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New photoantimicrobial films composed of porphyrinated lipophilic cellulose esters

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Abstract—Porphyrinated cellulose laurate esters have been prepared in homogeneous DMA/LiCl medium by 'one-pot, two-step' reactions starting from cellulose, protoporphyrin IX, and lauric acid, and using a TsCl/Pyridine system. The plastic films obtained after casting were shown to display photobactericidal activity against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria. This new photobactericidal polymer has potential for industrial, medical, or household applications.

Porphyrins and other tetrapyrroles are currently used as photosensitizers in photodynamic therapy (PDT) for the treatment of oncological diseases.[1,2] This photodynamic process, depending on the combined effects of a photosensitizer and visible light, leads to the formation of free radicals[3] (type I photochemical reactions) or more probably singlet oxygen[4,5] (type II photochemical reactions); these highly reactive species are able to interact with virtually every cell component, protein, lipid, and nucleic acid[6] and to generate a number of reactive by-products such as reactive oxygen species (ROS). As a consequence, induced damages can affect every cell compartment and lead to cell death. This efficiency, associated to a relative lack of cellular specificity, could give rise to a new and promising approach for the inactivation of micro-organisms[7–10] in an attempt to overcome the rampant bacterial multiresistance to antibiotics.[11–14] This technique, known as photodynamic antimicrobial chemotherapy (PACT), also relies on the accumulation of a photosensitizer into the target organism; usually Gram-positive bacteria prove to be more sensitive than the Gram-negative ones, and treatment of the latter often needs the additional use of a polymyxin nonapeptide that, by altering the outer membrane structure, helps the photosensitive drug to accumulate inside the bacterium.[15,16] It has been recently shown that polycationic porphyrin polyamines, acting on both Gram-positive and Gram-negative bacteria, display their strong photoactivity even in absence of the polymyxin nonapeptide helper when used against Gram-negative bacteria.[13]

Grafting π-systems like porphyrins to a polymer backbone should lead to a new material with interesting photobactericidal properties. On the other hand, esterification of cellulose, a natural polymer having stiff, shape-stable structure, by lauric acid allows the formation of plastic materials.[17] We thought to combine these two reactions to generate a novel photobactericidal surface. We report in this paper preliminary results on the synthesis of porphyrin-appended cellulose laurate polymers obtained by 'one-pot, two-step' esterification reactions. We show that protoporphyrin IX (PpIX), covalently bound to the polysaccharidic polymer, can efficiently kill bacteria. This new polymer could find beneficial household, industrial or medical applications.

According to previous work in our laboratory, esterification of cellulose was achieved in the homogeneous DMA/LiCl solvent system[17] following Scheme 1. Cellulose was dissolved in DMA containing 8 g LiCl/100 mL. Cellulose (20 g L^{-1}) and LiCl/DMA were kept at 70 °C under stirring until homogeneity. A limpid solution was obtained after cooling down to room temperature and overnight stirring.[18–20] The first step involves the esterification of cellulose by the system PpIX/TsCl/pyridine (Scheme 1).

Keywords: Porphyrin; Photobactericides; Cellulose; Polymer; Esterification; Plastic films.
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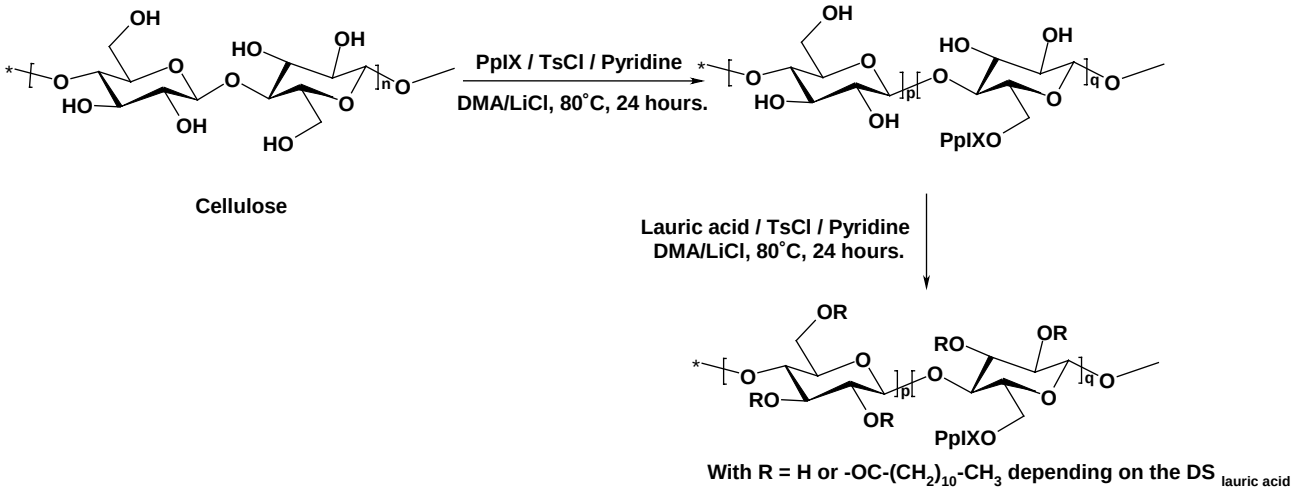

Scheme 1. 'One-pot, two-step' cellulose esterification (*p*: number of anhydroglucose units unesterified by PpIX; *q*: number of anhydroglucose units esterified by PpIX).

Equimolecular concentrations of TsCl, protoporphyrin IX, and pyridine were added with stirring at 80 °C; the three products remain soluble throughout the process, then the mixture was left for 24 h. The plastic porphyrin content was monitored by the amount of PpIX added. In a second step, esterification of cellulose-appended protoporphyrin with the lauric acid/TsCl/pyridine system was achieved under the same conditions of temperature and reaction time (Scheme 1).

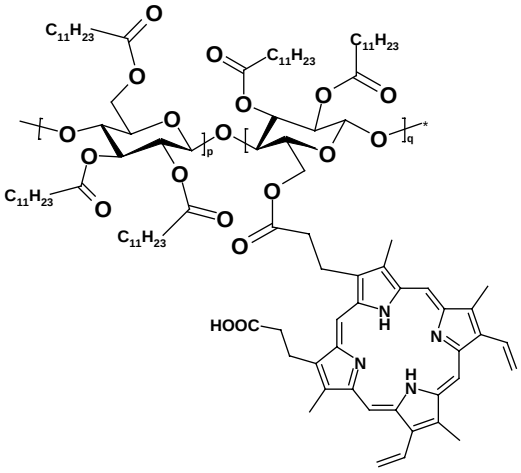

Figure 1. Most representative structure of porphyrinated cellulose laurate (for *p* and *q* meaning see Scheme 1).

After a total of 48 h of reaction, the modified cellulose was isolated by precipitation with MeOH. All samples were purified by reprecipitation, using chloroform as solvent and MeOH as precipitant, and then dried in vacuum in the presence of phosphorous pentoxide. After dissolution in $CHCl_3$ and filtration on glass wool, the plastic films were obtained by casting in a glass Petri dish. The structure of the cellulosic modified polymer is shown in Figure 1.

UV–visible absorption, ^{1}H NMR (400 MHz), and FT-IR spectra of these compounds showed the expected signals and absorptions.[21] $DS_{lauric\ acid}$ per anhydroglucose unit (AGU) were determined by a previously described method using ^{1}H NMR.[17] The plastic protoporphyrin IX contents were determined by using the molar extinction coefficient of the Soret band. Results as yields of each esterification step, plastic PpIX contents, and $DS_{lauric\ acid}$ of porphyrinated plastics are listed in Table 1.

Depending on the reaction condition, the fraction of anhydroglucose units esterified by PpIX varied from 0.19% to 1.1%, whereas $DS_{lauric\ acid}$ was found to be virtually constant throughout polymers **1**–**8**. We observed a decrease in the yields of plastic film obtained when porphyrin content exceeded 1.1%. This is probably due to the decrease in solubility of these high porphyrin content plastics. A view of a porphyrinated cellulose laurate is presented in Figure 2.

Table 1. Yields and DS of the porphyrinated cellulose laurate

Polymer:	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**[a]
mequiv PpIX/AGU[b]	3	6	9	1.2	1.4	1.9	2.3	0
Plactic PpIX content (PpIX/100 AGU)	0.19	0.38	0.52	0.73	1.1	1.05	1.1	0.00
$DS_{lauric\ acid}$	2.78	2.67	2.65	2.74	2.74	2.7	2.65	2.7
Yields of PpIX esterification	58	48	51	51	39	35	35	—
Yields of lauric acid esterification	86	71	83	79	66	63	63	86

[a] Plastic film **8** (cellulose laurate) serves as a reference.
[b] Amount of reacting protoporphyrin IX per anhydroglucose unit (AGU).

Figure 2. Porphyrinated cellulose laurate plastic film of 0.52% PpIX content.

Porphyrinated cellulose laurate films **1**–**7** were evaluated for their antimicrobial activity against two strains, obtained from 'Institut Pasteur, Paris': (a) *Escherichia coli* CIP 368548, class 2 and (b) *Staphylococcus aureus* CIP 35053156, class 2 whose antibiotic resistance has previously been described.[13] These two strains were grown for 24 h at 37 °C in test tubes containing 10 mL peptone water broth. 0.2 mL of 1/10 dilution of these broths was plated onto Müeller–Hinton agar.

A preliminary screening for photobactericidal activity was developed as follows. Disks (diam. 2 cm) were cut out of the porphyrinated plastic films and deposited onto the nutrient agar seeded with the target strain. Laurate cellulose ester plastic film disks (non-porphyrinated plastics with the same $DS_{\text{lauric acid}}$) were taken as references and used in the same conditions. Duplicate plates containing porphyrinated and non-porphyrinated disks were incubated in the dark and served as additional controls.

Test plates were turned upside down, placed on a bench, and incubated during 24 h at 37 °C under continuous illumination by four 150 W tungsten bulbs placed 60 cm above and 60 cm away from the flipped plates, totalling 1.7 mW/cm^2 fluence rate at the level of the bottom of the plates (Fig. 3). Each experiment was performed three times. The results are given in terms of positive (no colony) or negative response. The growth of the microorganisms was examined visually under the porphyrinated disks (Fig. 4). After removing the disks, the irradiated plated were incubated an additional 24 h in the dark, in order to check for any further possible appearance of colonies and to decide if the films have a bacteriostatic or a bactericidal photoactivity.

Seven porphyrinated films with different PpIX contents from 0.19% to 1.1% were tested with the two bacterial strains, allowing us to correlate the antimicrobial activities of films **1**–**7** with their porphyrin content (Table 2).

When illuminated, all the porphyrinated cellulose laurate films inhibit the growth of the Gram-positive bacteria *S. aureus*, whereas the unporphyrinated control **8**

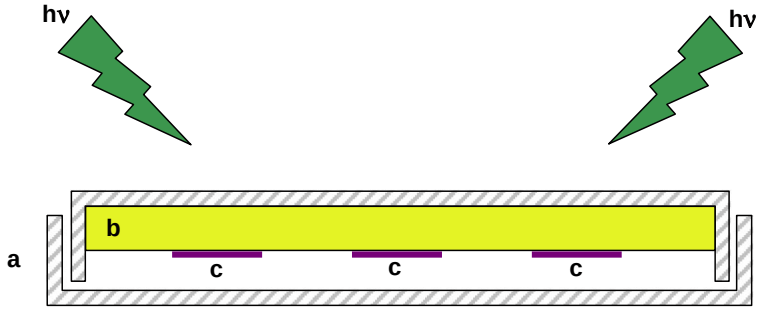

Figure 3. Photoinactivation of bacteria:[22] (a) flipped Petri dish; (b) nutrient agar plate seeded with target strain; (c) plastic film disks.

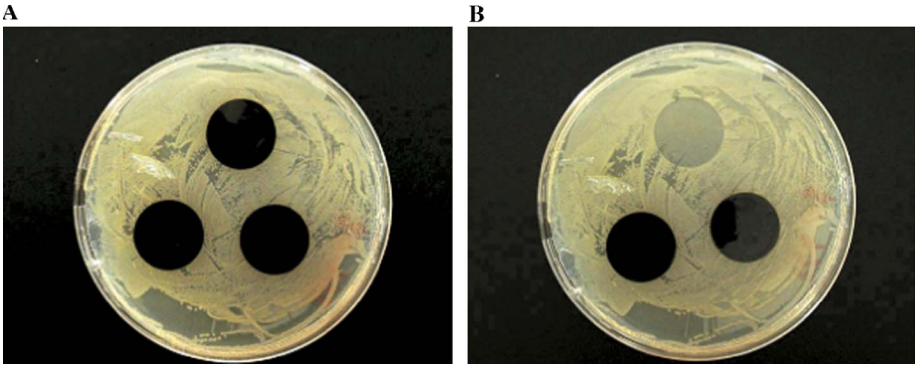

Figure 4. Results of photoinactivation of *Staphylococcus aureus*. The picture shows seeded Petri dishes after 24 h illumination at 37 °C. (A) Petri dish with porphyrinated cellulose laurate film PpIX/100 AGU = 0.73; (B) Petri dish with the upper porphyrinated cellulose laurate film removed showing the absence of colonies below the film.

Table 2. Photoactivity of plastic films as a function of their porphyrin contents

Polymer:	1	2	3	4	5	6	7	8
PpIX plastic content (PpIX/100 AGU)	0.19	0.38	0.52	0.73	1.1	1.05	1.1	0.00
S. aureus	−[a]	−	−	−	−	−	−	+[b]
E. coli	±[c]	±	−	−	−	−	−	+

[a] −, no colony observed under the disks.
[b] +, colonies observed under the disks.
[c] ±, presence of a small number of colonies (<5).

allows a full growth of bacteria. In the absence of light, no inhibition occurs. Further incubation of the treated plates does not lead to any changes, that is, no colony appears in the sterile areas.

As regards the photoinhibition of the Gram-negative bacteria *E. coli*, only polymers **3**–**7** are efficient. In this case, photoactivity needs a minimum porphyrin percentage content of 0.52. Again, no additional colony did appear in the photoinactivated areas after additional 24 h incubation at 37 °C and no inhibition has been observed in the dark.

Owing to the insoluble and immobilized character of the photosensitizer, mechanistic interpretations of these experiments must take into account the generation of a reactive species on the film surface, followed by its diffusion and eventual interaction with the target cell. Midden and co-workers[14] have already shown that such photoinhibition is due to the type II photochemical process implying singlet oxygen ($^1O_2$) that ultimately damages the cell membrane, since there is no penetration of the photosensitizer into the bacterial cell. This porphyrinic material has been proved to produce $^1O_2$ by ergosterol acetate photoperoxidation.[23] A solution of porphyrinic polymer in $CHCl_3$ was subjected to light illumination (150 W tungsten bulbs) in the presence of ergosterol acetate (10^{-5} M L^{-1}) for 2 h. At the end of the reaction all of the ergosterol acetate was converted to its endoperoxide. On the other hand continuous illumination of porphyrinic polymers by four 150 W tungsten bulbs during 48 h did not modify their UV-spectrum, so this surface presents the advantages of a long-living material since continuous light did not prove detrimental to the film structure or properties.

In conclusion, we have obtained protoporphyrinated plastic films starting from natural products such cellulose, PpIX, and lauric acid, in which the dye units were covalently bound to the cellulosic polymer. We have shown that these protoporphyrin-appended polymers are able to kill Gram-positive and Gram-negative bacteria. In addition, these films have user-friendly features. Indeed, their photogermicidal action would not generate harmful by-products, since non-reacting singlet oxygen would spontaneously revert to harmless triplet ground state oxygen. Such materials could be used in industrial, household, and medical environments, and more generally in areas that would benefit from permanent and efficient surface disinfection.

Acknowledgments

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21. Selected data: for compound **3**, ^1H NMR ($CDCl_3$, 400.13 MHz): δ (ppm) = 3.62 (m, 2.78H, CH-UAG), 2.32 (m, 2H, CH_2-laurate), 1.59 (m, 2H, CH_2-laurate), 1.26 (s_e, 16H, CH_2-laurate), 0.88 (t, 3H, CH_3-laurate). UV–vis spectrum in THF: λ_{max}, nm (ε, Lcm^{-1} $mol^{-1} \times 10^3$): 404

(150.1); 506 (18.2); 539 (13.6); 579 (8.5); 632 (6.4). Infra-red spectrum: ν, (cm^{-1}) = 2900 (C–H alkyl); 1750 (C=O ester).

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