



## Pyrrolidine-fused chlorin photosensitizer immobilized on solid supports for the photoinactivation of Gram negative bacteria



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### ABSTRACT

The emergence of resistant microorganism to conventional therapeutics prompted us to search new and better antimicrobial treatment modalities. In this work was envisaged an economically viable and environmental friendly approach to allow successive recovery and removal of the photosensitizer agent after photodynamic treatment. The photodynamic inactivation of bioluminescent *Escherichia coli* in the presence of an immobilized cationic chlorin photosensitizer and its reusability are described. The chlorin photosensitizer (PS) was immobilized on two commercial materials: a 3-bromopropyl-functionalized silica and a Merrifield resin. The new photosensitising materials were characterized by UV–vis spectroscopy, SEM and EDX. Their singlet oxygen generation capacities were also assessed. Previous studies showed that there is a direct relationship between the photoinactivation efficiency and the number of positive charges on such PS molecules. Therefore the number of positive charges on the new prepared materials was further increased by treatment of the PS-immobilized materials with 1-methylimidazole and pyridine. The efficiency of photoinactivation against bioluminescent *E. coli* was evaluated in the presence of the non-immobilized chlorin (20 μM) as well as in the presence of the new prepared materials positively charged or non-charged on the surface (20–200 μM) with white light at an irradiance of 4.0 mW cm<sup>-2</sup>. Overall, this study shows that the materials resulting from the immobilization of the chlorin on the silica surface and on the Merrifield resin, followed by further treatment with pyridine, have high potential as PS for the inactivation of Gram negative bacteria (3.0 log reductions) and are able to maintain its antimicrobial efficiency after at least 3 repeated cycles of photoinactivation. Their reusability, without loss of effectiveness, confirms a potential inexpensive and friendly application in clinic and environmental areas.

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### 1. Introduction

Natural heterocycles of the porphyrin-type are well known for their special role in certain vital functions, like respiration, photosynthesis and drug detoxification. The unique physico-chemical properties of these tetrapyrrolic pigments and of synthetic analogues are also responsible for their promising applications in different fields such as electronics, artificial photosynthesis, catalysis, sensors, and in the development of new drugs [1]. In medicine,

these compounds are receiving special attention due to their efficiency to treat oncological, cardiovascular, dermatological and ophthalmic diseases by a technique known as photodynamic therapy (PDT). More recently, some studies demonstrated that PDT can be effective in the selective photoinactivation of microorganisms and can become a potential alternative for the treatment and eradication of microbial infections [2,3].

The photodynamic inactivation of microorganisms (PDI) is based on irradiation with visible light in the presence of oxygen and of a photosensitizer (PS), with preferential affinity to target cells. It is generally accepted that cytotoxic agents such as singlet oxygen and reactive oxygen radicals generated by the PS action are the species causing cell disruption [2–5]. This approach is a potential alternative to conventional antibiotics and is effective *in vitro*

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against bacteria, viruses, fungi and protozoa [2,6–8]. The possibility of using this methodology, not only for applications in the clinical field, but also in environmental applications, more specifically for the inactivation of pathogenic microorganisms in water and wastewater was also investigated [9–13]. The main advantage of this method is that it is unlikely that mechanisms of resistance can be developed due to multi-target character of the photoinactivation process [14–17]. It is well known that cationic PS, namely porphyrins and analogues, cause effective photoinactivation (PI) against Gram-positive and Gram-negative bacteria [2,9,11,18].

The extension of the photodynamic principle to a new application, such as disinfection of blood, water and surfaces, can only become economically viable and environmentally friendly if the PS is covalently immobilized on solid supports to allow its successive recovery and removal after the photodynamic treatment [19–21]. Only a few studies have addressed this approach to photoinactivate microorganisms [20–28].

The selection of the solid supports must be based on: i) its compatibility with the PS; ii) easy and reproducible immobilization procedures; iii) mechanical strength and stability towards light; iv) good oxygen permeability for efficient  $^1\text{O}_2$  production with minimum quenching; v) high biocompatibility to maximize the interaction between the support and the microorganisms; vi) commercial availability and low cost [22]. In particular, the immobilization of a PS on a silicate matrix has some advantages when compared with organic matrices since it is insoluble in water, resistant towards microorganisms, easy to fabricate and might be successfully developed for the photo-disinfection of water or physiological fluids [2,20,29]. For instance, silicate sol–gel matrices immobilized with Photolon (a chlorin  $e_6$  derivative) have been used for antimicrobial PDT, sensing and medical therapy applications [19,24].

In a recent work we have shown that neutral and charged reduced porphyrin derivatives such as chlorins **2** and **3** (Fig. 1), and the corresponding isobacteriochlorins have better light absorbing features and singlet oxygen generating capacities than porphyrins. In addition, it was observed that their efficiency in the photoinactivation of Gram negative bacteria was influenced by the number of charges [30]. Therefore, in the present study, chlorin **2** (Fig. 1) was immobilized on a modified silica support and on a Merrifield resin and in order to increase the positive charges on the material surface, further treatments with 1-methylimidazole or pyridine were performed. The PS-materials were characterized by UV–vis spectroscopy, SEM and EDX and the antimicrobial activities of the new materials were assessed against bioluminescent *Escherichia coli*.

## 2. Experimental

### 2.1. Chemicals and instrumentation

All reagents were purchased from Sigma–Aldrich and were used as received without further purification. 4-Bromopropyl-

functionalized silica gel (200–400 mesh; extent of labelling: 1.5 mmol/g loading) and Merrifield's resin (100–200 mesh, extent of labelling: 3.5–4.5 mmol/g  $\text{Cl}^-$  loading, 1% cross-linked) were also purchased from Sigma–Aldrich.  $^1\text{H}$ ,  $^{19}\text{F}$  and  $^{13}\text{C}$  NMR spectra were recorded using either a Bruker Avance 300 spectrometer at 300.13, 282.38 and 75.47, respectively, or a Bruker Avance 500 at 125.77 MHz for  $^{13}\text{C}$ , using tetramethylsilane (TMS) as internal reference. The chemical shifts are expressed in  $\delta$  (ppm) and the coupling constants ( $J$ ) in Hz. MALDI mass spectra were recorded on a 4800 Maldi-TOF/TOF analyser and HRMS-ESI spectra on an APEXQe FT-ICR spectrometer (Bruker Daltonics, Billerica, MA). The UV–vis spectra were recorded on a Shimadzu UV-2501PC. Flash chromatography was carried out on silica gel (Merck, 35–70 mesh). Analytical TLC was carried out on silica gel precoated plastic sheets (0.2 mm thick, Merck).

### 2.2. Synthesis of porphyrin 1 and chlorin 2

The starting porphyrin derivatives **1** and **2** (Fig. 1) were prepared according to the literature [30–32]. After purification by chromatography (silica gel) the products were analysed by UV–vis,  $^1\text{H}$  NMR and MS. The spectra were consistent with literature data [30,32].

### 2.3. Synthesis of chlorin 3

A large excess of iodomethane (2 mL) was added to a dry toluene (5 mL) suspension of chlorin **2** (20 mg, 0.02 mmol). The reaction mixture was maintained under stirring for 24 h at 40 °C, in a closed vial. After this period, the mixture was cooled and the product was precipitated with diethyl ether. The precipitate was filtered and washed with diethyl ether. The solid was then dissolved in dichloromethane and re-precipitated from diethyl ether/dichloromethane. The methylated chlorin **3** was filtered, washed with diethyl ether and dried under vacuum to yield a green powder (16 mg, 77% yield); the spectroscopic data is in agreement with the literature [30,33].  $^1\text{H}$  NMR ( $\text{CDCl}_3 + \text{CD}_3\text{OD}$ ):  $\delta$  –1.98 (s, 2H, NH), 3.00 (s, 3H,  $\text{CH}_3$ ), 3.56–3.61 (m, 2H, pyrrolidine-H), 3.66 (s, 3H,  $\text{CH}_3$ ), 4.74–4.80 (m, 2H, pyrrolidine-H), 5.93–5.96 (m, 2H, H-2, 3), 8.41 (d, 2H,  $J = 4.9$  Hz,  $\beta$ -H), 8.54 (s, 2H, H-12, 13), 8.79 (d, 2H,  $J = 4.9$  Hz,  $\beta$ -H). UV–vis (DMF)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 402 (5.23), 500 (4.19), 525 (3.64), 594 (3.69), 647 (4.64) nm; HRMS (ESI):  $m/z$  calcd for  $\text{C}_{48}\text{H}_{20}\text{F}_{20}\text{N}_5\text{I}$   $[\text{M}-\text{I}]^+$ : 1046.1394; found: 1046.1388.

### 2.4. Immobilization of chlorin 2 on the silica support A and Merrifield resin B

Immobilization on the silica support (A) was adapted from literature [34]: a solution of chlorin **2** (10 mg) in *o*-dichlorobenzene (*o*-DCB, 5.0 mL) was added to 4-bromopropyl-functionalized silica gel (500 mg) and sodium iodide (20 mg). The mixture was refluxed for 48 h under nitrogen atmosphere and strong stirring (Scheme 1).

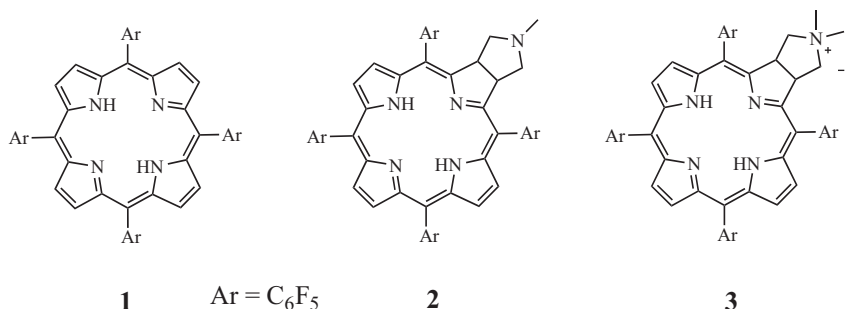


Fig. 1. Structures of porphyrin **1** and chlorins **2** and **3** prepared in this work.

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