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Maurício A. Caiut, Marie-Joëlle Menu, Christine Roques

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# Bifunctional silica nanoparticles for the exploration of *Pseudomonas aeruginosa* biofilm

L. Mauline<sup>2</sup>, M. Gressier<sup>2</sup>, P. Hammer<sup>3</sup>, S.J.L. Ribeiro<sup>4</sup>, J.M.A. Caiut<sup>5</sup> and M.-J. Menu<sup>2</sup>, C. Roques<sup>1\*</sup>

**Abstract**— Luminescent silica nanoparticles (LSNPs) are frequently employed for biotechnology applications mainly because of easy functionalization, photo-stability and biocompatibility. Bifunctional silica nanoparticles (BSNPs) are described here as new efficient tools for the understanding of a complex biological system such as biofilms. Photoluminescence is brought by the incorporation of a silylated ruthenium(II) complex, surface properties of the silica particles are designed by reaction with. BSNPs are fully characterized and Zeta potential and contact angle measurements exhibit various surface properties according to the functional groups. Confocal Laser Scanning Microscopy measurements show that the spatial distribution of these nanoparticles inside PAO1 biofilm depends more on their hydrophilic/hydrophobic characteristics than on their size.

## INTRODUCTION

A biofilm can be described as a community of adhering microorganisms, generally entrapped in a self-produced matrix of extracellular polymeric substances (EPS) [1]. Biofilm formation is a widespread phenomenon with a major economic impact in industrial [2, 3], medical [4, 5] and environmental fields [6, 7].

The particularity of biofilms comes from its high resistance towards antibiotic and disinfectant agents, greater than planktonic cells and which can be linked to the tridimensional structure and the EPS [8]. Knowledge about transfer limitation through biofilms would provide precious information for the control and/or the eradication of biofilm.

Luminescent silica nanoparticles (LSNPs) are frequently employed for biotechnology applications mainly because of easy functionalization, photo-stability and biocompatibility. Bifunctional silica nanoparticles (BSNPs) are described here as new efficient tools for the understanding of a complex biological system such as biofilms.

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<sup>1</sup>Laboratoire de Génie Chimique, UMR-CNRS 5503, Université Paul Sabatier, 35 chemin des maraîchers, 31062 Toulouse cedex 9, France;

<sup>2</sup>Centre Interuniversitaire de Recherche et de l'Ingénierie des Matériaux, UMR-CNRS 5085, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse cedex 9, France; <sup>3</sup>Laboratório de Espectroscopia de Fotoelétrons, LEFE, Institute of Chemistry, São Paulo State University, UNESP, CP 355-Araraquara – SP, 14801-970 Brazil; <sup>4</sup>Institute of Chemistry, São Paulo State University, UNESP, CP 355-Araraquara – SP, 14801-970 Brazil;

<sup>5</sup>Department of Chemistry, FFLCRP, University of São Paulo, Ribeirão Preto – SP, Brazil, <sup>5</sup>

E-mail addresses: mauline@chimie.ups-tlse.fr; gressier@chimie.ups-tlse.fr; menu@chimie.ups-tlse.fr; ch.roques@wanadoo.fr; peter@iq.unesp.br; caiut@ffclrp.usp.br; sidney@iq.unesp.br

## I. EXPERIMENTAL PART

Silica particles were synthesized by the Stöber method in reverse microemulsion. Luminescent characteristics were obtained by incorporation of the [Ru(bpy)<sub>2</sub>(bpy-Si)]Cl<sub>2</sub> silylated dye [9]. The microemulsions were stirred for 24 h and then broken by adding acetone (150 cm<sup>3</sup>). LSNPs were recuperated by centrifugation and several subsequent washing procedures were performed in order to remove surfactants and excess free dye. The isolated powder 130 mg (95%) was dried under vacuum for 2 h. LSNPs were activated by mild thermal treatment at 50°C under vacuum for 30 min to remove physisorbed water. LSNPs (100 mg) were dispersed in anhydrous acetonitrile (20 cm<sup>3</sup>) and different organosilanes bearing functional groups were introduced and the mixtures were allowed to reflux for 24 h. BSNPs were purified by cycles of centrifugation and washings then dried in vacuum for 2h. BSNPs were then characterized: <sup>13</sup>C and <sup>29</sup>Si Solid-state NMR spectra (Bruker Avance 400WB equipment using dipolar decoupling in combination with Cross-Polarization (CP) and Magic Angle Spinning (MAS) (100.356 and 79.391 MHz for <sup>13</sup>C and <sup>29</sup>Si respectively); Photoluminescence spectra (Jobin Yvon, Fluorolog-3 spectrophotometer model FL3-22); XPS measurements (UNI-SPECS UHV); TEM analyses (JEOL 2010 (200 kV)); Zeta potential (ζ) and dynamic light scattering (DLS) (MALVERN nanosizer ZS90 equipped with a red laser (633 nm)); Contact angle measurements using a CDD camera with water drop volume of 4.10<sup>-3</sup> cm<sup>3</sup>. Bacterial biofilms were grown at 37°C in 24-well or 6-well microplates containing 2 cm<sup>3</sup> or 6 cm<sup>3</sup> of MBB, respectively as previously described [10]. After initial inoculation with a 10<sup>3</sup> CFU cm<sup>-3</sup> bacterial suspension of PAO1, the medium MBB was renewed at 2, 4, 6, 20, 24 and 48 h (end 72h) After the period of growth, the wells were rinsed and 2 cm<sup>3</sup> or 6 cm<sup>3</sup> of a filtered (0.2 μm) aqueous suspension of BSNPs at a concentration of 0.5 or 0.05 mg cm<sup>-3</sup> was introduced. Incubation was performed 16 hours before confocal microscopy observations (Leica SP2 confocal laser scanning system). Staining was performed using ConA-Alexa Fluor@488 (Molecular Probe, Invitrogen, France), DNA-stain, SYTO45® (Molecular Probe).

## II. RESULTS AND DISCUSSION

Two adapted procedures have been used for the elaboration of BSNPs, a ternary (cyclohexane/Igepal CO 520/water) [11] and a quaternary (cyclohexane/hexanol/triton X-100/water) microemulsion [12], in order to isolate two sizes of nanoparticles (25±5 nm and 68±2 nm). Different techniques used to characterize LSNPs confirmed the

presence of silica and the ruthenium complex. Surface modification of LSNPs has been performed by grafting reaction of organosilanes on silica particles followed by control of chemical integrity. Figure 1 shows the images of formed drops in contact with the pellets of BSNPs. *Ru@Si*, *Ru@Si-A*, *Ru@Si-B* and *Ru@Si-C* are hydrophilic with contact angles between 31 and 38°, whereas *Ru@Si-D* appears very hydrophobic with a contact angle greater than 90°.





	$\tau$ mmol.g <sup>-1</sup>	Angle de contact	$\zeta$ mV (pH = 6)
		35°	-39 ± 5
	1 - 0,4	31°	43 ± 5
	0,18	38°	-47 ± 5
	0,3 - 0,5	93°	-32 ± 5

Fig. 1. Contact angle measurements ( $\theta$ ) formed by the water drop of BSNPs *Ru@Si-A*, *Ru@Si-B*, *Ru@Si-C* and *Ru@Si-D*.

#### Biofilm penetration.

**25 nm BSNPs.** Hydrophilic particles *Ru@Si*, *Ru@Si-A*, *Ru@Si-B* and *Ru@Si-C* were able to penetrate through the biofilm regardless of surface charge (Figure 2). The same 2-dimensional snapshots have been performed using hydrophobic *Ru@Si-D* nanoparticles, results are presented in figure 9 I and II. The behavior of *Ru@Si-D* differed from that of the hydrophilic nanoparticles since they aggregated around the biofilm.

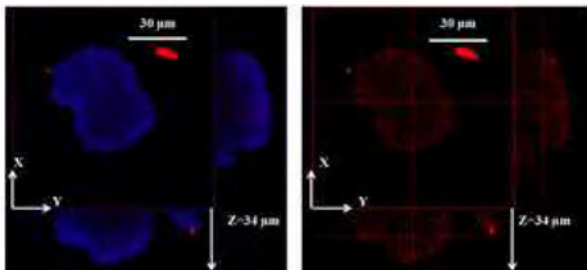


Fig. 2. 3-dimensional snapshot of *Ru@Si-B* particles in *P. aeruginosa* biofilm (III). DNA bacteria is labeled with SYTO45® (blue;  $\lambda_{exc}$  = 458 nm;  $\lambda_{em}$  = 468 – 488 nm) and *Ru@Si-B* particles (red;  $\lambda_{exc}$  = 543 nm;  $\lambda_{em}$  = 600 – 700 nm)

**68 nm BSNPs.** In both cases, hydrophobic and hydrophilic BSNPs, the snapshots and the penetration curves showed a significant decrease of the emission intensity with increasing particle size, indicating deeper penetration of the smaller hydrophilic particles.

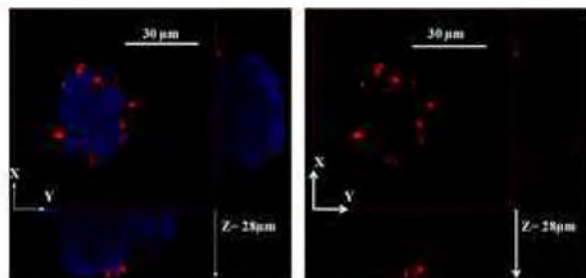


Fig. 3. 3-dimensional snapshot of *Ru@Si-D* particles in *P. aeruginosa* biofilm (III). DNA bacteria is labeled with SYTO45® (blue;  $\lambda_{exc}$  = 458 nm;  $\lambda_{em}$  = 468 – 488 nm) and *Ru@Si-D* particles (red;  $\lambda_{exc}$  = 543 nm;  $\lambda_{em}$  = 600 – 700 nm)

### III. CONCLUSION

The present study shows that regardless of size of the hydrophilic particles that they could penetrate throughout the biofilm but a steric effect was observed, the smaller particle diffusing in greater number than the larger ones. For hydrophobic nanoparticles, an accumulation at the periphery of the biofilm was observed regardless of the particle size; this observation suggests that the functionalization of particles could be a key factor in the penetration of particles in the biofilm. These results emphasize that BSNPs are powerful and innovative tools for the study of bacterial biofilms. Contrary to latex particles, silica has the advantage to display a great variety of functional surface groups.

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