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CeO_2 Nanoparticles for the Protection of Photosynthetic Organisms Immobilized in Silica Gels

Clémence Sicard,[†,‡,⊥] Mercedes Perullini,[§,⊥] Cecilia Spedalieri,[§] Thibaud Coradin,[†] Roberta Brayner,[‡] Jacques Livage,[†] Matías Jobbágy,*[,§,∥] and Sara A. Bilmes[§,∥]

[†]UPMC Univ Paris 06, CNRS, Chimie de la Matière Condensée de Paris (LCMCP), Collège de France, 11 place Marcelin Berthelot, F-75005 Paris, France

[‡]Interfaces, Traitements, Organisation et Dynamique des Systèmes (ITODYS) Université Paris Diderot-CNRS 7086, F-75205 Paris, France

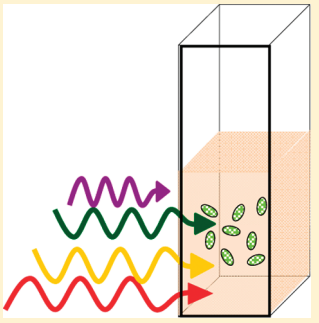
[§]Laboratorio de Superficies y Materiales Funcionales INQUIMAE-DQIAQF, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pab. II, C1428EHA, Buenos Aires, Argentina

[∥]Centro Interdisciplinario de Nanociencia y Nanotecnología, Argentina

Supporting Information

ABSTRACT: Synthetic cytocompatible CeO_2 nanoparticles embedded in transparent silica hydrogels are useful for creating inorganic supports favoring the long-term entrapment of living *Chlorella vulgaris* cells. The key protective role of nanoparticles is twofold: (1) They efficiently absorb harmful UV radiation without scattering the useful visible one. (2) They limit oxidative stress damage, such as that induced by H_2O_2, thanks to the surface redox $Ce^{3+}-Ce^{4+}$ reactivity of nanoparticles.

KEYWORDS: bio-materials, bio-mimetic materials, nanomaterials, nanoparticles, sol−gel chemistry, sol−gel processing



INTRODUCTION

Silica-based materials are now considered as useful robust matrices for living cell entrapment.[1−4] In recent years, efforts have been mainly focused on maximizing cell viability by means of milder sol−gel procedures, including the elaboration of organically modified hydrogels.[5−10] Because cells can remain physically isolated from the surrounding media in a stable and nondegradable film or hydrogel, these biomaterials have potential applications either as a platform for advanced biosensors[11,12] or as an optimized support for bioreactors.[13−15] More recently, silica biocomposites were also developed as active photosynthetic materials or even green hydrogen sources.[16−20] As the variety of entrapped strains and targeted applications is expanding, novel host materials are necessary, which may require the use of other metal oxides.[21−23] Cyanobacteria and photosynthetic algae base their life cycle on the absorption of visible light and require matrices with optimized optical properties in order to minimize the loss of incident light. In parallel, because higher energy photons belonging to the UV−B range decompose biomolecules and can strongly impact on cell viability, UV filtering properties would be highly desirable.[7,24] For instance, the inclusion of UV absorptive entities within the inorganic matrix could act as an inner cutoff filter. Those absorbing entities should be (photo)-chemically stable and must remain retained within the matrix. In principle, several metal oxide nanoparticles (below about 10 nm) fulfill all the aforementioned requirements, while minimizing undesired visible light scattering. However, most oxides, such as ZnO or TiO_2, release harmful radicals resulting from photogenerated electron−hole pairs.[25,26] In contrast, CeO_2 particles were reported to be less photoreactive, as well as biocompatible for a wide range of microorganisms.[27] Moreover, their inherent redox activity can contribute to the scavengeing of reactive free radicals, which exert severe stress or cell disruption.[27−30]

We propose here the use of CeO_2 nanoparticle-loaded silica hydrogels as protective matrices for long-term entrapment and growth of model photosynthetic microalgae *Chlorella vulgaris*. These expected properties were evaluated under harmful UV light, as well as in the presence of H_2O_2.

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EXPERIMENTAL SECTION

Cell Cultures. *C. vulgaris* were grown under controlled conditions at 20 ± 1 °C. Illumination was supplied, alternating 12 h light and 12 h darkness. Experimental cell cultures were initiated at 1.0×10^6 cells mL^{-1}, cultured in Bold's Basal medium, and shaken at 150 rpm in a rotary shaker.

Synthesis of Dextran Capped Nanoceria (DCNC). A total of 0.03 g of $Ce(NO_3)_3 \cdot 6H_2O$ (Sigma) and 0.430 g of Dextran (from *Leuconostoc mesenteroides*, average molecular weight 64−76 kDa, code D4751) were dissolved in 2.5 mL of H_2O and stirred for 30 min. Concentrated ammonia (25% w/w) was then added dropwise until a slightly basic pH (8−9) was reached (~3 μL). The solution was left to oxidize by atmospheric oxygen under 24 h stirring at room temperature. The particles were then washed through a dialysis membrane (Spectra/Por; MWCO: 3500) for 72 h. HRTEM experiments were performed on a JEOL 100CXII transmission electron microscope operating at 100 kV).

Silica Sol Synthesis. Silica sol was obtained by mixing 20 mL of tetraethoxysilane (TEOS 98%), 6.25 mL of H_2O, and 0.72 mL of HCl 0.6 M. The sol was vigorously stirred for 24 h. To obtain the aqueous sol, the hydrolyzed silica sol was diluted with an equal volume of H_2O, and ethanol removed from this mixture under vacuum (40 °C, 30 mbar) until the weight loss equaled that of ethanol generated by the hydrolysis reaction.

Alginate Silica Sol−Gel Encapsulation. In Step 1, *C. vulgaris* cells were pre-encapsulated in alginate beads formed on a polyacrylate sample holder. A 32 ± 1 μL aliquot of cells suspended in 4% sodium alginate was introduced in the cavities of the sample holder. A 0.2 M $CaCl_2$ aerosol was sprayed onto the sample holder for 2 min to initiate alginate cross-linking. This soft polysaccharide network was further consolidated by immersing the sample holder into a 0.1 M $CaCl_2$ solution for 10 min. In Step 2, the silica-based gels were obtained by mixing 1 mL of the aqueous silica sol, *x* mL of the DCNC solution, and *y* mL of water in order to reach a total volume $x + y = 0.235$ mL. Reference samples were prepared by adding 0.235 mL of H_2O. A total of 0.235 mL of phosphate buffer solution (0.2 M, pH 7.0) was further added, and 20 s after, the sample holder was carefully introduced along the wall of the measuring cell (Scheme 1 of the Supporting Information). The time elapsed between buffer addition and the introduction in the measuring cell is below the gel time.

Growth Rate of *C. vulgaris* Entrapped within Silicate−Ludox-Based Hydrogels. To evaluate the degree in which the scattering of visible light affects the encapsulated *C. vulgaris* growth rate, the number of cells developed in voids created inside silicate−Ludox hydrogels was measured as a function of gel thickness. The algal growth inside the voids was studied for individual cavities after 7 days of culture in BBM liquid medium. At the initial time, calcium alginate beads with a content of (100 ± 15) cells/bead were dispensed into acrylic molds, and the silica encapsulation procedure was performed mixing appropriate volumes of 1.25 M sodium silicate, commercial colloidal silica (LUDOX HS40, Aldrich), and succinic acid (5% w/w) to obtain hydrogels with a total SiO_2 content of 12% w/v and a silicate percentage in the range of 17−25%. After 1 week, the total number of cells inside individual cavities was determined by counting cells in a Neubauer chamber. To analyze cellular growth, voids were grouped according to their distance from the hydrogel surface.

Toxicity Analysis of DCNC and H_2O_2 with Simultaneous Evaluation of DCNC Protection against Oxidative Damage. Toxicity of both DCNC and H_2O_2 was evaluated by mixing 200 μL of a high-density *C. vulgaris* culture (1.5×10^7 cells mL^{-1} in Bold's Basal medium) with 80 mg of grinded dry silica gel (drying conditions: 37 °C during 12 h). The dry gels contain different amounts of ceria. A volume of H_2O_2 (1−10 μL) was added to the mixture in order to achieve the desired final H_2O_2 concentration. The cell survival at different times was evaluated over 26 h by monitoring the color intensity with the procedure described below. For the sake of comparison, additional experiments were carried out by counting the number of cells mL^{-1} in a Neubauer chamber. Proper controls to test the inherent decay observed in the absence of DCNC and/or H_2O_2 were also performed.

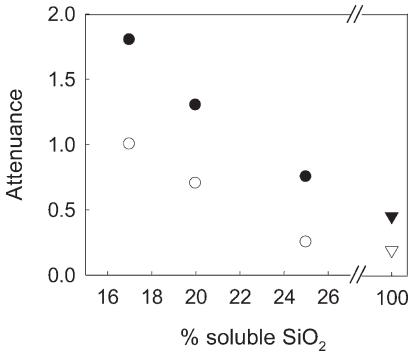

Figure 1. Attenuance of 1 cm thick silicate−Ludox-based hydrogels at 400 nm (●) and 500 nm (○), as a function of the percentage of sodium silicate, for a total SiO_2 content of 12% w/v. Attenuance of pure TEOS-based hydrogels (SiO_2 content of 12% w/v) at 400 nm (▼) and 500 nm (▽) is also presented.

UV Damage Test. Quartz or PMMA cuvettes containing the entrapped algae cultured 24 h in darkness were placed on a rotating platform (45 rpm) at 35 cm from the light source (150 W XBO Orem Ex. lamp fitted in a high-collection-efficiency housing and equipped with no cut off filters). An additional fan provided refrigeration in order to prevent excessive heating during the light exposure. The UV damage after increasing exposure times was estimated by image analysis with ImageJ free software.[31] For each sample, a selected area corresponding to the well containing the algae was digitally integered and decomposed in 3 main colors (red, green, and blue). Green intensity, related to the chlorophyll content of algal cells, was normalized as the ratio between the intensity of green color and the summed intensity of all the 3 colors. This value, which exhibits a linear dependence with the algae content, was properly tested with a calibration curve.

RESULTS AND DISCUSSSION

As a first step to optimize the preparation of algae−silica biomaterials, a series of experiments were conducted to explore to what extent the formulation of silica hydrogels affect light transmission and algal growth. Figure 1 presents the optical properties of several hydrogel formulations, evaluated in terms of the attenuance, corresponding to −log[*transmittance*], recorded at two representative wavelengths as a function of the percentage of soluble silica, i.e., the fraction of silicate present in the formulation. Among the assayed silicate−Ludox hydrogels,[5,6] the poorer optical properties limited the cell growth to more than 2 orders of magnitude, even for gels thicknesses of 3−4 mm (Figure S1 of the Supporting Information). In contrast, hydrogels obtained via an alkoxide route (i.e., using tetraethoxysilane, TEOS)[32] exhibited better optical and mechanical properties and were therefore chosen to fulfill the present requirements.[9,33]

After defining the composition of the silica hydrogel, a stable suspension of ceria nanoparticles was necessary for homogeneous dispersion through the matrix before gelation, preventing flocks that alter the light transmitance of the final hydrogel. To this aim, Dextran-capped CeO_2 nanoparticles (DCNC) of 3.2 ± 0.4 nm were prepared with a procedure based on the precipitation of Ce(III) salts followed by a mild oxidation under

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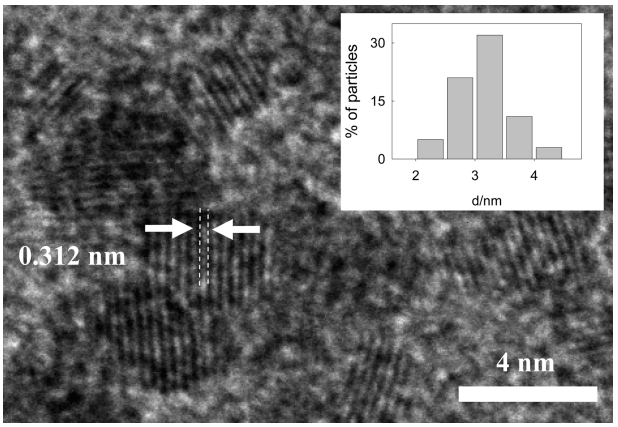

Figure 2. HRTEM image of several monocrystalline Dextran-capped CeO_2 nanoparticles. The *d*-spacing values of 3.12 Å, corresponding to the distance between (111) planes of the ceria fluorite lattice, are indicated. Inset: Size distribution derived from HRTEM images.

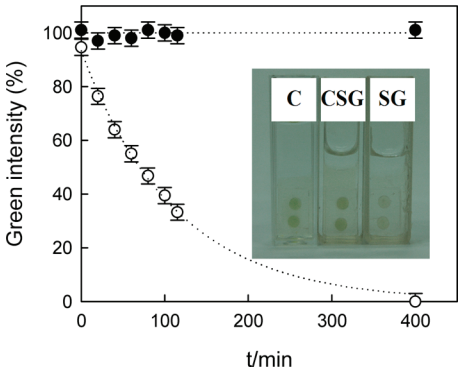

Figure 4. Green intensity of *C. vulgaris* entrapped inside TEOS hydrogel (SG, ○) and CeO_2-loaded TEOS hydrogel (CSG, ●) as function of UV exposure time. Inset: Quartz cells containing *C. vulgaris* cultures inside silica hydrogel (SG) and CeO_2 loaded silica hydrogel (CSG), after a UV irradiation period of 6.5 h. A nonirradiated SG control (C) is also presented.

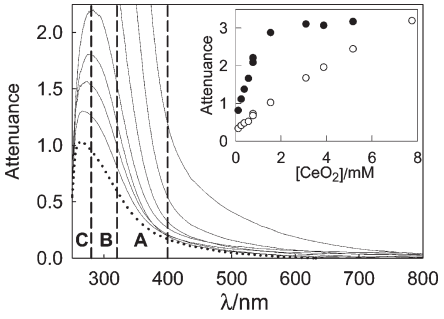

Figure 3. UV−vis attenuance spectra of 1 cm thick silica hydrogel with increasing CeO_2 contents. From right to left, dashed vertical line denotes the lower energy limits for UV A, B, and C radiations ranges, respectively. The spectrum of bare silica hydrogel with Dextran is represented with a dotted line. Inset: Attenuance at 299 (●) and 356 (○) nm as a function of CeO_2 concentration .

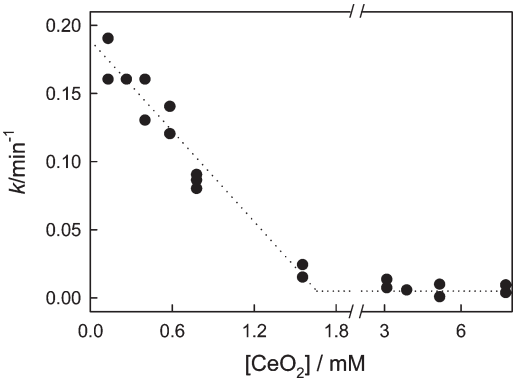

Figure 5. Bleaching rate constant of *C. vulgaris* entrapped in TEOS hydrogels exposed to UV for 400 min as a function of DCNC concentration in the gel.

atmospheric conditions.[34] HRTEM inspection revealed that the particles consisted of single crystalline entities exhibiting the typical interbasal lattice distances of cerianite (fluorite-like CeO_2) (Figure 2 and Figure S2 of the Supporting Information).

Several hydrogels with increasing DCNC contents were prepared and molded inside spectrophotometer cells with 1 cm path length. After an aging time of 24 h, the UV−vis spectra exhibit an increasing absorption band (Figure 3). The absorbance recorded at 299 and 356 nm taken as representative UV B and A, respectively, reveals that for DCNC concentrations below 2 mM in CeO_2, the UV−B filtering ability of the hydrogels tends to be proportional to the ceria content.

The gels were molded inside quartz cuvettes containing *C. vulgaris* culture entrapped in calcium-alginate gel (Scheme 1 of the Supporting Information) and exposed to a UV source. After 2 h of irradiance, a massive bleaching of algae entrapped in ceria-free hydrogel (SG) was observed, while the green intensity of those entrapped in the silica hydrogel containing 2 mM ceria nanoparticles (CSG) remained mostly unaltered. The decrease of green intensity gives a qualitative indication of photooxidative death, as several mechanisms are involved in the complex response of microalgae to light stress.[35,36] After a cumulative exposure time of 6.5 h, the totally bleached SG entrapped algae were no longer viable, while those immobilized in the presence of DCNC remained viable and almost unaltered compared to the nonirradiated control, demonstrating the effectiveness of the UV barrier. The composite gel is stable for 6 months, and the irradiated culture trapped in CSG are viable for the same period in the dark. These algae resubmitted to an equivalent UV dose after 6 months under a 12 h photoperiod exhibited the same behavior shown in Figure 4.

Because increasing DCNC loading results in significantly different UV filtering abilities, an extensive screening of the protective ability of hydrogels was performed. With this aim, the experiment shown in Figure 4 was repeated for formulations containing DCNC in a 0−8 mM concentration range, expressed as moles of CeO_2 per dm^3 of hydrogel. Each curve was fitted to an exponential decay, from which a bleaching rate decay constant was obtained. Figure 5 depicts the algae bleaching rate constant obtained as a function of DCNC loading. From the comparison of Figure 5 and the inset in Figure 3, it can be stated that the protective ability correlates with the UV B absorbance. For DCNC contents lower than 1.5 mM, the bleaching rate constant decays almost linearly with the ceria concentration, reaching the values of the decay rate constant observed for nonirradiated controls.

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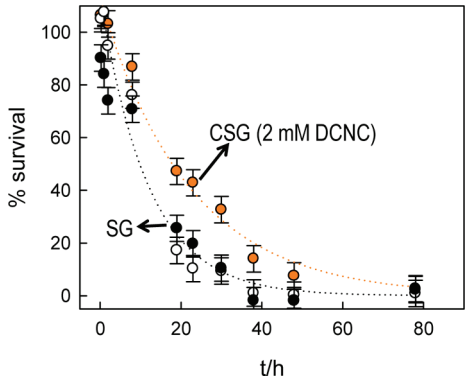

Figure 6. Percent survival of *C. vulgaris* exposed to 1% H_2O_2 in the presence of CSG and SG. Empty circles: data obtained for free algae.

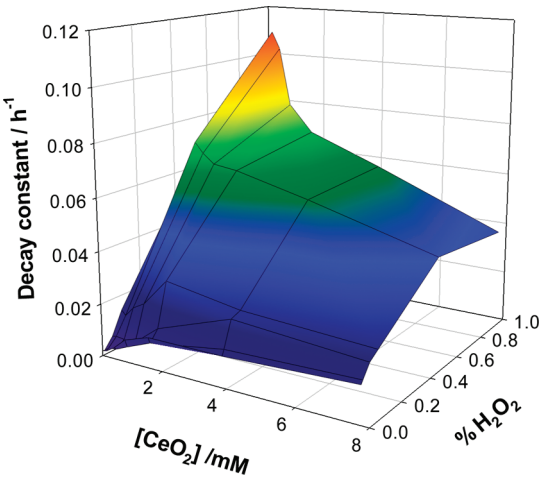

Figure 7. Survival (%) decay constant of *C. vulgaris* exposed to an increasing concentration of H_2O_2 in the presence of silica hydrogels containing increasing amounts of DCNC.

As stated above, DCNCs exhibit, in addition to UV filtering ability, efficient radical scavenging properties due to the surface $Ce^{3+}-Ce^{4+}$ redox couple, even in the presence of capping agents.[25,26,37] However, in the present case, ceria nanoparticles face an additional barrier to their surface reactivity due to the silica matrix in which they are entrapped. The antioxidant activity of DCNCs dispersed in CSG matrices was evaluated from the evolution of algae survival in contact with H_2O_2. In this experiment, algae were put in contact with dried silica gels for two reasons: (i) Pre-encapsulation in alginate could give ambiguous results arising from the diffusion of free radicals through the biopolymer. (ii) Direct encapsulation in wet silica may change the stress response due to direct contact of cells with silanol groups during the sol → gel → aging process. Figure 6 shows the fraction of living cells exposed to 1% H_2O_2 as a function of time for CSG (2 mM DCNC) and SG matrices. Decay curves obtained for SG are overimposable to those obtained for the free cell culture control, revealing that the bare silica gel cannot offer any protection toward free radicals. However, the survival decay of algae embedded in CSG is markedly slower, indicating that DCNC dispersed in the silica gel are able to limit the harmful effect of peroxide species that are known to be a possible source of cellular oxidative stress and may even lead to massive damage.

Although DCNC proved to be efficient scavengers of radicals, and shield against UV B radiation, ceria may be toxic for living organisms.[27,38] The evaluation of both harmful effects and radical scavenging of DCNC was performed by measuring the green color evolution as a function of time for a series of samples containing algae and gels with variable amount of DCNC in contact with H_2O_2, whose concentration lies in the 0% and 1% range. The control experiment with free algae in contact with free DCNC is shown in Figure S3 of the Supporting Information. Curves similar to those in Figure 6 were fitted to an exponential function; the corresponding decay constants are plotted in Figure 7 against DCNC concentration and percent H_2O_2. In the absence of H_2O_2, the effect of ceria is deleterious, saturating at concentrations close to the optimum for UV protection. In contrast, in the absence of DCNC, H_2O_2 exert a noticeable damage with an increase in the decay constant up to 0.11 h^{-1} when peroxide concentration reaches 1%. In the presence of ceria, there is a noticeable decrease in the decay constant indicating that DCNC suppress the damage produced by free radicals. From these results, it may be concluded that beneficial effects of DCNC toward both UV damage and free radical exposure exceeds the toxicity noticed for concentrations below 2 mM.

CONCLUSIONS

This work demonstrates the ability of CeO_2-loaded SiO_2 hydrogels to efficiently protect entrapped *C. vulgaris* cultures from harmful UV light, while ensuring their viability or even growth for periods of several months, without recording toxicity for the entrapped culture. Moreover, DCNCs dispersed in the hydrogel decrease the oxidative damage that may be induced by external sources.

The present findings open the gate to the formulation of more robust hydrogels containing photosynthetic cellular, subcellular or molecular entities that could contribute to the green energy field in a nearby future.[39] Moreover, they suggest that it is possible to further widen the structural and chemical diversity of inorganic matrices that are compatible with the encapsulation of living cells.[40]

ASSOCIATED CONTENT

Supporting Information. Details on experimental procedures for samples preparation and further sample characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: jobbag@qi.fcen.uba.ar.

Author Contributions

⊥Clémence Sicard and Mercedes Perullini contributed equally to the experiments presented here.

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