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Superior performance of macroporous over gel type polystyrene as support for the development of photo-bactericidal materials

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Hexanuclear molybdenum cluster $[Mo_6I_8Ac_6]^{2-}$ (**1**) has been ionically bound onto macroporous ($\mathbf{P_{mp}}$) and gel-type ($\mathbf{P_{gel}}$) resins and their performance as materials for the photodynamic inactivation of microorganism have been studied. It has been found that $\mathbf{1@P_{mp}}$ in combination with light is able to reduce 99.999999% the population of Gram-positive *Staphylococcus aureus* whereas the activity of $\mathbf{1@P_{gel}}$ is limited to a 99.99% reduction at the same light dose. The same trend is observed with Gram-negative *Pseudomonas aeruginosa*. A comprehensive study of both materials has been performed using confocal laser scanning microscopy, thermogravimetric analysis, nitrogen porosimetry, steady state and time resolved fluorometries and diffuse reflectance spectroscopy. The photochemical generation of singlet oxygen ($^1O_2$) has been assessed using 9,10-dimethylanthracene as a trap for this reactive oxygen species. It can be concluded that the nature of the polymeric support is of paramount importance for the development of surfaces with bactericidal properties.

Introduction

In the current context of increasing antibiotic resistance there is an urgent need for alternative ways to fight bacterial infections.[1,2] It has been estimated that in Europe healthcare associated infections cause 37,000 deaths every year, with an economic burden of €13-24 billion.[3] In the United States, the estimated death toll is about 99,000 every year, with an economic cost of $33 billion.[4] Although the general public is becoming slowly aware of this global threat, public institutions have been warning since long time ago about the imminent crisis on this regard. The problem could reach in the future unprecedented proportions since microbial resistance has been recently described both as a 'One Health Issue' and as a 'One World Issue'.[5] Antimicrobial photodynamic inactivation (aPDI) is becoming a promising tool to fight bacterial infections.[6-11] It is a technique consisting in the application of light to a compound capable to generate reactive oxygen species (ROS) cytotoxic to the pathogenic microbes. aPDI can be applied using soluble[12] or supported photosensitizers.[13-20] A broad number of chemical families have been proposed as effective generators of ROS, especially singlet oxygen ($^1O_2$).[21] Among those molecules, xanthenic dyes, phenothiazinium compounds, porphyrins and phthalocyanins have been studied.[22] Recently we have proposed a hexanuclear molybdenum cluster complex (**1**: $[Mo_6I_8Ac_6]^{2-}$, Ac is acetate; see structure in Figure 1) as photoactive agent for aPDI,[23] owing to its notable photochemical properties.[24-35] Regarding the supports for the photoactive agents, a variety of materials have been described in the literature, like polyurethane, cellulose, nylon, chitosan, silica and polystyrene.[13-20] This last polymer is one of the most popular supports employed so far, dating back to the pioneering works of Schaap and Neckers.[36] Polystyrene is a versatile system, used in applications as diverse as fluorescence sensing[37] or catalysis.[38] In catalysis, this polymer is the basic scaffold of many ion exchange resins[39] and as a matter of fact some of those resins were employed very early to support ionic photosensitizers for the generation of singlet oxygen.[40-46] The vast majority of reported systems consist of photosensitizers covalently attached (or embedded) onto the so called gel-type resins, comprised of polystyrene with very low porosity.[47-49] In contrast with the frequently used gel-type supports, there is another kind of resin of technological interest: macroporous resins are made of highly-crosslinked polystyrene and have the unique property of having permanent pores allowing effective mass transfer throughout the entire polymeric matrix.[39] This fact is known and used regularly in the fields of catalysis and also for separations, but has not been explored, to the best of our knowledge, in the aPDI context.

In this work we describe a comparative study of the photodynamic activity of two ion exchange resins used for immobilization of cluster **1** (Figure 1a), one gel-type ($\mathbf{P_{gel}}$) and the other one macroporous ($\mathbf{P_{mp}}$). Both resins were loaded with the same amount of photosensitizer **1** and the photodynamic activity towards representative examples of Gram-positive and Gram-negative bacteria (*Staphylococcus aureus* and

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† Electronic Supplementary Information (ESI) available: diffuse reflectance spectra, time resolved emission decays and full set of photoinactivation assays.

Pseudomonas aeruginosa respectively) has been tested. In our preliminary communication only one type of polymer ($\mathbf{P_{mp}}$) and one strain of bacteria (*S.aureus*) were tested, and the objective of the research was to check the ability of supported **1** to generate cytotoxic $^1O_2$.[23] Here we focus our attention on deciphering the role of the organic polymeric matrix in the antimicrobial activity. As it will be shown in the following lines, the solid support is a variable that critically matters and hence must be taken into account in the development of effective photobactericidal materials.

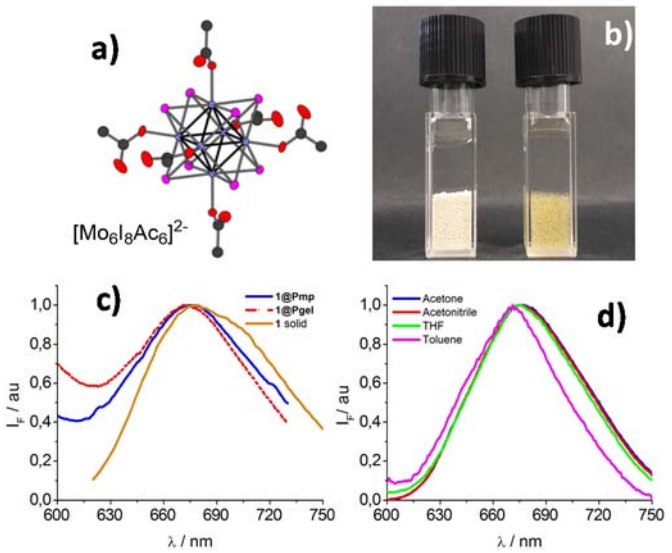


Figure 1.(a) Cluster **1**, $[Mo_6I_8Ac_6]^{2-}$, Ac is acetate; (b) picture of $\mathbf{1@P_{mp}}$ (left) and $\mathbf{1@P_{gel}}$ (right); (c) Phosporescence emission of solid powder of **1** (λ_{max}= 678 nm),1@Pmp(λ_{max} = 676 nm) and $\mathbf{1@P_{gel}}$ (λ_{max} = 671 nm); (d) Emission of **1** in ACN (λ_{max} =675 nm), acetone (λ_{max} =676 nm),THF (λ_{max} =674 nm) and toluene (λ_{max} =671 nm).

It must be noted that recently the research in the field of aPDI has focused its attention on the development of nanomaterials for the delivery of photosensitizers *inside* the pathogen cells.[50] However the polymer beads employed in this study are much larger (about 600-700 microns) than the bacteria to be killed (*S.aureus* are about half a micron in diameter and *P.aeruginosa* are few microns in length[51]). Hence, the materials here employed can be considered as surrogates of macroscopic surfaces, and thus as models for the development of bactericidal materials to prevent biofilm formation leading to nosocomial infections, for instance.

Experimental

Preparation of photoactive polymers

Washed resin (1g) Amberlite™ IRA-900 or Amberlite™ IRA-400 (both from Sigma-Aldrich, chloride form) was suspended in 50 mL of absolute EtOH containing dissolved cluster **1** (1.5 mg, as tetrabutylammonium salt). The suspension was stirred overnight at room temperature, filtered and the polymer washed with 100 mL of absolute EtOH. UV-vis absorption measurements before and after the overnight period showed complete exchange of chloride by the octahedral molybdenum anionic complex (no remaining cluster detected in the supernatant). Characterization of supported photosensitizers was performed by emission and diffuse reflectance spectroscopies, thermogravimetric analysis (TGA), porosimetry, confocal laser scanning microscopy (CLSM) (see below).

Steady state emission

The steady-state emission of the samples was recorded with a Spex Fluorolog 3-11 apparatus equipped with a 450W xenon lamp, operated in the front-face mode. The samples were introduced into quartz cells, sealed and purged with nitrogen prior to measurements. Five measurements were carried out for each sample in different areas of the solid and the results were averaged. Excitation was set at 400 nm.

Time resolved emission

Data was recorded using a Varian Cary Eclipse apparatus (75W pulsed lamp) with excitation at 400 nm and placing the solid samples (beads of supported photosensitizers) inside 1mL quartz cells (sealed and purged with nitrogen prior measurement) and using a holder for solids oriented at the appropriated angle to maximize the intensity of the signal.

Thermogravimetric Analyses

TGA experiments were performed on TG-STDA Mettler Toledo model TGA/SDTA851e/LF/ 1600 apparatus. For the determination of the water content of the polymers, 100 mg of $\mathbf{P_{gel}}$ and $\mathbf{P_{mp}}$ where hydrated with water for 30 min and dried with filter paper prior to perform the thermogravimetry from 25 to 200 ^{0}C.

Porosimetry

Measurements were performed on Micromeritics ASAP 2020 gas porosimeter equipped with degasification system Smart Vac. 1g of $\mathbf{P_{gel}}$ and $\mathbf{P_{mp}}$ were washed and dried at 60 ^{o}C vacuum oven for 24h prior to perform the measurements.

Diffuse reflectance Spectroscopy

Measurements were performed on Agilent Cary 400 UV-Vis-Nir spectrophotometer equipped with a diffuse reflectance accessory. The measurements were performed from 700 to 200 nm.

Confocal Laser Scanning Microscopy (CLSM)

Experiments were performed on an inverted confocal microscope Leica TCS SP8. Images where obtained with HCX PL APO CS 10x/0.40 DRY objective. Excitation of samples was done with a diode laser (488 nm) and images were acquired with a PMT detector. The polymers were observed directly on

sterilized Ibidi µ-Slide 8 Well Glass Bottom: # 1.5H (170 µm ± 5 µm) Schott glass.

Chemical trapping of singlet oxygen

Photo-oxygenation reactions were performed inside open Erlenmeyer flasks containing aerated acetonitrile solutions of the trap (50 mL, DMA 0.1 mM) and 500 mg of **1@P$_{mp}$** or **1@P$_{gel}$**. Irradiations were carried out, with continuous stirring, using two LED lamps (11W each, Lexman, ca. 400-700 nm emission output) placed 3 cm away from the Erlenmeyer flask. The evolution of the photoreactions was monitored over time by means of UV-vis absorption spectrophotometry (decrease of absorbance at 376 nm). The initial points of the kinetic traces were fitted to a pseudo-first order model ($\ln C/C_0 = - k_{obs} \cdot t$, where C is the concentration of DMA at a certain time t and C_0 is the initial concentration of DMA).

Antibacterial Photodynamic studies

Staphylococcus aureus ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Columbia Blood Agar (BA) was purchased from Oxoid. Microorganisms were grown aerobically in BA medium at 35^{0}C for 24 h. Stock inoculum suspensions were prepared in bi distilled water and adjusted to optical densities corresponding to 0.5 McFarland containing > 10^8 cell/mL. A Showtec LED Par 64 Short lamp was used for the irradiations. Irradiation was performed up to a dose of 200 J/cm^2 with a blue LED lamp (maximum emission at 460 nm, 0.013 W/cm^2) at a distance of 17 cm for 12 minutes and 49 seconds every 10 or 20 J/cm^2. Three groups of microorganisms were prepared for the irradiations (and other three as controls in the darkness): 5 mL of the initial suspensions with the desired McFarland value of *S. aureus* or *P. aeruginosa* were dropped into different RODAC plates and then (a) 200 mg of supported photosensitizer or (b) the same amount of control matrix (without photosensitizer) or (c) no polymer, were added. The final concentration in the experiments was 40 mg polymer/mL. All the groups were prepared and handled under light-restricted conditions. The six groups were shaken during the whole time of the photodynamic treatment. The antimicrobial effect was determined by counting the number of colony-forming units (CFU) / mL at different light doses up to a maximum of 200 J/cm^2. Bacterial cultures were incubated at 35 ^{0}C for 24 h. CFU counting was performed using Flash and Go automatic colony counter.

Results and Discussion

For the sake of reproducibility, **P$_{gel}$** and **P$_{mp}$** were obtained from commercial sources, since the properties of these ion exchange resins have been thoroughly studied from different viewpoints.[39] **P$_{gel}$** is Amberlite™ IRA 400 and **P$_{mp}$** is Amberlite™ IRA 900, both type I strong anion exchange resins (trimethylammonium chloride functionality) differing only in the crosslinking degree and the porosity of their structure (present in **P$_{mp}$**, negligible in **P$_{gel}$**). Supported photosensitizers (**1@P$_{gel}$** and **1@P$_{mp}$**) were prepared easily via chloride exchange by the dianionic complex **1** and isolated by filtration, as described earlier (final loading: 0.74 µmol of **1**/g of polymer).[23] The visual appearance of **1@P$_{gel}$** is translucent whereas **1@P$_{mp}$** is opaque, suggesting a different interaction with light (much different refraction index; see Figure 1b). Diffuse reflectance spectra of both materials showed unequivocally the presence of **1** (signal at ca. 405-485 nm; see ESI).

The optical properties of both materials were also examined by emission spectroscopy (Figure 1c). The hexanuclear molybdenum complex **1** has the valuable property of bright-red room temperature phosphorescence.[24-35] In our case solid powder of **1** emitted phosphorescence at λ_{max}= 678 nm, **1@P$_{gel}$** emitted at λ_{max}= 671 nm and **1@P$_{mp}$** displayed emission at λ_{max} = 676 nm. The intensity of both emissions are quenched by oxygen upon exposure of the samples to air. Also emission lifetimes are shortened (see ESI), thus confirming that the origin of the emission is the triplet state of **1**. For comparison purposes, phosphorescence of **1** in several solvents was recorded at λ_{max} = 671-675 nm (Figure 1d) in accordance with the literature.[24-35]

The performance of **1@P$_{gel}$** and **1@P$_{mp}$** as bactericidal materials was tested against *S.aureus* and *P.aeruginosa*. For both types of microorganisms notable differences were found between the gel-type and macroporous resins, especially in the case of gram positive bacteria. **1@P$_{mp}$** is more effective for the eradication of *S.aureus* than **1@P$_{gel}$** as it can be seen in Figure 2. At a light dose of 110 J/cm^2 the decrease in *S.aureus* population photoinduced by macroporous **1@P$_{mp}$**was 8 log units[23] of colony forming units (CFU) (hence 99.999999% killing). At the same dose, the reduction caused by gel-type **1@P$_{gel}$** is only about 4 log units. Control experiments showed that the photosensitizers in the dark or irradiation of the polystyrene matrixes alone caused no important reductions in the populations of *S.aureus* (see ESI). Hence, in the case of these gram positive bacteria, the effect of the matrix is very clear: the macroporous polymer favours the inactivation of the pathogens.

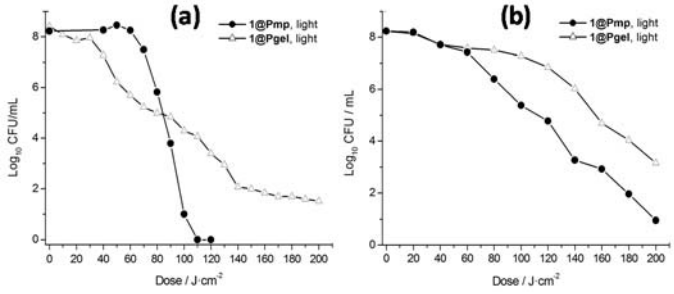


Figure 2. Comparison of antimicrobial photodynamic therapy of **1@Pgel** and **1@Pmp** in two different bacterial species (a) *S.aureus* and (b) *P.aeruginosa*.

In the case of *P.aeruginosa* the photobactericidal effect of the macroporous **1@P$_{mp}$** is again more pronounced than the action of thegel type **1@P$_{gel}$**, although in both cases less intense than for *S.aureus*, as expected for a gram negative bacteria.[6-11]

It is worth to note that for this microorganism, both the macroporous material **P_{mp}** and the gel polymer **P_{gel}** alone (both without **1)** have intrinsic photobactericidal effect against *P. aeruginosa*, which is potentiated by **1** (for instance, decrease of log CFU after 200 J/cm^2 irradiation is ca. 6 for P_{mp}, 7 for **1**@P_{mp}, 3 for P_{gel} and 5 for **1**@P_{gel}; see control experiments in the ESI). This striking performance could have practical implications for the development of inert surfaces without added photosensitizers, but the clarification of this effect falls out of the scope of this work and will be studied in detail in the future. The finding that a porous structure like Amberlite™ IRA 900 has a remarkable effect as bactericidal support for photosensitizer **1** is important from the technological perspective since it can point the way for future investigations with other sensitizers and matrixes. Among the reports on materials with photo-bactericidal properties, only few mention the importance of porosity, and none compares directly a porous system to a non-porous material loaded with the same photosensitizer. However, some porous materials with photosensitizing properties have been studied. The group of Orellana reported a porous silicone hosting photoactive Ru(II) complexes.[52,53] Greer and coworkers studied the generation of $^1O_2$ in a porous Vycor glass from the photophysical point of view, even envisaging the photobactericidal utility of such material.[54] The group of Hao found that a micro-patterned structure had some advantageous photobactericidal properties for the killing of *Escherichia coli* but with only a moderate 83% population reduction.[55]

The enhanced porosity of **1@P_{mp}** (as compared to **1@P_{gel}**), and concomitantly better mass transport, is suggested as preliminary hypothesis to account for the superior bactericidal properties of this material. In order to get a deeper knowledge of the studied resins, a comparative study was performed using thermogravimetric analysis (TGA), nitrogen porosimetry, and confocal laser scanning microscopy (CLSM). TGA characterization was performed in order to estimate the amount of water absorbed by the polymers, resulting in ca. 14 and 25 % (weight) for **1@P_{gel}** and **1@P_{mp}** respectively (Figure 3a). Porosimetry measurements were conducted in order to estimate the specific surface of both systems. For **1@P_{mp}** there resulted a value of 66.36 m^2/g (32 nm of pore diameter) and for **1@P_{gel}** the estimated value falls below the detection limit of the technique (Figure 3b). CLSM was envisaged as a mean to visualize the distribution of the photosensitizer in the polymers. Since the phosphorescence of cluster **1** is easily quenched by oxygen, direct estimation of its distribution in **1@P_{gel}** and **1@P_{mp}** was not possible by CLSM. However we employed an analogue of **1** for this task: fluorescein (another dianionic system, whose fluorescence is not quenched in air) was diffused in samples of **P_{gel}** and **P_{mp}** (1.2 µmol of dye /g of polymer). As it can be seen in Figure 3c-f, the differences between both polymers are remarkable. Whereas in the case of the porous matrix the dye is distributed almost uniformly throughout the material, in the case of the gel resin, the dye is exclusively confined to an outer shell of ca. 60 µm.

The ultimate importance of this distribution for the photobiological performance remains to be exactly determined, but it suggests that photosensitizer **1** must be spread out along a more extended volume, hence increasing the probability of interaction with oxygen or the microbes (or both). This idea must be considered with caution since it is well known that singlet oxygen diffusion in water is conditioned by the short lifetime of this species (3.5 µs), hence distances travelled are limited to a few microns.[56] Additionally, fluorescein is structurally very different from complex **1** and might not be reflecting exactly the behavior of the molybdenum complex in the polymers.

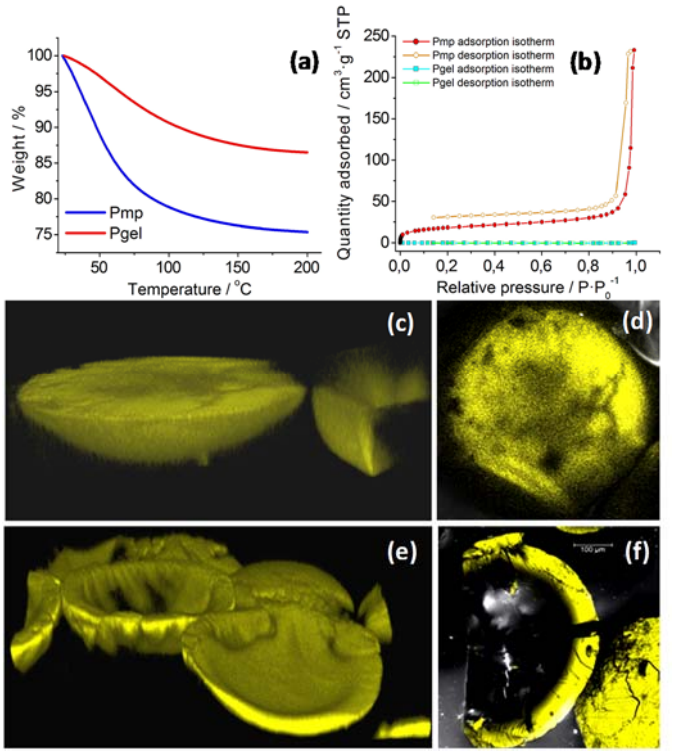
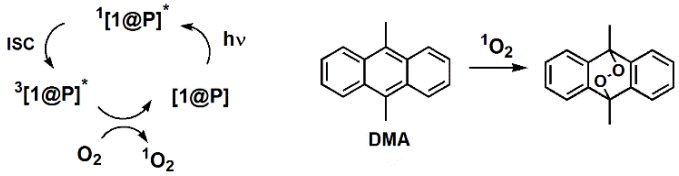


Figure 3. Characterization of **1@P_{mp}** and **1@P_{gel}**: (a) TGA of **1@P_{mp}** and **1@P_{gel}**; (b) porosimetry of **1@P_{mp}** and **1@P_{gel}**; (c)-(d) CLSM (λ_{exc} = 488 nm) of **fluorescein@P_{mp}** (3D reconstitution in (c) and section view in (d));(e)-(f) CLSM (λ_{exc} = 488 nm) of **fluorescein@P_{gel}** (3D reconstitution in (e) and section view in (f)).

Another proof of the enhanced generation of singlet oxygen in **1@P_{mp}** as compared to **1@P_{gel}** comes from the different photosensitizing activity displayed by them when used to sensitize a benchmark oxygenation reaction. Samples of both resins were submitted to irradiation in the presence of the well-known $^1O_2$ trap 9,10-dimethylanthracene (DMA),[57-61] in ethanol and in acetonitrile.



Scheme 1. 9,10-dimethylanthracene (DMA) oxidation by singlet oxygen photosensitized by **1@P**(**P** refers to **P_{mp}** or **P_{gel}**).

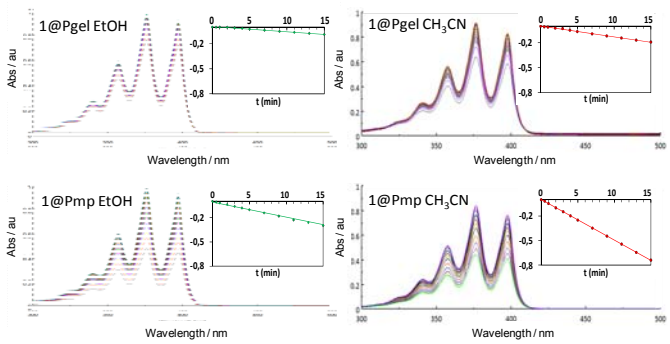


Figure 4. UV-Vis spectra monitoring the photooxidation kinetics of DMA by $^1O_2$ in ethanol (left) andacetonitrile (right) by **1@P$_{gel}$** (top) and **1@P$_{mp}$** (down); insets: pseudo-first order fittings (ln C/C$_0$ vs time).

As it can be seen in Figure 4 in both media, resin **1@P$_{mp}$** showed clear superiority for the generation of $^1O_2$ as evidenced by the higher calculated pseudo-first order kinetic constants (0.018 min^{-1} for **1@P$_{mp}$** and 0.006 min^{-1} for **1@P$_{gel}$** in ethanol; 0.049 min^{-1} for **1@P$_{mp}$** and 0.012 min^{-1} for **1@P$_{gel}$** in acetonitrile). A compilation of characterization data, including photosensitizing activity, is presented in Table 1.

Table 1. Characterization of **1@P$_{gel}$** and **1@P$_{mp}$**

Polymer	Water content (TGA)	Specific surface (m^2g^{-1})	Pore size (nm)	k_{DMA} ACN (min^{-1})	k_{DMA} EtOH (min^{-1})
1@Pgel	14%	(a)	(a)	0.012	0.006
1@Pmp	25%	66.36	32.0	0.049	0.018

(a) below the detection limit of the technique

To explain the superior performance of **P$_{mp}$** as support for photosensitizer **1** as bactericidal material we have focused our attention in the better capacity of the porous material to generate $^1O_2$, as it has been demonstrated above. However other factors could be playing key roles to rationalize the bactericidal performance. Specifically it is well known the cytotoxic effect for bacteria of cationic groups (like the ammonium moieties present in our polymers) and also the recently discovered importance of the roughness of the surfaces (**1@P$_{mp}$** and **1@P$_{gel}$** are very different in this regard).[62-66] Overall, the photodynamic effect of the materials here presented should be explained as a combination of all the above mentioned factors.

Conclusions

In summary, two readily available polystyrene matrixes (**P$_{gel}$** and **P$_{mp}$**) have been used as supports for singlet oxygen photosensitizer **1**, and the resulting supported sensitizers compared for the inactivation of microorganisms like Gram-positive *S.aureus* and Gram-negative *P.aeruginosa*. **P$_{gel}$** (Amberlite^{TM} IRA 400) is a gel structure with no permanent pores and **P$_{mp}$** (Amberlite^{TM} IRA 900) is a macro-reticular scaffold with permanent pores and high specific surface. The nature of the support has an enormous influence on the bactericidal action induced by light, in such a way that, at the same photosensitizer loading and light dose, **1@P$_{gel}$** induces the killing of 99.99% of bacteria, whereas **1@P$_{mp}$** is able to kill 99.999999% of such microorganisms. The case of the highly resistant *P.aeruginosa* is qualitatively the same, with better performance of the macroporous resin over the gel type support. The comparative study of these two types of resins, common place in the field of catalysis and separation science, is novel in the area of aPDI. Although this is a basic research, we hope that the findings here reported will help to develop in the future improved materials for the generation of singlet oxygen with applications not only in aPDI but also in related areas.

Conflict of interest

There are no conflicts of interest to declare.

Acknowledgements

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