fig. S2†). The supernatant obtained after the 20 h incubation period exhibited an additional emission band at around 580 nm. A sharp peak of the Soret band detected at 403 nm in the spectrum recorded at 580 nm (Fig. 1D) indicated the presence of metalloporphyrins, which are most likely to be formed from the water-soluble porphyrins.[28,29]

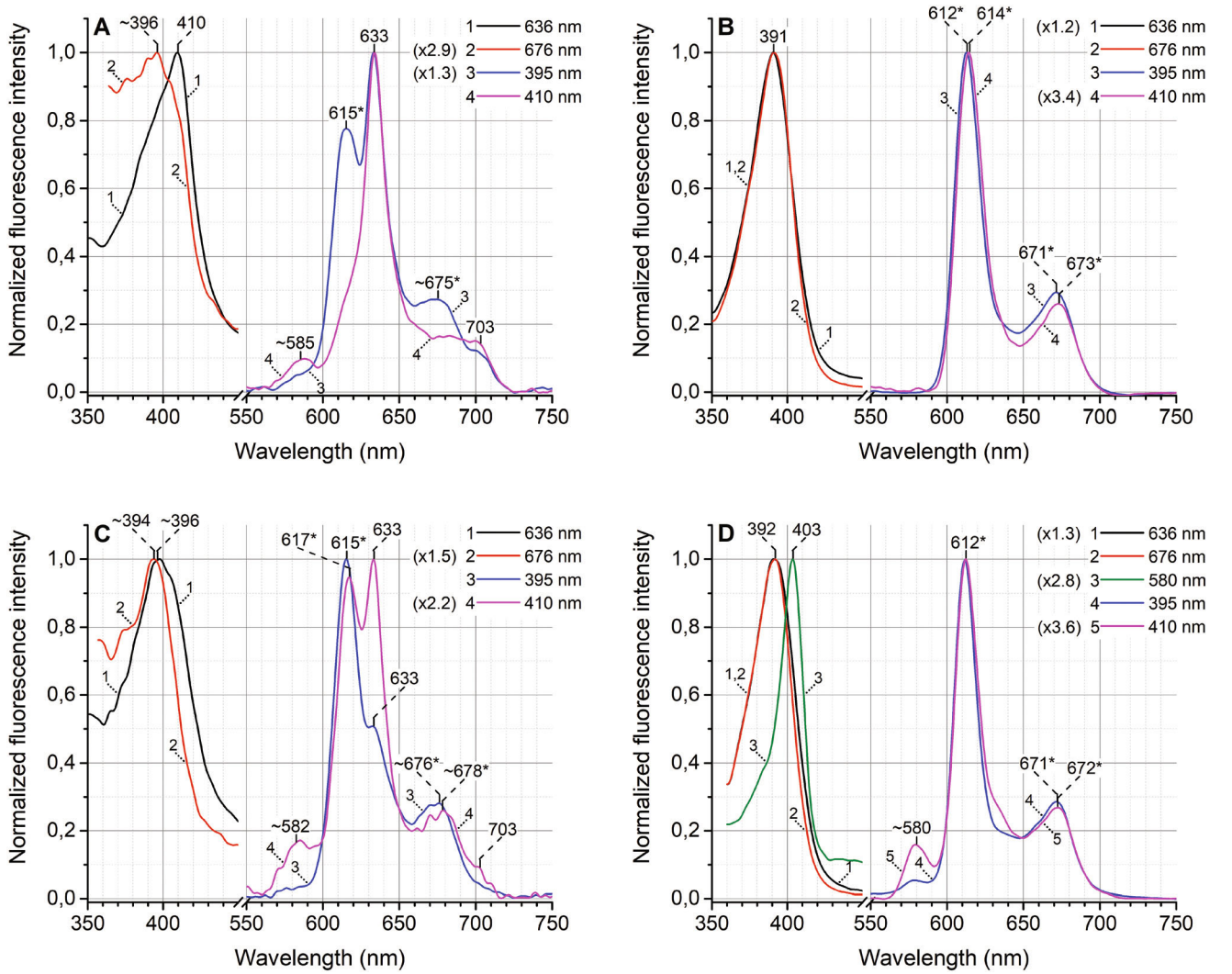

Fig. 1 Normalized fluorescence excitation and emission spectra observed in the samples of the resuspended bacterial cells (A and C) and the supernatants (B and D) after incubation of the bacterial culture with 5-ALA (fraction I) for 4 h (A and B) and 20 h (C and D) in PBS. Wavelengths used for the excitation and registration of the corresponding spectra are denoted in the legend. *Here and elsewhere a spectral peak position apparently represents a weighted sum of the fluorescence emission intensities belonging to different types of porphyrins.

To determine the relative proportions of PP IX and different water-soluble porphyrins present in the bacterial cells after 4 h and 20 h of incubation with 5-ALA, a detailed spectroscopic analysis of the registered fluorescence spectra was performed. The spectra of porphyrins (CP I, UP I and PP IX), which had been registered in representative control medium under the same experimental conditions, were used as the standards to fit the recorded fluorescence emission spectra manually by applying accurately the estimated ratios for each standard (see S2† for more details). The spectroscopic evidence for the presence of different types of porphyrins is shown in Fig. 2. The modeling demonstrates that every fluorescence emission spectrum recorded in the bacterial cells comprises a mixture of the spectra of at least three different types of porphyrins. Excitation at 395 nm and at 410 nm revealed the variation in the detected proportions of distinct porphyrins after each incubation period. After 4 h, the predominant porphyrin was PP IX, the other two being water-soluble CP I and UP I, with a higher contribution of the latter. The residual spectrum (Fig. 2A) also revealed a noticeable shoulder at approximately 621 nm and a small band at about 685 nm. After 20 h, the relative contribution of UP I and CP I as well as porphyrin(s) with a fluorescence band at 621 nm was slightly higher than that after the shorter incubation period, but the opposite trend was observed for PP IX (Fig. 2C and D).

3.2. Spectroscopic evaluation of endogenously synthesized porphyrins in fraction II

The spectra, which had been registered in the samples of the bacteria from fraction II by using different excitation and emission wavelengths, are shown in Fig. 3, in separate panels for the resuspended bacterial cells and the supernatants prepared

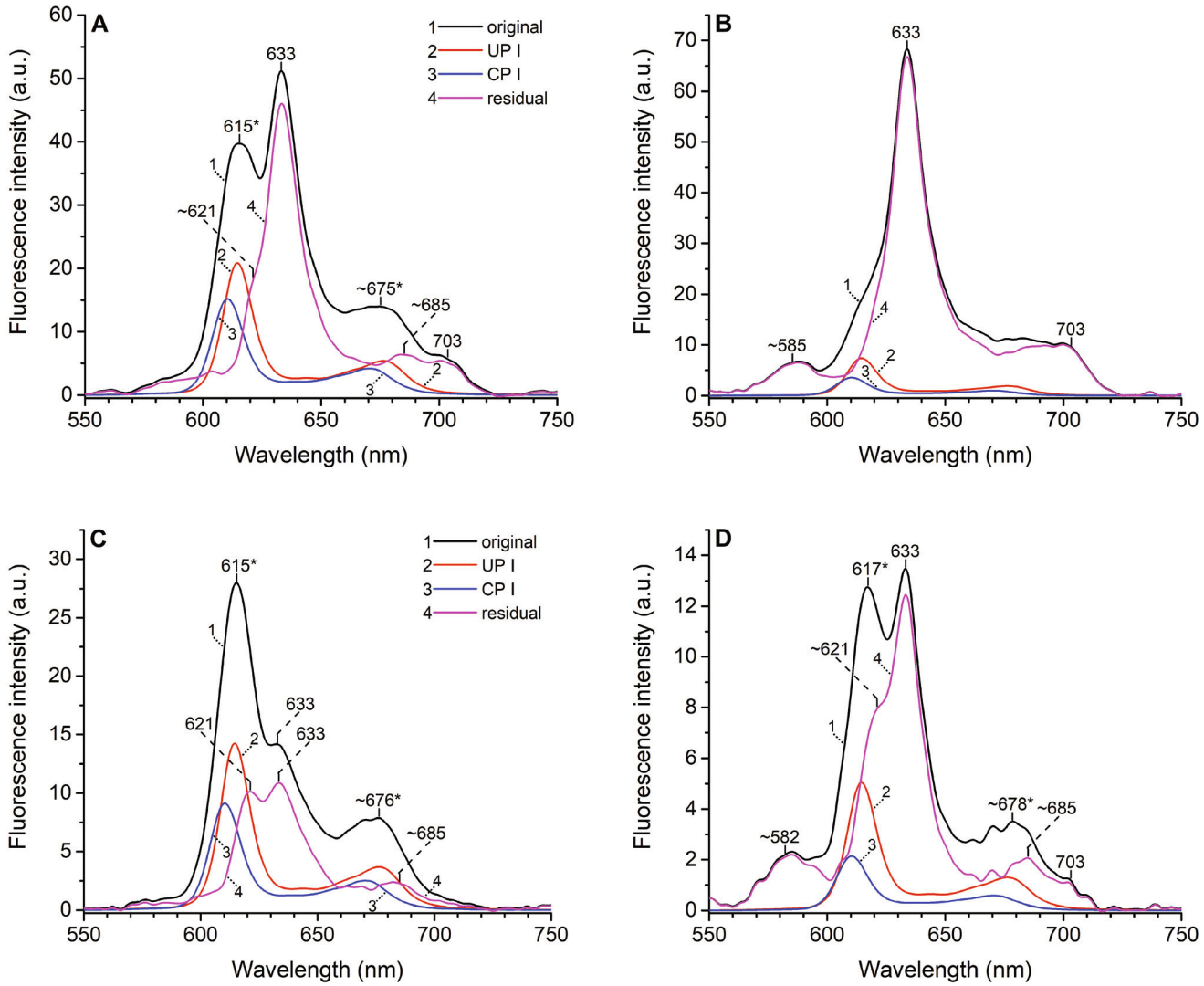

Fig. 2 Modeling of the fluorescence emission spectra detected in the samples of the resuspended bacterial cells after incubation of the bacterial culture with 5-ALA (fraction I) for 4 h (A and B) and 20 h (C and D) in PBS; excitation at 395 nm (A and C) and at 410 nm (B and D).

after both incubation periods of 4 h and 20 h. After adding HCl to the samples, the general shape of the fluorescence emission spectra of the porphyrins changed, and the peak positions were shifted to the blue spectral side, which is the typical indication of the presence of dicationic species. After the incubation period of 4 h, the spectra measured under excitation at 390 nm and 405 nm (Fig. 3A) showed a broad fluorescence band at 600 nm and a second band at 652 nm, which is a sign of different types of porphyrins being synthesized in the cells. The former excitation also revealed a band at about 623 nm. However, excitation at 420 nm resulted in a shift of both the major and the second fluorescence bands to 604 nm and 661 nm, respectively, indicating the presence of PP IX in a dicationic form.[30] The heterogeneous nature of the accumulated endogenous porphyrins was also supported by the fluorescence excitation spectra monitored at different wavelengths. While the spectral position of the Soret band at 404 nm remained unchanged, with the exception of a slight relative enhancement at the blue side (in the spectrum recorded at 620 nm), its spectral features and the spectral position of the fluorescence bands with excitation at 405 nm did not resemble any pair of the fluorescence excitation and emission spectra of the three standard porphyrins (Fig. S1†). In contrast, the properties of the fluorescence excitation and emission spectra recorded in the supernatant medium closely resembled those of the dicationic form of CP I. The exception was the additional fluorescence band at about 621 nm (Fig. 3B), the spectral features of which were similar to those detected in the bacterial cells, and the corresponding broader Soret band in a fluorescence excitation spectrum. Slight changes were registered in the spectra of the samples prepared after the incubation period of 20 h implying that the redistri-

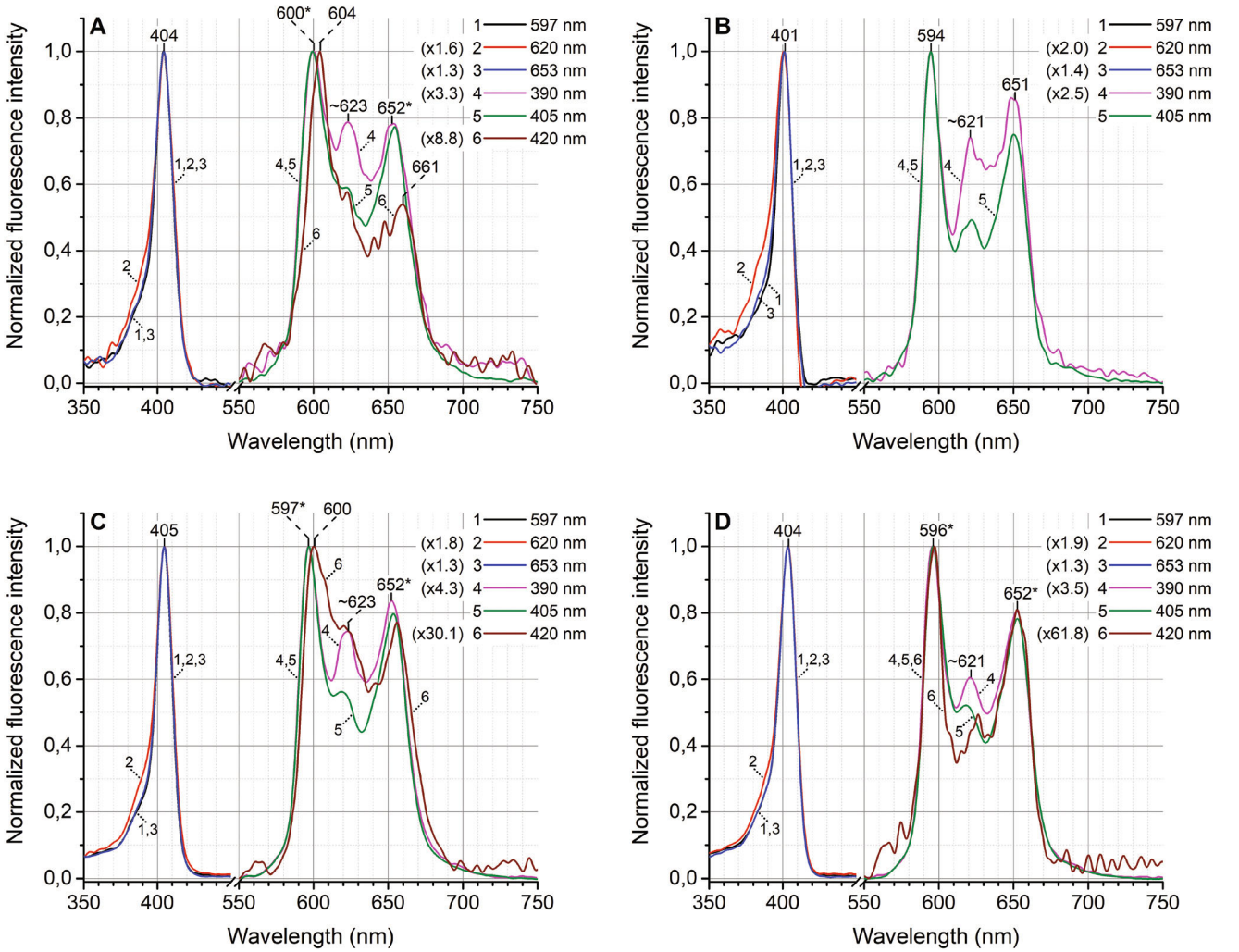

Fig. 3 Normalized fluorescence excitation and emission spectra observed in the samples of the resuspended bacterial cells (A and C) and the supernatants (B and D) after incubation of the bacterial culture with 5-ALA (fraction II) for 4 h (A and B) and 20 h (C and D) in PBS and the subsequent treatment with HCl (its final concentration in the samples was 1.5 M). Wavelengths used for the excitation and registration of the corresponding spectra are denoted in the legend.

bution between different porphyrins is dependent on time. The bands of the fluorescence spectra registered in the resuspended bacterial cells underwent a slight hypsochromic shift (Fig. 3C), while the opposite trend was observed in the supernatant medium (Fig. 3D). The Soret band became narrower in the bacterial cells, but broadened and shifted from 401 nm to 404 nm in the case of the supernatant.

The detailed spectroscopic analysis of the registered fluorescence emission spectra was performed in the same way as in the case of fraction I, using the spectra of standard porphyrins registered in acidic medium (Fig. S1†). The distribution of the diverse types of porphyrins accumulated in the bacterial cells of fraction II after 4 h and 20 h periods of incubation with 5-ALA and also those porphyrins being excreted into the medium is shown in Fig. 4. The excitation at different wavelengths (390 nm – Fig. 4A, C and E and 405 nm – Fig. 4B, D and F) showed the time-dependent variation in spectral proportions of the synthesized porphyrins that was predefined by their distinct absorption properties in the spectral region of the Soret band. The spectrum of the samples of the bacterial cells corresponding to the 4 h period of incubation was found to be a weighted sum of the spectra of PP IX and UP I dications with a smaller contribution of the residual spectrum (Fig. 4A and B), which was very similar to the spectra registered in the supernatant medium with the same incubation period (Fig. 4B, inset). The spectral position of the fluorescence emission bands at 594 nm and 651 nm allowed us to assign it to the dicationic form of CP I. After the longer incubation period (Fig. 4C and D), the bands of a UP I dication dominated in the registered spectra of bacterial cells. However, instead of PP IX, the residual spectrum with fluorescence bands at about 590 nm and at about 647 nm was observed. An additional broad fluorescence band at about 625 nm was also observed under excitation at 390 nm (Fig. 4C), but was absent under

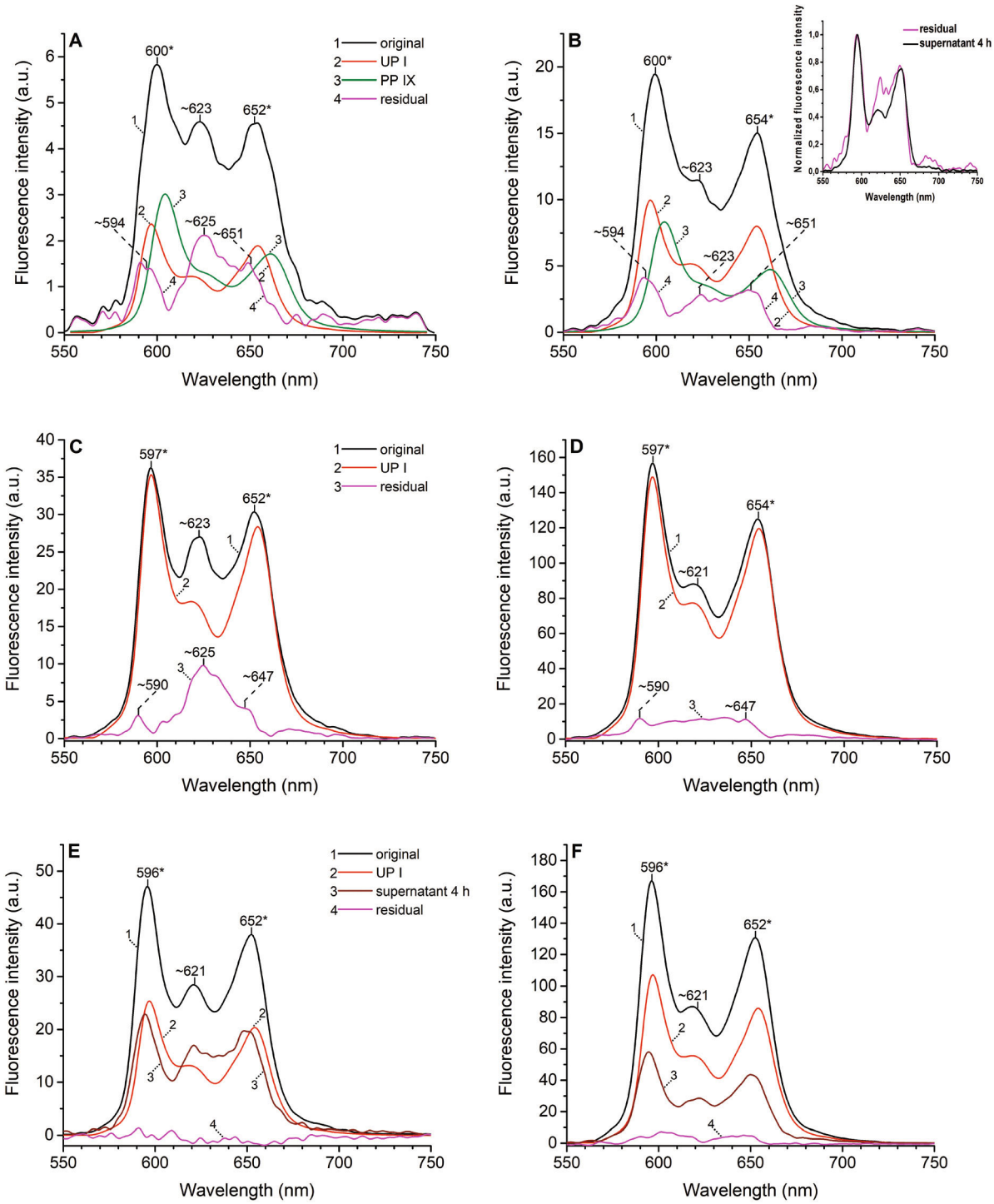

Fig. 4 Modeling of the fluorescence emission spectra detected in the samples of the resuspended bacterial cells (A–D) and the supernatants (E and F) after incubation of the bacterial culture with 5-ALA (fraction II) for 4 h (A and B) and 20 h (C–F) in PBS and the subsequent treatment with HCl (its final concentration in the samples was 1.5 M); excitation at 390 nm (A, C and E) and at 405 nm (B, D and F).

excitation at 405 nm (Fig. 4D). In the case of the supernatant medium after the prolonged incubation period (Fig. 4E and F), the increased contribution of UP I fluorescence was the main observed difference with respect to the spectrum registered after 4 h incubation.

4. Discussion

The ability of bacterial cells to synthesize extensive amounts of porphyrins after the application of exogenous 5-ALA is well established. Different types of porphyrins have been documen-

ted, such as the water-soluble uroporphyrins, coproporphyrins, intermediates containing 5 to 7 acetyl chains and the final product of the synthesis chain – hydrophobic protoporphyrin IX.[22,31]

Amongst the factors influencing the quantities and relative distribution of these porphyrins, one has to mention the strain of the studied bacteria, the applied concentration of 5-ALA and the incubation conditions such as the duration, the type of the medium used, and the cell density. In addition, the bacterial cells have a capacity to get rid of extra amounts of porphyrins by extruding it into the surrounding medium,[18] which can be affected by various external and internal factors too. The diversity in the published findings related to the types and the relative amounts of the detected porphyrins might be explained by the fact that bacteria utilize 5-ALA in a complex synthesis pathway[9,32,33] consisting of two related branches, which compete with each other for the same substrate.

The recorded fluorescence spectra of the samples of *Salmonella enterica* cells sensitized with endogenously produced porphyrins evidenced the involvement of both the branches of heme synthesis, although the duration of incubation seemingly had an impact on their output. Despite the initial differences between the samples of fractions I and II in the applied concentration of 5-ALA and the chosen bacterial cell density in the medium, the common tendency reflected by the spectral features was a time-dependent reduction of the intracellular content of PP IX. The application of the spectra of the standard porphyrins for modeling of the fluorescence features of intracellular porphyrins revealed a notable decrease in the fluorescence intensity of PP IX after the prolonged incubation period in the case of fraction I (Fig. 2), while PP IX fluorescence was undetectable in the spectra of fraction II measured by using two excitation wavelengths (Fig. 4C and D). Another finding made after modeling in the spectra registered after the prolonged incubation period was the relatively increased fluorescence intensity of UP I.

The longer incubation period also caused the relative increase of the fluorescence band at 621 nm in the residual spectra of resuspended cells (Fig. 2C and D). The bathochromic shift of the fluorescence bands of porphyrins is a usual sign of their interactions with the surrounding biomolecules. In accordance with ref. 34, the interaction of coproporphyrins I and III with certain cellular proteins results in a shift of the peak position to about 618–620 nm. While discrimination between these isomers can be done by using chromatography methods,[35] the spectroscopic properties of interacting coproporphyrin isomers do not allow distinguishing between them unambiguously. Since the fluorescence spectral bands of uroporphyrins are red-shifted with respect to those of coproporphyrins, we suppose that the band at 621 nm could be assigned to the intracellular uroporphyrins strongly interacting with the surrounding biomolecules.

Another time-dependent effect that was reflected in the spectral properties of porphyrins present in bacterial cells and the supernatants of fraction I was the increased relative amounts of metalloporphyrins. It is well documented[36] that the spectral position of a fluorescence band of Zn-PP IX corresponds to the lower energies in comparison with metal complexes of the water-soluble porphyrins. The difference in the peak position at the blue side of the major fluorescence band indicates that porphyrin–metal complexes located inside the cells (Fig. 1A and C) contained relatively more PP IX, while those of the water-soluble porphyrins dominated in the supernatant medium (Fig. 1D).

The incubation time-dependent changes observed in the spectra of fraction II revealed a tendency for increasing intracellular accumulation of UP I (Fig. 4A–D). On the other hand, the bands detected at about 590 nm and at about 647 nm in the residual fluorescence spectra (Fig. 4C and D) were found to be very similar to the bands characteristic of metalloporphyrins.

The comparison between the fluorescence spectra of the bacterial cells of fraction II with respect to the excitation wavelengths showed a relative increase of a band at 623 nm under excitation at 390 nm (Fig. 3). A shifted broad band at about 625 nm was revealed in the residual fluorescence spectra of the bacterial cells after both incubation periods (Fig. 4). Despite the acidic environment, the spectral position of this band is close to that detected at 621 nm in the case of the residual spectra of fraction I (Fig. 2). The broadening of the Soret band towards the shorter wavelengths, which was detected in the fluorescence excitation spectra monitored at 620 nm (Fig. 3), implies that the fluorescence band at 625 nm might reflect the presence of porphyrin(s) in the neutral state. We suppose that a porphyrin molecule can maintain this state in acidic medium when it forms a strong complex with biomolecules such as certain cellular proteins, and that such complexes can also be excreted by bacterial cells (Fig. S2†). Similar to the relative changes observed for the band at 621 nm in fraction I, the prolonged incubation period of fraction II with 5-ALA resulted in noticeably increased fluorescence intensity of the putative porphyrin–protein complexes in the cell samples (Fig. 4A–D).

A modeling analysis of the fluorescence emission spectra of the supernatants also showed that the distribution of the porphyrins was affected by the incubation duration. In the case of fraction I, the dominant porphyrin type after the prolonged incubation period became coproporphyrin I (Fig. S2†) being followed by the spectral bands at 621 nm, which was similar to those observed in the samples of the resuspended cells (Fig. 2). In addition, the modeling revealed the presence of UP I, while the band of metalloporphyrins and even the peak of PP IX were present in the residual fluorescence spectrum. The highest intensity of the CP I dication fluorescence was also detected in the acidic supernatants of fraction II after 4 h of incubation, while a band at about 621 nm implied the presence of uroporphyrins. The time-related spectral changes in the acidic supernatant medium of fraction II prepared after 20 h incubation mostly indicated the presence of UP I fluorescence bands (Fig. 4E and F). It should be noted that the acidic medium is not favourable for the stability of metalloporphyrins.[37]

The observed time-related redistribution of porphyrins might reflect the changes in the enzymatic activity of the bifurcated synthesis pathway. In general, the relative intracellular content of the water-soluble porphyrins increased in contrast to that of PP IX over time, while the products of a non-canonical pathway, such as UP I and CP I, were interfered with by additional by-products, such as metalloporphyrins and, presumably, strongly interacting porphyrin–protein complexes.

The ability of the bacterial cells to extrude excess porphyrins, which subsequently remain in the supernatants, might be related to cellular internal protective mechanisms. Certain non-iron metalloporphyrins were shown to be toxic for some bacterial strains, as they can be taken up through the same pathway as heme.[38] On the other hand, a non-specific enzymatic activity of a ferrochelatase located in the membrane has been reported, which can readily incorporate other metal cations such as Zn^{2+} into PP IX.[39] Such activity would play a protective role against the sensitization of bacterial cells in the case of increased accumulation of endogenous porphyrins, leading to intracellular accumulation of metalloporphyrins, especially, when the access to iron ions is limited, since the Zn-porphyrins were found to be non-phototoxic for bacteria.[40] The next step could be the excretion of extra porphyrins into the surrounding medium *via* dedicated complexes of proteins such as a TolC-dependent efflux system.[19] Certain proteins participating in the transmembrane transportation of substances can not only regulate the extracellular transport, but also involve in direct interaction with the transported molecules.[41] Such transport-related interactions, leading to the formation of strong porphyrin–protein complexes, might provide an explanation for the spectral shift of the main porphyrin fluorescence band towards 621–625 nm, and its persistence in the acidic environment.

5. Conclusions

Various types of endogenous porphyrins were produced in *Salmonella enterica* cells after incubation with 5-ALA in buffered aqueous medium. The fluorescence spectroscopy measurements of intracellular photosensitizers revealed a time-dependent decrease in the accumulation of protoporphyrin IX, while the major water-soluble porphyrin was found to be uroporphyrin I. The ability of bacterial cells to extrude the produced porphyrins was evidenced by the detection of characteristic fluorescence bands in the supernatant medium, which indicated the dominance of coproporphyrins, presumably, coproporphyrin I. The relative increase in the intensity of the fluorescence spectral bands attributed to metalloporphyrins and the presumed porphyrin–protein complexes and their redistribution from bacterial cells towards the supernatant medium after the prolonged incubation period imply that 5-ALA-induced sensitization triggered a complex protective mechanism of *Salmonella enterica* cells. The obtained data can be useful in seeking efficient ways of 5-ALA-based PDT antibacterial application that account for the time-dependent production and redistribution of endogenous photosensitizers in bacterial cells.

Conflicts of interest

There are no conflicts of interest to declare.

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