Antibacterial Nanoparticles with Natural Photosensitizers Extracted from Spinach Leaves

Pavel Ludačka, Pavel Kubát, Zuzana Bosáková, and Jiří Mosinger*

ABSTRACT: We prepared antibacterial polystyrene nanoparticles (NPs) with natural photosensitizers from chlorophyll (Chl) extract via a simple nanoprecipitation method using the same solvent for dissolution of the polystyrene matrix and extraction of Chls from spinach leaves. A high photo-oxidation and antibacterial effect was demonstrated on Escherichia coli and was based on the photo-generation of singlet oxygen \( \text{O}_2(1\Delta_g) \), which was directly monitored by NIR luminescence measurements and indirectly verified using a chemical trap. The photoactivity of NPs was triggered by visible light, with enhanced red absorption by Chls. To reduce the quenching effect of carotenoids (\( \beta \)-carotene, lutein, etc.) in the Chl extract, diluted and/or preirradiated samples, in which the photo-oxidized carotenoids lose their quenching effect, were used for preparation of the NPs. For enhanced photo-oxidation and antibacterial effects, a sulfonated polystyrene matrix was used for preparation of a stable dispersion of sulfonated NPs, with the quenching effect of carotenoids being suppressed.

INTRODUCTION
Chlorophylls (Chls) are widely abundant photosensitizers in nature that enable conversion of solar energy to biochemically useful forms. They are very cheap, commercially available, main group metal-based, and provide excellent efficiency for singlet oxygen-mediated chemistry. The photosensitization process from the \( S_1 \) states after excitation with red and/or blue radiation competes with a very efficient intersystem crossing to the \( T_1 \) state and the formation of highly reactive singlet oxygen \( \text{O}_2(1\Delta_g) \). In general, \( \text{O}_2(1\Delta_g) \) is generated during photosynthesis in the photosystem II center. It is a signal molecule, but at high concentrations, it can oxidize and destroy target structures and can be applied for photodynamic inactivation of bacteria and viruses and for photodynamic therapy to treat tumors.

Chl extract from spinach (ChlE) and other plants is highly promising for applications in reactions photosensitized by \( \text{O}_2(1\Delta_g) \). A recent paper reported quantum yield of singlet oxygen \( \Phi_\Delta = 0.58 \) for a Chl-based extract, similar to the value of \( \Phi_\Delta = 0.51 \) for Chl \( a \). Note that \( \Phi_\Delta \) values for Chl-based compounds in different solvents were summarized in previous comprehensive reviews published more than 20 years ago. Application of the extract is limited by the presence of carotenoids that dramatically decrease photo-oxidation efficiency, which is a harmful process for natural or artificial photosynthesis. Both Chl triplet states and \( \text{O}_2(1\Delta_g) \) can be quenched by carotenoids via energy transfer (physical quenching). Additionally, carotenoids (carotenes and xantho-
noids that are diluted in solutions in comparison with their level in plants.

There are plenty of organic and inorganic nanocarriers under investigation for transport of photosensitizers, some of them have good photocatalytic and/or antibacterial properties. In our previous studies, we prepared different nanofiber materials and nanoparticles (NPs) with a porphyrin photosensitizer, which generated O$_2$(1Δg) “on demand” after excitation with visible light and exhibited antibacterial properties. In general, NPs with a high surface/volume ratio can move in close proximity to the biological targets to be destroyed, which can overcome the O$_2$(1Δg) diffusion limitations. The encapsulated photosensitizer is well protected against external quenchers, which allows quenching of triplet states by oxygen, exclusively in the interior of NPs. These antibacterial NPs are considered an alternative to antibiotics and have strong potential to solve the problem of bacterial multidrug resistance.

Among the variety of nanocarriers suitable for photodynamic inactivation, polystyrene NPs are superior due to their biocompatibility, low toxicity, high oxygen permeability/diffusion coefficient, and negligible leakage of nonpolar encapsulated photosensitizes to aqueous media.

This report describes a simple method for preparation of an aqueous dispersion of polystyrene NPs with encapsulated ChlE from spinach (Spinacia oleracea). In addition to Chl, the extract also contained carotenoids, which have a negative influence on photoactive NPs with respect to their photo-oxidation, antibacterial activity, and photostability. We used three simple methods to suppress the quenching effect of carotenoids: working with diluted ChlE (i), using ChlE preirradiated with light to photo-oxidize the carotenoids (ii), and/or using small sulfonated polystyrene NPs with a higher surface/volume ratio to suppress the effect of carotenoids inside the NPs (iii). The main advantages of this approach in the synthesis of photoactive NPs are the application of a “green” photosensitizer abundant in nature with absorption in both the red and blue regions of the visible spectrum and using the same solvent for extraction of Chl and preparation of NPs. Moreover, glassy polystyrene NPs prepared by the nanoprecipitation method are more stable than liposome- or polymeric micelle-based NPs and should preserve favorable photophysical properties not influenced by photo-electron transfer from excited Chl molecules to the metal core typically for gold and silver NPs.

## RESULTS AND DISCUSSION

### ChlE High-Performance Liquid Chromatography Analysis

Chl $a$ and Chl $b$ are highly abundant in green leaves and are accompanied by protective carotenoids. HPLC analysis revealed that the retention times and mass spectrometry (MS) profiles of compounds found in ChlE correspond to Chl $a$, Chl $b$, β-carotene, and lutein standards (Table 1, Figure S1, panel B, and Figure S2 in Supporting Information).

For HPLC quantitative estimation, a mixture of standards (Chl $a$, Chl $b$, β-carotene, and lutein) at concentrations of 0.5, 1, 2.5, 5, and 7.5 mg/L, respectively, in MeOH/THF 9:1 (v/v) was applied and used to construct calibration plots for each component (peak area vs. concentration). Then, ChlE diluted 10X in MeOH was tested. The concentration of each photosensitizer, which generated O$_2$(1Δg) close proximity to the biological targets to be destroyed, which have good photocatalytic and/or antibacterial properties. In our previous studies, we prepared different nanofiber materials and nanoparticles (NPs) with a porphyrin photosensitizer, which generated O$_2$(1Δg) “on demand” after excitation with visible light and exhibited antibacterial properties. In general, NPs with a high surface/volume ratio can move in close proximity to the biological targets to be destroyed, which can overcome the O$_2$(1Δg) diffusion limitations. The encapsulated photosensitizer is well protected against external quenchers, which allows quenching of triplet states by oxygen, exclusively in the interior of NPs. These antibacterial NPs are considered an alternative to antibiotics and have strong potential to solve the problem of bacterial multidrug resistance.

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### Table 1. Retention Time of Standards and Concentration of Corresponding Compounds in ChlE

| pigment   | Chl $a$ | Chl $b$ | β-carotene | lutein |
|-----------|---------|---------|------------|--------|
| $t_R$ (min) | 17.6 | 9.8 | 56.7 | 4.3 |
| $c$ (mg/L) | 154 (190) | 54 (62) | 38 | 28 (39) |
| content in w/w % | 56 (58) | 20 (19) | 14 (11) | 10 (12) |

“Values in parentheses include the isomers of the respective compounds.

ChlE consists of a Q$_a$ band of Chls at 664 nm corresponding predominantly to the Chl $a$ spectrum and of a Soret band in the blue region of the spectrum partially overlaid with the absorption band of other species (Chl $b$, carotenoids, etc.). The absorption spectrum allows efficient harvesting of red and blue radiation in the solar spectrum.

We measured the weak near infrared luminescence of singlet oxygen, O$_2$(1Δg) at ~1270 nm (Figure S3 and S4 in Supporting Information) and estimated the quantum yield ($\Phi_{\Delta}$) by comparing the amplitudes of singlet oxygen decay kinetics after excitation with a dye laser (664 nm and 28 ns pulse length) at the same absorbance at the excitation wavelength (Table 2). Taking into account $\Phi_{\Delta} = 0.53$ for ZnPc in THF, the calculated values of $\Phi_{\Delta}$ were 0.64 and 0.66 for ChlE and Chl $a$, respectively. This result corresponds to previous literature data for ChlE prepared from spinach.

The concentration of photosensitizers (predominantly Chl $a$) used in experiments was proportional to the absorbance at the Q$_a$ band of Chl. The estimated concentration was $10^{-5}$ mol/L$^{-1}$ (Table S1 in Supporting Information) or lower taking into account the literature value for the extinction coefficient of Chl $a$ ($\sim 9 \times 10^3$ L mol$^{-1}$ cm$^{-1}$). The singlet oxygen lifetime ($\tau_{\Delta}$) increased with the dilution of ChlE due to the lower concentration of quenchers (carotenoids, Figure 1B).
To remove the $O_2(1\Delta_g)$ quenchers from the extract, purification by chromatographic methods was needed. Alternatively, we irradiated raw ChlE with visible light, under which the quenching effect of carotenoids was suppressed by their oxidation with $O_2(1\Delta_g)$ to form photochemically inactive endoperoxides.\(^\text{17}\) The value of $\tau$ also increased with increasing irradiation time up to a value of $\sim 20.6\ \mu s$ measured for the ZnPc standard, which corresponds with the literature data for THF (Figure 1B).\(^\text{36}\) This effect corresponds with degradation of the quenchers through the reaction of $O_2(1\Delta_g)$ with the double bonds of carotenoids. Note that carotenoids exhibit complex behaviors and can be degraded by other mechanisms, for example, by free radical reaction.\(^\text{37,38}\) The changes in the absorbance of $Q_y$ bands at 664 nm also indicate partial photodegradation of ChlE (proportional to changes in the absorbance of the $Q_y$ band).

We also measured transient absorption spectra to evaluate the kinetics of the photosensitizer (predominantly Chl $a$) triplet states—a precursor of $O_2(1\Delta_g)$ (Figure S5 in Supporting Information). The kinetics of the triplet states in THF show very efficient quenching of the triplets by dissolved oxygen (Table 2), leading to $O_2(1\Delta_g)$ formation. We also found a lower lifetime of the photosensitizer triplet states for ChlE ($167\ \mu s$) in argon-saturated THF in comparison with the Chl $a$ standard ($294\ \mu s$); however, more than 99% of triplets were quenched by oxygen in an air-saturated solution of ChlE, indicating that quenching by oxygen was dominant in comparison with quenching by carotenoids. Overall, the basic processes photosensitized by ChlE are summarized in Figure 2.

**Photostability.** A relatively photostable ChlE from the cyanobacterium *Spirulina maxima* was recently suggested for application in photodynamic therapy after purification.\(^\text{39}\) We tested the photostability of our raw ChlE from spinach after irradiation with a 500 W Xe lamp with a long pass filter ($\lambda > 400$ nm), and the kinetics of decomposition were followed at 664 and 459 nm (Figure 3). The first wavelength (664 nm) corresponds to the absorption bands of Chl, and the second wavelength (459 nm) corresponds to the absorption band of $\beta$-carotene. The photodegradation of $\beta$-carotene dominated, and the amount of $\beta$-carotene was negligible after 20 min of irradiation both in ChlE and in a two-component model, where standards of Chl $a$ and $\beta$-carotene were mixed. The photodegradation of both Chl and $\beta$-carotene is a complex process, including oxidation by photogenerated $O_2(1\Delta_g)$ and energy transfer from excited Chl $a$ to $\beta$-carotene (Figure 2).\(^\text{40}\)

A sample of ChlE after 50 min of irradiation was analyzed by HPLC. Chl $a$ and Chl $b$ were present in the sample, but no lutein or carotene was found (Figure S1, panel C in Supporting Information).

**Sulfonated and Nonsulfonated Polystyrene Nanoparticles. Preparation and Characterization.** Sulfonated and nonsulfonated polystyrene NPs were prepared by simple
nanoprecipitation of THF solutions of sulfonated or non-sulfonated polystyrene enriched with THF spinach extract or standards/quenchers (see Experimental Section and Figure S6 in Supporting Information). Sulfonation of the polystyrene matrix increased NP stability, even in aqueous environments with high ionic strengths.

The dispersions of sulfonated/nonsulfonated polystyrene NPs with spinach extract (ChlE@NPs) and NPs without and with the chlorophyll standard and carotene quencher were characterized by transmission electron microscopy (TEM) (Figure 4B,C), UV/vis absorption and fluorescence spectroscopy (Figure 4E), and dynamic light scattering (DLS) (Figure 4F).

The nanoprecipitation method led to mostly spherical NPs, as observed in the TEM images, where larger NPs were more visible in comparison to the bulk of small NPs revealed by DLS. The basic properties of the NPs are summarized in Table 3.

Dispersions of NPs prepared from nonsulfonated polystyrene nanofiber membranes displayed hydrodynamic diameters of approximately 230–280 nm. The sulfonated NPs had average hydrodynamic diameters of approximately 60–64 nm with a low polydispersity (PDI index of 0.2–0.3), in contrast to nonsulfonated NPs, where the freshly prepared nonsulfonated NPs were polydispersed with a tendency to precipitate. The size and zeta potential of NPs showed their high stability over time (Table S2 in Supporting Information).

According to HPLC analysis, the composition of encapsulated compounds in NPs corresponded to the composition of ChlE. Generally, chlorophylls are not stable in an acid environment and can undergo demetalation to the corresponding pheophytin but under our experimental conditions, no demetalation occurs (Figure S1, panel C in Supporting Information).

Also, no absorption or fluorescence of Chls was found in the filtrate (Figure S7 in Supporting Information), which indicates no leaching to aqueous media. The Soret absorption maximum of Chl a in the absorption spectrum of ChlE was shifted from 436 nm in THF solution to 415 nm in ChlE@NP dispersion.

Photophysical Properties. In contrast to a solution, individual components of ChlE (Chls and carotenoids) are fixed at specific places inside glassy ChlE@NPs, and only oxygen can diffuse through the polystyrene matrix with a relatively high oxygen diffusion coefficient [D(O2)∼3×107 cm−1 s−1]. The lifetime of singlet oxygen (τΔ) measured from weak NIR luminescence is controlled by the size of the NPs, by different values of τΔ in the NP interior and outside in the aqueous environment, and by additional quenching by carotenoids (Figure 5a). Note that only O2(1Δg) that diffuses from the NP interior to the exterior environment can be used for photo-oxidation of chemical substrates and bacterial structures (see photo-oxidation tests and photodynamic inactivation of Escherichia coli). For this reason, polystyrene with a high oxygen diffusion coefficient was selected as the starting material for NP preparation.

Prolongation of O2(1Δg) luminescence kinetics for sulfonated ChlE@NPs prepared from extract preirradiated with visible light from a Xe lamp, Figure 5c, τΔ = 13 μs in
Comparison of the SODF signals generated a dramatic increase in the I3/Δg concentration for nonsulfonated ChlE@NPs (a) and preirradiated sulfonated ChlE@NPs (c). The SODF signal for preirradiated sulfonated ChlE@NPs showed a much higher SODF signal (Figure S9 in Supporting Information). Experiments were carried out in an oxygen-saturated water dispersion to increase the amount of O2(1Δg) generated by the photosensitization process. The red lines are single exponential fits to the experimental data. Note that the value of τA is influenced by slow decay kinetics of Chl triplets in nanoparticles (Figure S8 in Supporting Information).

Figure 5. Simplified behavior of O2(1Δg) in the NP interior and outside NPs in an aqueous environment (a). Normalized singlet oxygen kinetics after excitation of ChlE in sulfonated ChlE@NPs (b) and preirradiated sulfonated ChlE@NPs (c). The SODF signal for preirradiated sulfonated (d) and nonsulfonated ChlE@NPs (e). Experiments were carried out in an oxygen-saturated water dispersion to increase the amount of O2(1Δg) generated by the photosensitization process. The red lines are single exponential fits to the experimental data. Note that the value of τA is influenced by slow decay kinetics of Chls triplets in nanoparticles (Figure S8 in Supporting Information).

**Table 3. Basic Properties of Sulfonated and Nonsulfonated NPs**

| Properties                | nonsulfonated NPs | sulfonated NPs |
|---------------------------|-------------------|----------------|
| @NPs                      | ChlE@NPs          | @NPs           |
| Diameter (DLS) (nm)       | 278               | 64             |
| Zeta potential (mV)       | −34               | −33            |
| Number of NPs in 1 mL dispersion | 2.60 × 10^10          | 1.41 × 10^13   |
| Surface (nm^2)            | 243 000           | 13 000         |
| Surface/Volume (nm^-1)    | 0.02              | 0.09           |
| Appearance                | Milky             | Transparent    |

Figure 6. Photo-oxidation of Chemical Substrates. The dispersions of NPs were irradiated with a 36 W red light-emitting diode (LED) grow light bulb (λ = 662 nm) in iodide detection solution to observe O2(1Δg) photogeneration (see Experimental Section). A linear increase in the I5^- concentration (following the UV/vis absorbance change at 287 or 351 nm) proportional to the generation of O2(1Δg) was found (Figure S11 in Supporting Information). Alternatively, O2(1Δg) photogeneration was confirmed by photodegradation of uric acid at 293 nm (Figure S12 in Supporting Information). The experimental data indicate that oxidation of both I^- and uric acid substrates is due to oxidation by O2(1Δg) for several reasons: a) formation of O2(1Δg) was confirmed from luminescence measurement (see photophysical measurements above), b) the oxidation did not take place in inert gas-saturated samples and/or in aerated samples in the dark, and c) a strong physical quencher of singlet oxygen (sodium azide) inhibited oxidation of both substrates. A fluorescence assay using terephthalic acid did not reveal the generation of any other ROS (H2O2, O2^-, and OH^-). Also, no post-irradiation effect, for example, dark oxidation of iodide (in iodide test), typical for accumulation of H2O2 due to presence of OH and O2^- was found. However, we cannot exclude the minor formation of these species; the quantitative evaluation of data for such complex systems may be limited.

To estimate the relative efficiency of I^- photo-oxidation to I5^- by O2(1Δg), absorbance-matched dispersions of different NPs were irradiated with a red LED bulb (λ = 662 nm). Figure 6 shows that smaller sulfonated ChlE@NPs were much more effective producers of O2(1Δg) than larger, nonsulfonated ChlE@NPs and exhibited approximately the same photo-oxidation efficiency as Chlα@NPs (using a Chl α analytical standard) of a similar size.
The presence of β-carotene reduces the photo-oxidation response. Surprisingly, we found that sulfonated ChlE@NPs (originating from 4 mg of β-carotene) have the same photo-oxidation kinetics as a sample with a half concentration of β-carotene (2 mg, not shown). This observation may correspond with the small size of NPs: \(O_2(\Delta g)\) photo-generated near the surface of NPs is directly released into aqueous media and does not meet any β-carotene (carotenoid) quencher located inside the NPs. In contrast, \(O_2(\Delta g)\) photo-generated inside larger nonsulfonated NPs can be quenched even by a low concentration of β-carotene. This efficient quenching reduces the number of \(O_2(\Delta g)\) molecules that diffuse into the environment of the NPs toward target structures and inhibits total photo-oxidation (Figure 6).

To evaluate the preirradiation effect on the relative efficiency of photo-oxidation of chemical/biological targets by \(O_2(\Delta g)\), aqueous dispersions of sulfonated ChlE@NPs and nonsulfonated ChlE@NPs were irradiated with a 36 W red LED bulb before addition of iodide detection solution and photo-oxidation tests. Preirradiation of sulfonated ChlE@NPs led only to a decrease in photo-oxidation efficiency, attributed to the common photodegradation of Chl photosensitizers (Figure S13 in Supporting Information). The large nonsulfonated ChlE@NPs exhibited a more complex profile with limited reproducibility.

In our previous study,\(^{41}\) we found that polystyrene NPs can be easily removed by filtration through a 0.03 mm thin hydrophilic electrospray polycarbonate (Tecophilic) nanofiber membrane. The removal of sulfonated ChlE@NPs was followed by decreased red fluorescence in the supernatant (Figure S7 in Supporting Information). After one filtration, approximately 90% of fluorescent ChlE@NPs were removed. Repeated filtration yielded complete removal of all NPs. No fluorescence or absorption was observed, and only a background DLS signal was observed. This test also verified that the hydrophobic compounds in ChlE are fixed in the glassy polyacrylate matrix, which efficiently prevents release of the encapsulated compounds into the aqueous medium. In short, only components of ChlE encapsulated in ChlE@NPs exhibited an efficient photoantibacterial effect due to the formation of cytotoxic \(O_2(\Delta g)\).

\textbf{Photodynamic Inactivation of \textit{E. coli}.} Previous photo-oxidation experiments revealed that \(O_2(\Delta g)\)-generating NPs can easily oxidize external substrates in aqueous media. To test photodynamic inactivation (PDI), a suspension of Gram-negative \textit{E. coli} was mixed with a dispersion of @NPs or sulfonated ChlE@NPs and irradiated with a 36 W red LED bulb (\(\lambda = 662\) nm) for 0, 10, and 20 min. No significant photodegradation of Chls during irradiation (monitored by changes in the absorbance at 664 nm) was observed (Figure S10 in Supporting Information). In contrast to controls (suspension of \textit{E. coli} alone or with sulfonated @NPs), a substantial decrease in the colony forming unit (CFU) ratio was observed when sulfonated ChlE@NPs were used (Figure 7). ChlE@NPs kept in the dark also exhibited no antibacterial effect (Table S3 in Supporting Information). In contrast to the frequently observed antibacterial effect of visible light itself (especially in the blue region), we found a slight increase in the CFU ratio. This stimulation effect can be attributed to the temperature increase in the cuvette chamber (0.5–1.0 °C) during irradiation.

\textbf{CONCLUSIONS}

Polystyrene nanoparticles with Chl photosensitizers from green plants prepared by simple nanoprecipitation methods can be inexpensive and green alternatives to photoactive antimicrobial NPs with many applications, for example, for cleaning contaminated water or for antibacterial treatment of biofilms. The high quantum yield of singlet oxygen generation for ChlE (close to that of pure Chl a) allows direct application in photodynamic treatment without a complicated purification process to prepare individual photosensitizers. The quenching effect of carotenoids in ChlE can be suppressed by preirradiation with visible light, under which carotenoids decompose more efficiently than Chl photosensitizers and lose their quenching ability due to the reaction of \(O_2(\Delta g)\) with the conjugated double bonds of carotenoids. Encapsulation of Chl photosensitizers from ChlE in polystyrene NPs led to higher photostability. NPs prepared from sulfonated polystyrene with small sizes (diameter of \(\sim 60\) nm) exhibited more efficient photo-oxidation and antibacterial properties than those prepared from nonsulfonated polystyrene. Moreover, efficient...
removal of the NPs together with the inactivated bacteria after their use by simple filtration through an electrospun membrane (a precursor of NPs) is also an important benefit.

**EXPERIMENTAL SECTION**

**Materials.** Chl (Chl a and Chl b), lutein, and \( \beta \)-carotene standards; uric acid sodium salt; tetraethylammonium bromide (TEAB); hydrogen peroxide; ampicillin; potassium iodide and other inorganic salts (all Sigma-Aldrich); phosphate-buffered saline (PBS); agar and LB medium (Lennox) (all Carl Roth GmbH); cyclohexanone; and sulfuric acid (both Lach-Ner, Czech Republic).25,43,50

Electrospinning process was described in detail in our previous studies.25,43,50

**Preparation of Nanofiber Material and NPs.** A mixture of 0.07 wt % TEAB and 99.93 wt % polystyrene was dissolved in cyclohexanone to prepare a 17% solution for fabrication of electrospun polystyrene nanofiber material. The conductivity of the solution was enhanced by TEAB (0.12 g/kg). The electrospinning process was described in detail in our previous papers.25,43,50

Sulfonated NPs were prepared from electrospun polystyrene nanofiber material fixed on quartz substrates treated with immersion in 96% sulfuric acid at room temperature for 48 h. The materials were washed with distilled water until a neutral pH was reached and were then stored between two pieces of filter papers.25,43,50

**Photophysical Properties.** UV/vis absorption spectra were recorded using Unicam 340 and Varian 4000 spectrometers. The steady-state fluorescence spectra were monitored using an FLS 980 (Edinburgh Instruments, UK) spectrophotometer. For time-resolved measurements, the samples were excited with a Lambda Physik FL3002 laser (\( \lambda_{\text{exc}} = 644 \) nm and pulse length \( \sim 28 \) ns) that matched the Qy band of Chls.25

The optimized MS/MS conditions are shown in Table S1 in Supporting Information. THF was removed by evaporation under a slight vacuum (60 °C, to a final volume of approximately 15 mL). The resulting dispersion of NPs in water was centrifuged for 10 min at 3070 g to remove microparticles and was dialyzed using Float-A-Lyzer G2 with a molecular weight cutoff of 50 kDa for 15 h in distilled water at room temperature to remove traces of sulfuric acid and THF. Non sulfonated NPs were prepared under the same protocol (see above) without sulfonation.

**HPLC Analysis.** HPLC analyses were performed on an Agilent Infinity 1290 liquid chromatographic system coupled with a Triple Quad 6460 tandem mass spectrometric detection system (Agilent Technologies, Germany). A SunFire C18 column (150 mm × 4.6 mm, 5 μm particle size), (Waters, Ireland), was thermostatted at 20 °C, was used for separation. The mobile phase consisted of methanol (MeOH) with 0.5% formic acid at a flow rate of 0.8 mL/min. The injection volume was 20 μL, and samples were kept at 20 °C. The MS/MS measurements were performed in the multiple reaction-monitoring (MRM) mode using positive electrospray ionization (ESI). The gas flow was 10 L/min, gas temperature was 350 °C, nebulizer pressure was 55 psi, and capillary voltage was 5500 V. The optimized MS/MS conditions are shown in Table S4 in the Supporting Information. Total-ion current (TIC) chromatograms with MRM chromatograms of the individual analytes obtained for a mixture of standards are shown in Figure S1, panel A (Supporting Information), and the same TIC chromatograms for ChlE are shown in Figure S1, panel B (Supporting Information).

Characterization of NPs and Electrospun Polymeric Membranes. Nanofiber and NP morphology was studied with a scanning electron Quanta 200 FEG microscope (FEI, Czech Republic). The nanofiber diameters were measured using NIS Elements 4.0 image analysis software (Laboratory Imaging, Czech Republic). The NP size and size distributions in water were determined by dynamic light scattering on a Zetasizer Nano ZS particle size analyzer from Malvern (United Kingdom).

**Antibacterial Assays.** A culture of *E. coli* DH5α (Invitrogen, CA, USA) with the plasmid pGem112 (Promega, WI, USA) was incubated at 37 °C while stirring in LB medium after addition of ampicillin. Incubation was terminated when the absorbance at 560 nm reached approximately 1.2. The prepared culture was diluted 5000× to the desired concentration in PBS. NPs from stock suspensions of sulfonated NPs were added to each well of a 96-well plate, and membranes were placed on top. The solution and the inactivated bacteria after its use by simple filtration through an electrospun membrane (a precursor of NPs) is also an important benefit.
(≈2.0 × 10¹³ NPs/mL) with or without encapsulated ChlE were mixed with diluted bacterial culture at a ratio of 1:1. Two milliliters of this suspension was placed in a thermostatted 10 mm quartz cell (25 °C). While stirring, the cell was irradiated with red light produced by a stabilized deep red LED grow light bulb (36 W and 662 nm). At regular time intervals, 150 μL of the irradiated suspension was placed on an agar plate. The plates were incubated for 20 h in darkness at 37 °C to allow the individual bacteria to grow and form colonies.

**ASSOCIATED CONTENT**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c06229.

HPLC analysis, absorption and fluorescence spectra, time-resolved luminescence of O₂(1Δg), transient absorption data, delayed fluorescence of ChlE from spinach, and nanoparticles prepared from ChlE from spinach (PDF)

**AUTHOR INFORMATION**

**Corresponding Authors**
Pavel Kubát – J. Heyrovský Institute of Physical Chemistry of the Academy of Sciences, 182 28 Prague 8, Czech Republic; orcid.org/0000-0002-7861-9212; Email: pavel.kubat@jh-inst.cas.cz

Jiří Mosinger – Faculty of Science, Charles University, 128 43 Prague 2, Czech Republic; orcid.org/0000-0001-5173-2744; Email: mosinger@natur.cuni.cz

**Authors**
Pavel Ludačka – Faculty of Science, Charles University, 128 43 Prague 2, Czech Republic
Zuzana Bossáková – Faculty of Science, Charles University, 128 43 Prague 2, Czech Republic

Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acsomega.1c06229

**Notes**
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by the Czech Science Foundation (19–09721S) and by the OPVV “Excellent Research Teams”, project no. CZ.02.1.01/0.0/0.0/15_003/0000417−CUCAM. The authors thank Dr. Miroslav Štěpanek for TEM evaluation of NPs and Dr. Pavel Engst for help with the photophysical measurements.

**ABBREVIATIONS**

Chl a  chlorophyll a  
Chl b  chlorophyll b  
ChlE  chlorophyll extract from spinach in THF  
ChlE*  designates ChlE preirradiated with a Xe lamp with a long pass filter (λ > 400 nm)  
NPs  nanoparticles  
@NPs  nanoparticles without a photosensitizer  
Chlx@NPs  polystyrene nanoparticles with chlorophyll a or chlorophyll extract (x = a, E)  
ChlαCar@NPs  polystyrene nanoparticles with both chlorophyll a and β-carotene

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