Photophysical Properties of Fluorescent Self-Assembled Peptide Nanostructures for Singlet Oxygen Generation

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ABSTRACT: In this work, a drug delivery system for perillyl alcohol based on the peptide self-assembly containing 3-(2-benzothiazolyl)-7-(diethylamino)coumarin (C6) as a fluorescent additive is obtained, and its photophysical characteristics as well as its release dynamics were studied by steady-state and time-resolved fluorescence spectroscopy. Results proved the dynamics of drug release from the peptide nanostructures and showed that the system formed by the self-assembled peptide and C6, along with perillyl alcohol, presents unique photophysical properties that can be exploited to generate singlet oxygen (\(1^{{\text{O}}_2}\)) upon irradiation, which is not achieved by the sole components. Through epifluorescence microscopy combined with time-correlated single photon counting fluorescence spectroscopy, the release mechanism was proven to occur upon peptide structure interconversion, which is controlled by environmental changes.

INTRODUCTION

Perillyl alcohol, a monoterpene derived from limonene, is intensively studied because of its anticancer properties. It has been successfully used in brain cancer therapy, administered by intranasal route, because it improves the drug penetration through the blood–brain barrier\(^1\) to\(^5\) and in topical administration for treating skin tumors.\(^4\),\(^5\) In addition, the antitumor activity of perillyl alcohol was reported in a variety of other cancer cells, such as breast, liver, and pancreatic cancer cells,\(^6\) to\(^8\) and its antitumor action is related to its radical scavenging activity, by which reactive oxygen species (ROS) in the intracellular medium are eliminated.\(^9\) A variety of other mechanisms of action of perillyl alcohol are reported, related to its penetration to the plasma membrane bilayer and induced stress in the endoplasmic reticulum,\(^10\) a pro-oxidant effect, involving the disruption of the mitochondria transmembrane potential that leads to intracellular buildup of ROS,\(^11\) among others.\(^12\) to\(^18\)

Nevertheless, the use of perillyl alcohol has been restricted to the inhalation and topical methods due to its gastrointestinal side effects incurred by oral administration.\(^12\) An intensively studied strategy that has promised to optimize drug efficacy is the use of drug delivery systems, which offer the possibility of drug encapsulation and controlled release at the desired site for therapeutic action. Drug encapsulation is responsible for providing in route stability, by avoiding contact of the drug with the physiological medium, and increased affinity and permeability to the targeted cells, thereby greatly improving selectivity, minimizing side effects, and efficacy of the drug, once a prolonged and sustained release can be achieved at the targeted location. Drug carrier systems also minimize intoxication by preventing the spread of the drug in the organism,\(^4\) and, because of this, it is inferred in this work that potential therapies with perillyl alcohol against tumors in distinct regions of the organism can be achieved by the use of delivery systems.

A variety of these carriers are investigated, such as organic nanoassemblies,\(^19\) micelles,\(^20\) target receptor nanocarriers,\(^21\) and biopolymer nano/microparticles\(^22\) to\(^24\) and also explored for encapsulation of perillyl alcohol.\(^4\),\(^5\) One particularly interesting class of materials researched for drug delivery is organic supramolecular entities comprising peptide nanostructures.\(^25\) to\(^27\) Diphenylalanine (Phe–Phe) nanostructures are protagonists in this class of self-assembling nanomaterials because of their biocompatibility, their remarkable thermal and chemical stability,\(^28\) to\(^30\) and their easily controllable self-assembly mechanisms, which provide a variety of nanoarchitectures, such as nanotubes, vesicles, and nanowires, by simply varying the concentration or modifying the chemical environment.\(^31\) to\(^37\)

Drug delivery systems can be efficiently combined with photodynamic approaches to result in selective and non-
invasive therapies. Photodynamic therapy (PDT) consists of a local administration of a photosensitizer, activated by electromagnetic irradiation. Excitation of the photosensitizer by light triggers photochemical processes that result in ROS generation, such as superoxide and hydroxide radicals and molecular oxygen in its singlet electronic excited state, $^{1}O_2$, which are responsible for the death of tumor cells only at the vicinity of photosensitizer activation.\(^{38}\) Allied with drug delivery systems, current third generation photosensitizers are able to improve even further the efficacy of the PDT treatments, which also enhance cell assimilation of the short-lived $^{1}O_2$.\(^{39}\)

In a previous work,\(^{40a}\) we have successfully prepared self-assembled Phe–Phe nanotubes containing C6, a fluorescent coumarin derivative, as an optical sensor for dissolved oxygen, which showed improved sensitivity, selectivity, and reproducibility as compared to the response of the dye alone.\(^{40b}\) The enhanced sensitivity toward oxygen verified in our sensor also makes it a potential $^{1}O_2$ generator useful for PDT. Also, this work showed that photophysical properties of coumarin derivatives can be modulated by solvent election and combination with other materials. Because of this property, a variety of formulations containing coumarin molecules for PDT have been proposed, some of which incorporate the benefits of drug delivery systems and anticancer drugs, aiming a combination of PDT and other therapeutic approaches.\(^{21,41–49}\) Additionally, several biological activities of coumarin compounds, including anticancer properties, are also reported.\(^{50–55}\)

In this work, a peptide-based system for perillyl alcohol-controlled release is obtained, presenting unique photophysical properties that enable it to generate singlet oxygen because of the formation of an exciplex. Herein, we prepared fluorescent Phe–Phe vesicles doped with coumarin-6 for perillyl alcohol encapsulation, which also resulted in o, a system with unique photophysical properties that are not achieved by the sole components, as we demonstrated through epifluorescence microscopy combined with steady-state and time-resolved fluorescence spectroscopy. These combined techniques also showed that drug release is accomplished by induced structural interconversion of the vesicles to nanotubes by changing the chemical environment of Phe–Phe assemblies. We also investigated the pro-oxidant properties of the vesicle system in indirect singlet oxygen measurements, seeking both drug delivery and PDT applications.

\section*{RESULTS AND DISCUSSION}

The system in study is a combination of three major components, that is, self-assembled Phe–Phe vesicles, formed in the presence of a fluorophore, coumarin-6 (C6), containing perillyl alcohol encapsulation. Such combination results in unique photophysical characteristics that are the main achievement of this work. In order to understand the properties of this system, the sole components as well as their combination two-by-two were also investigated. Therefore, in this section, Results and Discussion are presented in terms of the experiments as they were executed: (1) system preparation to drug release; (2) the morphological analysis by epifluorescence microscopy and scanning electron microscopy (SEM); (3) steady-state fluorescence spectroscopy and time-resolved fluorescence spectroscopy of the system and sole components, which were analyzed together and in combination with the microscopic study to understand the photophysical properties of such system; and (4) singlet oxygen formation by the system, as an unique property of the system, not characteristic of any of the sole components or combination two-by-two.

\textbf{Drug-Controlled Release Experiments}. The kinetics of the release of perillyl alcohol from the Phe–Phe vesicles were determined by the interpretation of distinct analytical methods combined together, such as epifluorescence microscopy, SEM, steady-state fluorescence spectroscopy, and time-resolved fluorescence spectroscopy. These results are presented separately for comprehension.

\textbf{Epifluorescence Microscopy and SEM}. Perillyl alcohol release from Phe–Phe vesicles was promoted by a chemical environment change, which promotes the conversion of Phe–Phe vesicles into nanotubes, resulting in the release of the encapsulated drug. It is achieved by adding 20 $\mu$L of a mixture of ethanol/water in the ratio 1:1 to a glass substrate where Phe–Phe vesicles were previously deposited. Epifluorescence micrographs were recorded in a time range of 0–105 min, after ethanol/water addition, to investigate the conversion dynamics from vesicles to nanotubes as it occurs with solvent change. Images are shown in Figure 1.

Figure 1A shows the microvesicles obtained in acetone before addition of the 1:1 ethanol/water solvent mixture.
Figure 1B shows smaller vesicles dispersed in the solvent mixture after 10 min of solvent addition. In contrast to what is observed in acetone, vesicles tend to gain directionality, a pattern that is more evident in Figure 1C, recorded after 20 min from solvent addition, in which vesicles are more aligned. After 80 min, new growing patterns resembling tubes can be visualized in Figure 1D and, as shown in Figure 1E, nanotubes are numerous after 105 min. A scanning electron micrograph recorded for Phe−Phe structures grown after addition of ethanol/water mixture, showed in Figure 1F, reveals features characteristic of Phe−Phe microtubes, such as the opened edges of the structure and the hexagonal symmetry P6_3_56.

Because epifluorescence micrographs evidenced the steps of the interconversion occurring after the environment change, it is possible to infer that there is a balance of intermolecular forces between the dipeptide assemblies and the solvent molecules that favors the dipeptide self-assembly to structures distinct from the vesicles which are less stable in this environment than in acetone.

Although microscopy provided the evidence for the interconversion, it is not conclusive to the drug release approach. In order to corroborate these findings and prove the perillyl alcohol release, steady-state and time-resolved fluorescence studies were carried out.

**Steady-State Fluorescence Spectroscopy.** Perillyl alcohol does not show fluorescence and, to perform the steady-state fluorescence spectroscopy, the fluorophore C6 was added to the perillyl alcohol-containing Phe−Phe vesicles. If released, perillyl alcohol would interact with C6, which, in turn, is less polar than the alcohol and interacts with the nanostructure’s external walls and cannot be located at the inner portion of the vesicles, leading to a change in the fluorescence pattern of the fluorophore. The recorded steady-state fluorescence spectra are shown in Figure 2A.

In Figure 2A, the fluorescence spectrum recorded before solvent addition is labeled as time zero (t = 0; black, solid line). The maximum of fluorescence occurring at 407 nm is characteristic of the self-assembled dipeptide emission and the maximum at 494 nm is characteristic of C6. A decrease in the fluorescence intensity is immediately observed (t = 1 min; red, solid line) after addition of the ethanol/water mixture, along with a bathochromic shift of 10 nm in C6 fluorescence. This red-shift indicates a lowering of the electronic excited-state energy, which is caused by a change in the fluorophore microenvironment upon addition of a new solvent. Also, a gradual decrease of the fluorescence intensity of C6 until 36 min is observed, and then, a fast loss of intensity occurs from 38 min and stabilizes after 60 min. A hypsochromic shift of 5 nm (from 506 to 501 nm) is also observed, accompanying the intensity decrease. The fluorescence peak at 407 nm, on the other hand, does not undergo any significant change.

In order to make a clear assessment of the photophysical processes acting on each fluorophore in the system, the intensity ratios of peaks at 505 and 407 nm for each spectrum were calculated, and they were plotted against time (Figure 2B). Quenching of C6 emission is proved by the decrease in the I_505/I_407 ratio, and it stabilizes at approximately 60 min. A hypsochromic shift of 5 nm (from 506 to 501 nm) is also observed, accompanying the intensity decrease. The fluorescence peak at 407 nm, on the other hand, does not undergo any significant change.

In order to make a clear assessment of the photophysical processes acting on each fluorophore in the system, the intensity ratios of peaks at 505 nm and at 407 nm for each spectrum were calculated, and they were plotted against time (Figure 2B). Quenching of C6 emission is proved by the decrease in the I_505/I_407 ratio, and it stabilizes at approximately 60 min, beyond which the relative intensity oscillates, showing that there is no preferable change in any of the fluorophores of the system. C6 fluorescence quenching, along with the observed blue-shift until 60 min, shows a progressive association between C6 in the electronic excited state and another chemical species in the environment, which shall be the perillyl alcohol gradually released. However, further time-
resolved fluorescence spectroscopy (time-correlated single photon counting—TCSPC) studies are needed to characterize the formation of an excited-state complex, and they are presented later on. Also, some other features of this system may influence C6 photophysical response and deviate it from the expected, such as fluorophore aggregation or changes due to Phe–Phe nanostructures interconversion. To eliminate such influence exerted by aggregation of C6 or Phe–Phe interconversion, the TCSPC experiment was carried out on a control sample comprising Phe–Phe vesicles and C6 in the absence of perillyl alcohol. The recorded fluorescence spectra are presented in Figure 2C,D, which shows a small decrease of intensity of the fluorescence peak at 407 and at 505 nm and an insignificant change in the $I_{405}/I_{505}$ ratio (Figure 2D), showing that both fluorophores exhibit the same behavior. Therefore, there is no suppression of fluorescence occurring in this control sample that would arise from the energy transfer process in the electronic excited state, in contrast to that observed for the system containing the drug. These results show an exciplex formation with participation of the nonfluorescent perillyl alcohol, giving rise to distinct exited-state photophysical processes in the system. Nevertheless, the exciplex needs to be characterized by time-resolved fluorescence spectroscopy, as we show in further sections.

**Epifluorescence Microscopy and Steady-State Fluorescence Spectra.** Epifluorescence micrographs and steady-state fluorescence spectra were recorded to enable the drug release dynamics characterization. For that, the detector of the spectrophotometer was connected to the output of the microscope using an optical fiber, and fluorescence micrographs and steady-state fluorescence spectra were obtained from the same region of the sample.

Fluorescence micrographs from different regions of the sample, obtained in increasing intervals of time after addition of water/ethanol, are shown in Figure 3. They were recorded using a 420–490 nm excitation filter, which results in the strong greenish fluorescence of C6. In Figure 3C–F, patterns resembling microtubes were observed. Although the change to these patterns are present in images obtained starting from 20 min after induction of the interconversion (Figure 3C), an overall change occurred after 30 min. It is noteworthy that at 3 min after the addition of the solvent mixture, vesicles seem to align in order to enable the further structure changes that are enforced by the changes in environmental polarizability. Contours are observed in the vesicles, which are not observed in the first 15 min. These contours are related to the rupture of the larger vesicles and the coalescence of smaller ones, which might be accompanied by the release of the perillyl alcohol. In fact, the process start to occur at the first instant of the solvent addition to the vesicles. Vesicles immediately align, and from this point, the conversion to nanotubes takes place.

Steady-state fluorescence spectra obtained simultaneously to the epifluorescence micrographs were recorded at the occurrence of the interconversion and are presented in Figure 4A. Excitation was performed with the UV excitation filter of the microscope (340–380 nm). Also intensity ratios of peaks at 525 and 407 nm were plotted against time and are presented in Figure 4B. These experiments were performed simultaneously, also with time-resolved fluorescence spectroscopy, which will be discussed later on.

An intense fluorescence peak at 450 nm and another of a lower intensity at 525 nm are observed. In order to study the interactions of the chromophores of the system with the nonfluorescent perillyl alcohol, the intensity ratios of these peaks were obtained. Intensity ratios, shown in Figure 4B, tend to decrease with time, revealing a progressive quenching of the band at 525 nm, characteristic of C6; after 30 min and at 90 min, the intensity of the peak at 450 nm is the highest, which is due to the patterns resembling grown tubes in Figure 3D. These findings corroborate the decrease in the intensity ratio observed from the spectroscopy measurements alone, discussed in section Steady-State Fluorescence Spectroscopy, in which a significant quenching starts at approximately 30 min (Figure 2D). Thus, drug release is proven by the quenching effect of the fluorescence at 525 nm, due to an increase in the released perillyl alcohol concentration with time. Images in Figure 3 showed the interconversion of Phe–Phe vesicles into nano/microtubes, occurring in the same time range in which these changes in fluorescence spectra were detected. Upon vesicle elimination, the released perillyl alcohol quenches fluorescence of the Phe–Phe/C6 system at 525 nm, which results in the dark contour feature, better observed in Figure 3C. In addition, in Martins et al., it was shown by fluorescence spectroscopy and cyclic voltammetry that perillyl alcohol is efficiently encapsulated by Phe–Phe vesicles.

**Time-Correlated Single Photon Counting.** Time-resolved emission spectra (TRES) experiments were carried out to prove the role of the perillyl alcohol in the electronic excited-state processes of the system as well as to identify the groups of fluorophores active in the system. In this experiments, decay curves are successively recorded in a range of emission wavelengths, upon the selected excitation wavelength. The fluorescence decay curves obtained for distinct systems are shown in Figure 5, and lifetimes are in Tables 1–3.

From fluorescence lifetimes (Tables 1–3), fluorescence decays are biexponential from 395 to 415 nm, the characteristic region of emission of Phe–Phe structures. As longer emission wavelengths are monitored, a multieponential behavior is adopted by the system, from 435 to 475 nm, which is the evidence for the presence of more than one fluorescent moiety contributing to the total decay. At this emission region, there is no efficient emission of either one of
the Phe–Phe/C6 system-isolated participants. Emission, thus, must be from an exciplex formed between the Phe−Phe structure and C6, resulting in the multiexponential decay recorded at this region.

Figure 4. (A) Fluorescence spectra ($\lambda_{exc} = 340–380$ nm) of Phe–Phe vesicles containing perillyl alcohol and submitted to the drug release regime (i.e., addition of ethanol/water mixture) and (B) $I_{525}/I_{450}$ intensity ratios, with relative standard deviations of 0.001–0.01%.

Figure 5. Fluorescence decay curves recorded during TRES measurements, obtained for (A) sample 1; (B) sample 2; (C) sample 3; (D) sample 4; and (E) sample 5, as they were described in Table 6.

Table 1. Fluorescence Lifetimes, Contributions, and $\chi^2$ from TRES Measurements of Sample 2

| $\lambda$ (nm) | $\tau_1$ (ns) | $\tau_2$ (ns) | $\tau_3$ (ns) | $\chi^2$ |
|---------------|---------------|---------------|---------------|---------|
| 395           | 6.78 (16%)    | 1.06 (84%)    |               | 1.34    |
| 415           | 4.94 (20%)    | 0.74 (80%)    |               | 1.06    |
| 435           | 9.06 (13%)    | 2.19 (33%)    | 0.46 (54%)    | 1.19    |
| 455           | 9.01 (5%)     | 2.07 (17%)    | 0.36 (77%)    | 1.18    |
| 475           | 10.15 (3%)    | 2.26 (28%)    | 0.32 (68%)    | 1.22    |
| 495           | 2.38 (69%)    | 0.29 (31%)    |               | 1.19    |
| 515           | 2.35 (57%)    | 0.34 (43%)    |               | 1.29    |
| 535           | 2.35 (57%)    | 0.34 (43%)    |               | 1.52    |

Table 2. Fluorescence Lifetimes, Contributions, and $\chi^2$ from TRES Measurements of Sample 3

| $\lambda$ (nm) | $\tau_1$ (ns) | $\tau_2$ (ns) | $\tau_3$ (ns) | $\chi^2$ |
|---------------|---------------|---------------|---------------|---------|
| 395           | 3.43 (14%)    | 0.76 (86%)    |               | 1.28    |
| 415           | 3.30 (21%)    | 0.74 (79%)    |               | 1.12    |
| 435           | 6.18 (4%)     | 1.75 (24%)    | 0.50 (71%)    | 1.05    |
| 455           | 6.21 (3%)     | 1.61 (17%)    | 0.38 (79%)    | 1.05    |
| 475           | 6.84 (6%)     | 2.14 (35%)    | 0.44 (58%)    | 1.08    |
| 495           | 2.63 (64%)    | 0.48 (36%)    |               | 1.38    |
| 515           | 2.66 (70%)    | 0.60 (30%)    |               | 1.20    |
| 535           | 2.71 (68%)    | 0.61 (32%)    |               | 1.16    |
Table 3. Fluorescence Lifetimes, Contributions, and $\chi^2$ from TRES Measurements of Sample 1

| $\lambda$ (nm) | $\tau_1$ (ns) | $\tau_2$ (ns) | $\tau_3$ (ns) | $\chi^2$ |
|---------------|---------------|---------------|---------------|--------|
| 395           | 5.61 (27%)    | 1.24 (73%)    |               | 1.08   |
| 415           | 6.23 (22%)    | 1.39 (78%)    |               | 1.14   |
| 435           | 7.26 (7%)     | 1.86 (22%)    | 0.34 (71%)    | 0.98   |
| 455           | 7.26 (7%)     | 1.86 (22%)    | 0.34 (71%)    | 1.16   |
| 475           | 2.54 (59%)    | 0.31 (41%)    |               | 1.24   |
| 495           | 2.44          |               |               | 1.54   |
| 515           | 2.52          |               |               | 1.20   |
| 535           | 2.50          |               |               | 1.12   |

It is known that the dispersion forces responsible for the dipeptide conjugation and stability of the Phe−Phe assemblies give rise to unique photophysical properties. It is expected that the structural transition caused by the self-assembly process is thermodynamically driven by a delicate balance of noncovalent interactions between the solvent molecules and the peptide moieties, resulting in a configuration of minimum free energy. Comparing lifetimes obtained for partial systems (Table 1), it is possible to relate them to the lifetimes obtained for the whole system and characterize an exciplex, if formed, between all components of the system (Table 3). At all samples, at the range from 395 to 415 nm, which corresponds to the emission range of Phe−Phe structures and C6 and PA, in the presence of the ethanol/water mixture, as shown previously by the epifluorescence micrographs (Figure 3) because the self-assembly process is thermodynamically driven by a delicate balance of noncovalent interactions between the solvent molecules and the peptide moieties, resulting in a configuration of minimum free energy. Comparing lifetimes obtained for partial systems (Table 1), it is possible to relate them to the lifetimes obtained for the whole system and characterize an exciplex, if formed, between all components of the system (Table 3). At all samples, at the range from 395 to 415 nm, which corresponds to the emission range of Phe−Phe structures, the longer lifetime contributes with 20% in average to the total decay curve, whereas the shorter lifetime corresponds to 80%. Longer lifetimes are of 3.5 ns for the nanotube sample (Table 2) and are of 5–6 ns for vesicle samples (Tables 1 and 3). Nevertheless, shorter lifetimes are very similar in sample 2, which is Phe−Phe vesicles containing C6 and PA, in the presence of the ethanol/water mixture, as shown in Table 1, and sample 3 (Phe−Phe nanotubes, containing C6, after PA release), being 0.74±1 ns (Tables 1 and 2), whereas they are around 1.2–1.4 ns in the sample of Phe−Phe vesicles loaded with perillyl alcohol (Table 3). As longer wavelengths are monitored, decay curves became multieponential for all systems and became biexponential again from 495 to 515 nm, which corresponds to a region at only C6 is emitting. Therefore, the region from 435 to 475 nm is related to the exciplex emission region. In this region, the longer lifetimes become even longer at all samples, being 6–7 ns in sample 1 (Table 3), which consists of the complete system, kept in acetone and in sample 3 (Table 2), which has no PA, and is in the presence of the ethanol/water mixture, but it is 9–10 ns in the complete system, prepared in acetone, which had received the ethanol/water mixture to convert into nanotubes. Nevertheless, these longer lifetimes contribute with less than 10% to the total decay curve. Major components are the shorter wavelengths that arise at this emission region of 0.4–0.5 ns and contributing with around 70% to the total decay curve and an intermediary lifetime of around 2 ns and presenting 20–30% of contribution to the total decay curve is observed at all samples. From region from 495–515 nm, which corresponds to the emission region of the fluorophore and no emission from Phe−Phe structures is expected, a biexponential behavior is again observed. Lifetimes recorded are again around 2 ns with 30% of the contribution and 0.3–0.7 ns, contributing with around 70% to the total decay curve. Interestingly, curves obtained for the vesicles loaded with perillyl alcohol (prior to drug release) are monoeXponential at this emission region (Table 3), with lifetimes of 2 ns as well. Despite perillyl alcohol does not present fluorescence, it is involved in the exciplex formation; these findings reveal that perillyl alcohol plays an important role in the photophysical properties of the self-assembled dipeptide, which results in stabilization of electronic excited states of the system.

Table 4. Fluorescence Lifetimes, Contributions, and $\chi^2$ from TRES Measurements of Samples 4 and 5

| $\lambda$ (nm) | $\tau_1$ (ns) | $\tau_2$ (ns) | $\tau_3$ (ns) | $\chi^2$ |
|---------------|---------------|---------------|---------------|--------|
| 435           | 2.21 (23%)    | 0.78 (77%)    |               | 1.03   |
| 455           | 2.62 (37%)    | 0.94 (63%)    |               | 1.06   |
| 475           | 2.52          |               |               | 1.18   |
| 495           | 2.53          |               |               | 0.91   |
| 515           | 2.53          |               |               | 1.02   |
| 535           | 2.53          |               |               | 1.12   |

| $\lambda$ (nm) | $\tau_1$ (ns) | $\tau_2$ (ns) | $\tau_3$ (ns) | $\chi^2$ |
|---------------|---------------|---------------|---------------|--------|
| 435           | 2.52 (22%)    | 0.63 (78%)    |               | 1.48   |
| 455           | 8.17 (4%)     | 1.88 (24%)    | 0.49 (71%)    | 1.18   |
| 475           | 5.50 (6%)     | 1.82 (40%)    | 0.21 (53%)    | 1.18   |
| 495           | 2.30 (79%)    | 0.36 (21%)    |               | 1.13   |
| 515           | 2.28 (83%)    | 0.47 (17%)    |               | 1.05   |
| 535           | 2.34 (69%)    | 0.46 (31%)    |               |        |

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alcohol (Table 2), prepared in the same chemical environment of the drug release (Table 1), lifetimes recorded at the spectral range of C6 (435–535 nm) are higher in the control sample (Table 2), which indicates the occurrence of a nonradiative electronic energy transfer between the released perillyl alcohol and C6. Interestingly, the characteristic fluorescence lifetime of C6 in acetone, of around 2.5 ns (Table 4), is similar to those of the complete vesicle system, in acetone, prior to drug release (Table 3), recorded in the same spectral range, which further supports a stronger influence of the perillyl alcohol only after the release event.

**Generation of Singlet Oxygen.** In a previous photophysical study of the Phe−Phe/C6 system,40 it was inferred that this system could generate singlet oxygen, 1O2, upon irradiation with light of a proper wavelength, aiming new applications of this drug delivery system, such as PDT.

To determine its ability to generate 1O2, the indirect method for determination of 1O2 quantum yield, employing uric acid (UA) as the 1O2 sensor is used, which has proven to be a reliable, accurate, and low-cost method.59−61,65,66 By this method, the solution containing the vesicle system and UA was irradiated at 450 nm, which is the maximum absorption wavelength of the system, and electronic absorption spectra were recorded in intervals from 0 to 1320 s (Figure 6A). As control measurements, spectra were also recorded in similar experiments containing the Phe−Phe structure (Figure 6B); C6 (Figure 6C); perillyl alcohol (Figure 6D); a combination of C6 and perillyl alcohol (Figure 6E); and a combination of C6 and Phe−Phe structure (Figure 6F). The determination of 1O2 quantum yield is carried out by evaluating the intensity decrease of the UA absorption peak at 287 nm.

In Figure 6A, the absorption spectra of UA in the presence of the 1O2 standard generator, MB, are shown. A fast decrease in the absorption of UA at 280 nm occurs within 100 s of irradiation, a result of high concentrations of 1O2 produced by the MB standard. In Figure 6D–H, in which the absorption of UA in the presence of the vesicle system and its isolated components are shown, photodegradation of UA also occurs in the presence of Phe−Phe vesicles containing C6 and perillyl alcohol (sample 7), the Phe−Phe/C6 system (sample 8) and fluorophore alone (sample 9), however, with distinct rates.

For the control samples shown in Figure 6J–M, in which the effect of the isolated compounds over the absorption of the sensor UA is evaluated, no significant changes were observed at the absorption wavelength related to UA (280 nm), which was expected because it is known that both Phe−Phe and the perillyl alcohol exhibit antioxidant properties.13,67 Figure 6M also shows that the combination of C6 and perillyl alcohol presents no effect in the absorption of UA as well, which demonstrates that the oxidant effect as shown in Figure 6D can be only achieved by the system composed of Phe−Phe, C6 and perillyl alcohol, even though the isolated components are not able to promote UA degradation. In order to prove this synergistic effect and to relate it to the formation of an electronic excited-state complex that culminates with the increase of 1O2 generation, 1O2 formation quantum yields, ΦΔ, were calculated by an indirect method using MB as standard. According to Rabello et al.,60 UA undergoes a biexponential, two-step first-order degradation kinetics, in which the first step is characterized by the reaction of UA with 1O2 and the second step related to an intermediate product, which can be identified by its increasing absorption at 390 nm. In this mechanism, however, only the first-step kinetic constant
is suitable for $\Phi_\Delta$ calculation. The rate constant of UA photodegradation is extracted from the linear relationship between the natural logarithm of UA absorption at 290 nm and irradiation time.

For the sample of UA with the MB standard, which is the appropriate model for $\Phi_\Delta$ determination, prepared in the ethanol/water solvent system, the plot of absorption of UA versus irradiation time is exponential, as expected (Figure 6B). However, a linear fitting of the natural logarithm of absorption was unsuccessful (Figure 6C), which is due to solvation effects and the rate of diffusion of $1O_2$ factors that are affected by the chemical environment, ultimately reflecting in the degradation kinetics. For the systems of interest of this work, the plots of the absorption resulted in a linear relationship (Figure 6D through 6I) and were used for $\Phi_\Delta$ determination.

The respective UA photodegradation kinetic constants used in $\Phi_\Delta$ calculations and $\Phi_\Delta$ values are shown in Table 5.

Table 5. Photodegradation Kinetic Constants of UA, Autocorrelation Parameters ($R^2$), and $\Phi_\Delta$ Values Obtained for the Samples Combined to UA. MB is the Control

| sample | kinetic constant $k$ ($s^{-1}$) | $R^2$ | equation | $\Phi_\Delta$ (%) |
|--------|-------------------------------|------|----------|---------------|
| 6      | $5.85 \times 10^{-3}$         | 0.992| $[\text{Abs.}]_t = [\text{Abs.}]_0 + [\text{Abs.}]_0 e^{-kt}$ | 52 (in ethanol) |
| 7      | $3.24 \times 10^{-4}$         | 0.992| $[\text{Abs.}]_t = [\text{Abs.}]_0 e^{-kt}$ | 3.07 |
| 8      | $1.35 \times 10^{-4}$         | 0.990| $[\text{Abs.}]_t = [\text{Abs.}]_0 e^{-kt}$ | 0.82 |
| 9      | $1.30 \times 10^{-4}$         | 0.997| $[\text{Abs.}]_t = [\text{Abs.}]_0 e^{-kt}$ | 0.71 |

As showed in Table 5, $\Phi_\Delta$ of the Phe−Phe/C6 system is slightly higher than that of C6. However, the value of $\Phi_\Delta$ obtained from the Phe−Phe/C6/PA vesicle system is significantly higher than that of the C6 control sample. These results demonstrate that the association of self-assembled Phe−Phe with C-6 and perillyl alcohol yields a product of distinct electronic excited-state dynamics, characterized by the formation of the exciplex mentioned earlier, which improves the pro-oxidant activity inherent of the fluorophore.

Based on the steady-state and time-resolved fluorescence spectroscopy data, presented in the previous sections, it is implied that the mechanism of action of such exciplex involves its nonradiative deactivation. It concerns the intersystem crossing to populate the triplet electronic excited state of the complex, a process otherwise negligible in the isolated compounds, which is a requirement for conversion of oxygen to its reactive singlet electronic excited state, responsible for the PDT activity. The electronic energies involved in the proposed mechanism is shown in Scheme 1.

These results show that the vesicle system formed by the C6 fluorophore, perillyl alcohol, and Phe−Phe structure presents unique photophysical properties that enables it to be used as a drug delivery system with potential to generate $1O_2$ and, therefore, with potential for PDT applications. This opens great perspectives to the uses of perillyl alcohol to fight cancer because it is well-known that this compound is a potent drug that exerts cancer preventive and therapeutic activity in a variety of tumors, such gliomas, lung, and upper respiratory tumor, but the actual difficulties are faced to administer this drug prevent it from being widely used. In fact, until now, only administration by inhalation has proved satisfactory. With the perspective of encapsulation and its use in PDT, proved in this work, new possibilities of treatments based on perillyl alcohol use, can be considered.
CONCLUSIONS

The drug delivery system for perillyl alcohol based on self-assembled peptides containing C6 as a fluorophore described herein presented a prolonged and sustained release of perillyl alcohol because it was extended to over an hour. It is achieved via an induced disruption of the vesicles by simple modifications of environmental properties. The fluorescent additive, C6, was successfully used as the fluorescent probe to study the dynamics of drug release, but it also takes part, along with perillyl alcohol, in interesting photophysical processes favored by the exciplex formation involving the components of the system, as proved by TCSPC. Such photophysical processes are responsible for the enhanced pro-oxidant activity presented by this system, which is important for PDT applications.

In addition, the drug delivery system can extend the anticancer activity of perillyl alcohol to more than a single therapeutic approach, that is, a system that can be the combination of the antitumor properties of the encapsulated perillyl alcohol, and when activated by light, it can act as a photosensitizer in PDT or even be useful in combined therapy of both approaches.

MATERIALS AND METHODOLOGY

1,1,3,3,3,3-Hexafluoro-2-isopropanol (HFIP), CAS no. 920-66-1; L-diphenylalanine (Phe–Phe), CAS no. 2577-40-4; 3-(2-benzothiazolyl)-7-(diethylamino)coumarin (coumarin-6) CAS no. 38215-36-0; and perillyl alcohol, CAS no. 18457-55-1, purchased from Sigma-Aldrich, were used as received. Figure S1 in the Supporting Information presents their structures.

Preparation of Phe–Phe Vesicles and Drug Encapsulation. Phe–Phe (Sigma-Aldrich, M = 241.11 g mol⁻¹) vesicles containing perillyl alcohol (Sigma-Aldrich, d = 0.96 g cm⁻³; M = 152.23 g mol⁻¹) were prepared by dissolving 10 mg of the lyophilized dipeptide (4.0 × 10⁻² mmol) and 6 mg of perillyl alcohol (4.0 × 10⁻² mmol) in 50 μL of HFIP (Sigma-Aldrich, 99.8% pure, M = 168.05 g mol⁻¹). This fresh solution was diluted in a 10⁻⁶ mol L⁻¹ solution of C6 (Sigma-Aldrich) in acetone, resulting in a solution of final peptide concentration within the range of 1–2 g L⁻¹ and a proportion of 10⁻⁵ mmol of C6 to 4 × 10⁻² mmol of Phe–Phe. In acetone, the dipeptide spontaneously assembles in nano/microvesicles, encapsulating the perillyl alcohol. These concentrations were used in accordance to previous works.²⁹–⁵²,⁴⁰,⁶⁹,⁷⁰

Drug Release Experiments. Perillyl alcohol-controlled release from the peptide vesicles was evaluated by epifluorescence microscopy combined with steady-state and time-resolved fluorescence spectroscopy, as described below.

Epifluorescence Microscopy. Epifluorescence micrographs were recorded in a Leica DMIRBE inverted microscope, using 200× magnification objective lenses and 340–390 nm and 420–490 nm excitation filters. Micrographs were recorded from liquid samples to follow the Phe–Phe self-assembled interconversion at solvent polarizability changes.

Micrographs were recorded from the initial solution deposited on a glass substrate, at a time marked as zero for drug release, followed by the addition of 20 μL of a mixture of 1:1 of ethanol and water. At the same time, micrographs were recorded from several regions of the sample with time control of each shot, to monitor the interconversion kinetics. Based on our previous studies,⁴⁰ water alone is sufficient to promote interconversion; however, a mixture of ethanol and water at neutral pH was added to the dried sample, to promote C6 and perillyl alcohol dissolution. At each time interval, an aliquot was taken and deposited in a glass substrate to perform electron scanning microscopy (SEM) studies.

Electron Scanning Microscopy. SEM images were obtained in a JEOL scanning electron microscope, model JSM 6610, equipped with a Thermo Scientific NSS Spectral Imaging. Images were acquired at a spot size set at 40 mm, working distance at 14 mm, and with an accelerated voltage of 4 keV. A volume of 20 μL of the freshly prepared vesicles dispersed in acetone was deposited onto a glass substrate, and the solvent was evaporated in an oven at 40 °C for 48 h. Samples were placed in a copper sample holder, and metallization was carried out in a Bal-Tec MED 020 sputtering chamber.

Steady-State Fluorescence Spectroscopy Measurements. Steady-state spectroscopy was performed in a Horiba Fluoromax 4 spectrofluorimeter, equipped with a xenon lamp. A sample holder for cuvettes adapted for solid samples was used to place liquid samples in position for fluorescence detection at 90° and in solid samples, to place them in a 45° right-angle with respect to the incident radiation. Fluorescence was detected at the range of 250–850 nm. Narrow slits were employed to ensure resolution of 0.50 nm in measurements. A volume of 2 mL of the self-assembled system dispersed in acetone was placed in a quartz cuvette and had its fluorescence spectrum recorded, at distinct periods of time. This recording marked the time as zero. Then, the same volume of an ethanol/water mixture of 1:1 was added, and the fluorescence intensity of the band at 500 nm was monitored by recording successive fluorescence spectra in increasing intervals.

Time-Resolved Fluorescence Spectroscopy Measurements. Time-resolved spectroscopy was performed in an Edinburgh Instruments F900 TCSPC Analytical spectrophotometer, equipped with an Edinburgh Hamamatsu R3809U-50 TCSPC detector and a 351 nm pulsed LED of 77 ps of pulse width. An excitation and emission slit aperture of 1 mm was used. Samples were sealed in quartz cuvettes and placed in the sample holder to register the fluorescence lifetimes. Data of the sample signal over the instrumental response were deconvoluted and analyzed by the exponential series method.⁷⁷ Ludox (Sigma-Aldrich) was used as the scatterer. Experimental data correspond to expected theoretical values when χ² is close to 1.

TRES experiments were performed for the samples described in Table 6. Fluorescence decay curves were recorded in the spectral range from 370 to 520 nm, with a 20 nm step. The decay data were analyzed in Fluortools Decayfit free software, v. 1.4.⁵⁸

Samples compositions are presented in Table 6.

| Table 6. Description of the Samples Studied by Time-Resolved Fluorescence Spectroscopy²⁶ |
| sample | description |
| 1 | Phe–Phe vesicles + perillyl alcohol (PA) + coumarin 6 (C6) in acetone |
| 2 | Phe–Phe vesicles + PA + C6 in acetone, after addition of ethanol/water |
| 3 | Phe–Phe nanotube (PNT) + C6 without perillyl alcohol in ethanol/water |
| 4 | C6 in acetone |
| 5 | C6 in acetone after addition of ethanol/water |

²⁶Determination of singlet oxygen O₂."
In the pro-oxidant activity assessment, UA was used as the O_{2}^{1} sensor.\textsuperscript{59,60} UA was dissolved in 2 mL of 1:1 of ethanol and water mixture to result in an absorbance between 1.0 and 1.8. Phe–Phe vesicles were dispersed in 2 mL of UA solution of UA and in a quartz cuvette. The sample was irradiated with the appropriate excitation source at increasing intervals of time. After each irradiation, absorption spectra were recorded in a Hitachi U2900 spectrophotometer. The absorption intensity decrease of UA was monitored at 290 nm. This experimental protocol was repeated for samples listed in Table 7, with excitation at 650 nm (100 mW Laserline Izi laser source) for sample 1, and for samples 2–6, excitation was carried out at 450 nm using a portable array of 20 mW LEDs assembled at the laboratory. Samples compositions, prepared for these experiments, are presented in Table 7.

Oxygen singlet (\textsuperscript{1}O_{2}) quantum yield (\Phi_{\text{O}_{2}}(fs)) was indirectly determined using MB as standard.\textsuperscript{59,60} Quantum yield was calculated by eq 1:

$$\Phi_{\text{O}_{2}}(fs) = \Phi_{\text{O}_{2}}(0) \frac{k(fs) \int_{\lambda_{1}}^{\lambda_{2}} Abs(0)E(\lambda)d\lambda}{k(0) \int_{\lambda_{1}}^{\lambda_{2}} Abs(fs)E(\lambda)d\lambda}$$

In which, \Phi_{\text{O}_{2}}(fs) and \Phi_{\text{O}_{2}}(0) are \textsuperscript{1}O_{2} quantum yield of the system under study and of the MB standard, respectively; k(fs) and k(0) are the photodegradation rates of UA in the presence of the system and MB, respectively; E(\lambda) is the emission spectra recorded upon excitation at wavelengths \lambda_{1} e \lambda_{2}; Abs(fs) and Abs(0) are the absorptions of the system and MB, respectively. The rates of UA photodegradation by \textsuperscript{1}O_{2} were obtained from the slope of the plot of UA absorption intensity at 287 nm versus irradiation time.

### Table 7. Description of Samples Produced to Investigate Pro-oxidant Activity

| Sample | Description |
|--------|-------------|
| 6      | Methylene blue (MB) + UA |
| 7      | Phe–Phe vesicles + perillyl alcohol (PA) + coumarin 6 (C6) + UA |
| 8      | Phe–Phe + C6 + UA |
| 9      | C6 + UA |
| 10     | Phe–Phe + UA |
| 11     | PA + UA |
| 12     | C6 + PA + UA |

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**Notes**

The authors declare no competing financial interest.

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