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LETTER

A monolayer of a Cu^{2+} -tetraazamacrocyclic complex on glass as the adhesive layer for silver nanoparticles grafting, in the preparation of surface-active antibacterial materials†‡

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A silane-derivatized tetraaza Cu^{2+} macrocyclic complex is prepared, which forms monolayers on glass surfaces, capable of allowing the further deposition of a stable monolayer of silver nanoparticles, obtaining by this, surfaces that display an enhanced antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

Bacterial infections involving the surface of medical devices such as catheters, artificial prosthetics and subcutaneous implants are a serious nosocomial problem.¹ In the past twenty years huge efforts were made to obtain antibacterial coatings on the surfaces of such devices.² In this area, the use of silver nanoparticles (NP) has been widely studied,³ and it is accepted that it is the release of Ag^+ cations from their surface that exerts the antimicrobial action.⁴

The “layer-by-layer” (LbL) approach is a straightforward technique to obtain monolayers of silver NP on a chosen surface: first a molecular self-assembled monolayer (SAM) is formed on the surface, then a monolayer of NP is grafted on the SAM's terminating groups.⁵ We have recently shown that with easy wet procedures, a monolayer of Ag NP is prepared on flat glass surfaces, first forming a covalently-bound SAM of mercaptopropyltrimethoxysilane and then grafting citrate-protected Ag NP ($d = 7$ nm) on it.⁶ The Ag NP are firmly

grafted on the surface, and they exert an efficient microbicidal effect against *Escherichia coli* and *Staphylococcus aureus* by slow release of small Ag^+ quantities, avoiding NP detachment.⁶ The use of a thiol-terminated SAM is the method of choice to graft Ag NP on a surface by the LbL approach,^{5,6} although amine–silver bonds⁷ and electrostatic interactions have also been exploited.⁸ Regarding the latter, it should be stressed that the interaction of positively charged groups with noble metals nanoobjects is a common feature in the surfactant-mediated syntheses of nanorods.⁹ The interaction is electrostatic, and it takes place on the anionic layer adsorbed on the nanoobject surface.¹⁰ In the effort of attaining new antibacterial glass surfaces we have now found a LbL approach that uses a covalently-bound monolayer terminating with a Cu^{2+} macrocyclic complex to graft Ag NP.

Some recent papers have indagated the grafting of cyclam macrocycles (cyclam = 1,4,8,11-tetraazacyclotetradecane) on porous and mesoporous silica substrates, and the formation of their Cu^{2+} complexes in the perspective of obtaining metal sequestering materials.¹¹ In all cases, one, two or four propyl-trialkoxysilane groups were appended to the N atoms on the macrocyclic framework. A certain degree of instability was always noted, with partial detachment of the macrocyclic units from the silica surface.^{11a,b} Here we have used the Cu^{2+} complex of a thirteen membered macrocycle, 13aneN4 (1,4,7,10-tetraazacyclotridecane), bearing a methylamino function on a C atom.¹² We have bound it to a propyltrimethoxysilane moiety by means of a straightforward coupling reaction with commercial isocyanatopropyl trimethoxy silane, obtaining the $\text{PTS}[\text{Cu}(\text{13aneN4})]^{2+}$ conjugate (Scheme 1A). The amines in the ring remain thus secondary, allowing to maintain the original coordinating features of the plain 13aneN4 ring. We have already reported that inside plain 13aneN4 Cu^{2+} is bound with a square pyramidal geometry with an apical solvent molecule, as the $-\text{NH}_2$ on the side chain cannot fold and coordinate intramolecularly the Cu^{2+} cation in the ring, for geometrical reasons.¹³ The complex is kinetically inert in the 3–11 pH range.¹³

$\text{PTS}[\text{Cu}(\text{13aneN4})]^{2+}$ displays a d–d absorption band in ethanol solution at 560 nm ($\epsilon = 185 \text{ M}^{-1} \text{ cm}^{-1}$, Fig. 1A, inset),

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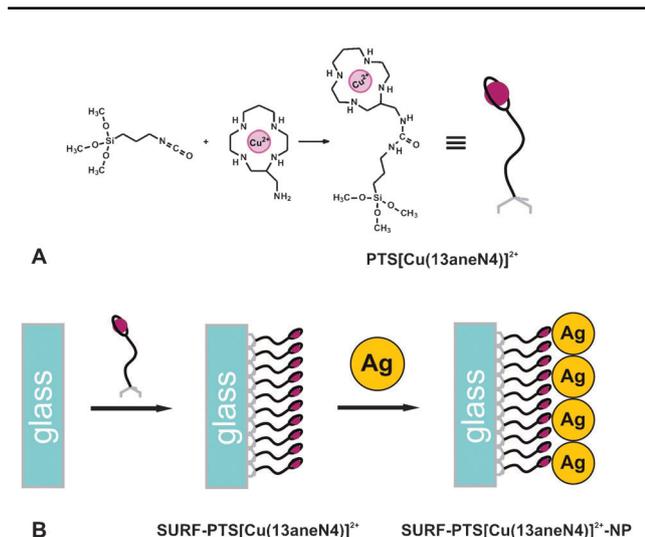
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‡ Electronic supplementary information (ESI) available: Further experimental details; ellipsometry, AFM and ATR-FTIR on $\text{SURF-PTS}[\text{Cu}(\text{13aneN4})]^{2+}$; molecular modelling for $\text{PTS}[\text{Cu}(\text{13aneN4})]^{2+}$ UV-Vis variations vs. time in PBS for $\text{SURF-PTS}[\text{Cu}(\text{13aneN4})]^{2+}$ -NP; AFM imaging on $\text{SURF-PTS}[\text{Cu}(\text{13aneN4})]^{2+}$ -NP. See DOI: 10.1039/c0nj00829j



Scheme 1 (A) Synthesis of the silane-functionalized Cu²⁺ macrocyclic complex; (B) two-steps synthesis of surfaces bearing a Ag NP monolayer grafted on a PTS[Cu(13aneN4)]²⁺ self-assembled monolayer.

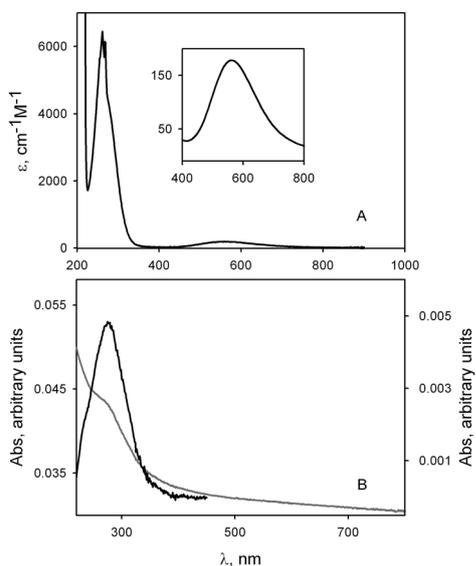


Fig. 1 (A) UV-Vis absorbance spectrum of a 10^{-4} M solution of **PTS[Cu(13aneN4)]²⁺** (CF_3SO_3^- salt) in ethanol, quartz cuvette; inset: zoom of the vis range, evidencing the d-d absorption; left axes: molar extinction coefficient. (B) UV-Vis spectrum of **SURF-PTS[Cu(13aneN4)]²⁺** *i.e.* a quartz slide coated with a SAM of **PTS[Cu(13aneN4)]²⁺** (grey spectrum, absorbance on the left axis). The black spectrum (absorbance on the right axis) is obtained by background subtraction (the scale of the axis is smaller, in order to evidence the band).

almost identical to what we reported for derivatives bearing *n*-alkyl chains on the side amino group,¹³ thus excluding inter- or intra-molecular interactions between Cu²⁺ and the urea group. An intense band is found in the UV region ($\lambda_{\text{max}} = 265$ nm, $\epsilon = 6450 \text{ M}^{-1} \text{ cm}^{-1}$, Fig. 1A), that in polyamino ligand complexes is commonly attributed to the Ligand to Metal charge transfer (LMCT) from the amines to the metal centre. Formation of a SAM of **PTS[Cu(13aneN4)]²⁺** on glass or quartz is carried out by dipping slides (pre-cleaned, ESI†) in

a 0.1 wt% solution of the complex in ethanol (although what is obtained is more properly to be defined a covalently-bound monolayer, the acronymous SAM is used for simplicity). Filling with the same solution a pre-cleaned quartz spectroscopy cuvette leads to the functionalization of its internal walls.

The obtained modified surface, **SURF-PTS[Cu(13aneN4)]²⁺** (Scheme 1B) has a static contact angle of $49^\circ(\pm 3)$. SAM thickness is 1.3 nm, determined by ellipsometry (as described in ESI†), comparable with the 1.5 nm length of **PTS[Cu(13aneN4)]²⁺** calculated with PM3 molecular modelling (distance between the Si atom and the most distant C atom of the macrocyclic ring, ESI†), indicating an almost perpendicular disposition of the SAM on the surface. UV-Vis spectra were recorded on quartz surfaces bearing the **PTS[Cu(13aneN4)]²⁺** SAM and corrected by background subtraction.^{14,15} The obtained spectra, Fig. 1B black profile, show the LMCT band at 265 nm. From the absorbance value at λ_{max} (A) by using the molar extinction coefficient found in solution (ϵ) and the relation $n_s (\text{cm}^{-2}) = 6 \times 10^{20} A/2\epsilon$, the number of chromophores per square centimetre (n_s) can be calculated^{14,15} (the factor 2 is needed as slides are coated on both sides). We calculated $n_s = 1.5(\pm 0.7) \times 10^{14} \text{ cm}^{-2}$ (average on 8 samples), that is in the same order of magnitude of what we¹⁴ and others¹⁶ have reported for similar coating procedures with trialkoxysilanes. The Cu cm^{-2} concentration in the SAM was also directly measured by demetallating the coated glass slides with 0.1 M HNO_3 (48 hours) and measuring the total copper with inductively coupled plasma atomic emission spectroscopy (ICP-OES). We found $1.3(\pm 0.1) \times 10^{14}$ Cu atoms cm^{-2} on freshly prepared slides, in agreement with optical methods. In addition, we let functionalized glass slides age in water for 48 hours at $\text{pH} = 7.5$ and measured by ICP-OES the Cu²⁺ released. We found that only $< 8\%$ Cu²⁺ is released from cuvette walls. This may apparently contrast with the instability found for cyclam complexes on porous or mesoporous silica^{11a,b} in water at a similar pH. Although the profoundly different surface nature could also be considered, the increased stability found here is most probably due to the grafting on the surface of pre-complexed macrocycles instead of the void ones. Leaching of cyclam from porous silica was reported to be due to amine-assisted hydrolysis of siloxane bonds^{11a,b,17} that is prevented in our case by using the kinetically inert **PTS[Cu(13aneN4)]²⁺** complex, where amine lone pairs are engaged in Cu²⁺ coordination. FTIR-ATR spectroscopy (ESI†) and Atomic Force Microscope imaging (AFM, showing homogeneous surface, no significant features, ESI†) confirm the nature of **SURF-PTS[Cu(13aneN4)]²⁺**. FTIR-ATR bands in the 1500–1700 cm^{-1} range are identical to those found with FTIR on bulk **PTS[Cu(13aneN4)]²⁺**, excluding urea-urea intermolecular interactions.

Grafting of silver NP was obtained by dipping the **SURF-PTS[Cu(13aneN4)]²⁺** surfaces in a freshly prepared aqueous suspension of citrate-stabilized Ag NP for 24 h at 30 °C. The interaction between the Cu²⁺ centres on the glass surface and the anionic citrate layer of Ag NP may either be purely electrostatic or involve coordination on the Cu²⁺ centre of one of the three carboxylates of the citrate group, as we have already demonstrated that the fifth coordination position of

Cu^{2+} in 13aneN4 is available for anion coordination¹³ (the counter ion of Cu^{2+} is the non-coordinating CF_3SO_3^- , in order to avoid any competition). The obtained coated surface, **SURF-PTS[Cu(13aneN4)]²⁺-NP**, Scheme 1B, has a pale yellow color, due to the LSPR band of the grafted silver NP. With the adopted preparation procedure, the citrate-stabilized Ag NP have a diameter of 7 nm ($\sigma = 4$ nm),⁶ with a LSPR centered at 394 nm in aqueous solutions. When grafted on the **PTS[Cu(13aneN4)]²⁺**-covered surfaces the Ag NP LSPR does not shift significantly (393 nm on dry surfaces, average on 36 slides, $\sigma = 4$ nm, see Fig. 2A for a representative spectrum). Also the FWHM remains substantially unchanged (full width at half maximum, 51 nm, average on 36 slides, $\sigma = 6$ nm). This indicates a not too dense disposition of the Ag NP on the adhesive **SURF-PTS[Cu(13aneN4)]²⁺** surfaces, as in a two-dimensional disposition of noble metal NP, for low $D/2r$ values (D = distance between adjacent particles centers and $2r$ = diameter of the particles) both the LSPR position and its line shape are affected.¹⁸ AFM images, Fig. 2B, confirm this picture, disclosing a monolayer of distanced nanoparticles (see also ESI S7† as regards apparent vs. real NP dimensions in AFM imaging).

ICP-OES analysis further confirms this picture, as we found a concentration of $1.5 \times 10^{-9} \pm 0.2 \times 10^{-9}$ mol cm^{-2} ($1.6 \times 10^{-7} \pm 0.2 \times 10^{-7}$ g cm^{-2}) of Ag. An Ag sphere of 7 nm diameter has a 1.897×10^{-18} g mass. The mass cm^{-2} data from ICP correspond thus to $\sim 8.4 \times 10^{10}$ cm^{-2} Ag NP that fits reasonably well with the counted 290 NP number in the 500 × 500 nm window of Fig. 2B.

Cu cm^{-2} concentration was measured also in the **SURF-PTS[Cu(13aneN4)]²⁺-NP** samples by ICP-OES, obtaining values unchanged with respect to samples pre-NP grafting. The obtained NP SAMs are stable in air. UV-Vis spectra display no change in peak intensity or FWHM. A λ_{max} red shift of 4 nm is observed in one week, due to humidity adsorption.⁶

Cuvettes with **SURF-PTS[Cu(13aneN4)]²⁺-NP** walls were filled with water, stoppered and monitored in a UV-Vis spectrophotometer ($T = 25$ °C) to verify their stability and Ag^+ release in H_2O . At $t = 0$, λ_{max} is 400 ± 1 nm (average on 12 experiments). The small shift with respect to air is due to the obvious change of the environment of the exposed part of NP.⁶ No significant shift of λ_{max} was observed with time, but the band intensity decreased, see Fig. 3 (inset = Abs_{400} vs. time). After 9 days the solution was removed from the cuvette and its spectrum was recorded in a clean cuvette, revealing no LSPR

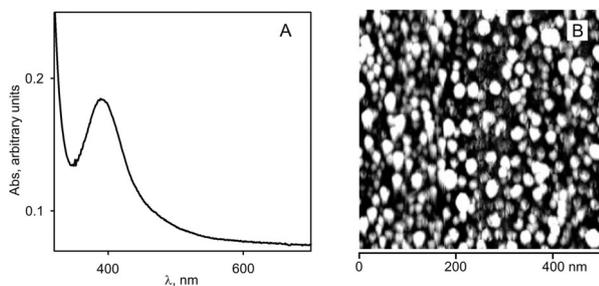


Fig. 2 (A) UV-Vis spectrum of a glass slide modified as **SURF-PTS[Cu(13aneN4)]²⁺-NP** (air exposed); (B) AFM image (500 × 500 nm) of the same slide.

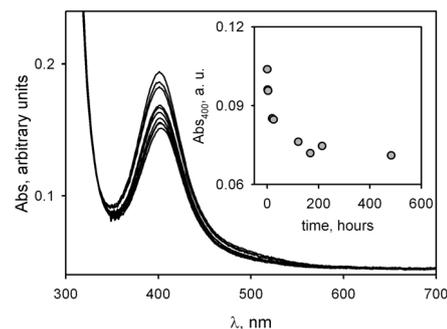


Fig. 3 UV-Vis spectra as a function of time on a cuvette modified on the internal walls as **SURF-PTS[Cu(13aneN4)]²⁺-NP**, filled with water. Inset: variation of absorbance at λ_{max} vs. time (Abs values are from background-subtracted spectra).

signal, *i.e.* no NP release. The Ag and Cu content in the solution was determined by ICP, finding values corresponding to 30.3% Ag release from the surface (average on 3 experiments, $\sigma = 7.5$) and no released Cu. The value is double with respect to what we found for Ag NP grafted on MPTS.⁶ The LSPR vs. time variation has a similar trend with what was observed in the latter case.⁶ The peak changes are due to the formation or the thickening of a Ag_2O layer on the water-exposed surface of Ag NP (LSPR depends also on the oxidation of metal atoms on NP surfaces).¹⁹ The plateau reached after ~ 80 hours is in agreement with the already observed⁶ formation of an oxidized silver layer on the exposed NP surface in an initial 3 days period, reaching a steady-state in which the slowly released Ag^+ ions are replaced by Ag oxidized from the bulk. The same experiments were repeated in PBS (Phosphate Buffered Saline), to mimic the physiological pH and ionic strength. The trend of the absorbance spectra is almost identical to that found in water (see ESI†).

Antibacterial activity tests were run both on glass slides bearing only a **PTS[Cu(13aneN4)]²⁺** SAM and on glass slides further coated with Ag NP. In order to evaluate the efficiency of these materials in preventing the development of local infections, we have used a test developed in our laboratories.⁶ This is capable of measuring the ME (microbicidal effect)²⁰ by simulating real-life conditions of use, *i.e.* it allows the evaluation of the bactericidal effect in a thin liquid film in contact with the surface (experimental setup in ESI†).²¹

S. aureus and *E. coli* were used as commonly considered representative strains for the evaluation of antibacterial activity of drugs,⁴ and for the goal of comparing the results with what we obtained with the same strains on Ag NP SAM grafted on MPTS-modified glasses.⁶ ME effects are summarized in Table 1.

In the tests proposed by EN 13697²² the microbicidal activity of a disinfectant is considered acceptable when ME is at least equal to 4 after 5 min of contact. We checked ME on the 5–24 hours interval, as interested, to materials exerting long-lasting antibacterial effects. ME is not negligible but poor in surfaces coated with the Cu^{2+} complex only. This activity is reasonably related to the removal from the macrocycles and uptake by bacteria of Cu^{2+} (a cation that is well known to have an antibacterial effect).²³ It has to be stressed that the available Cu^{2+} is a very small quantity ($\sim 1.6 \times 10^{-9}$ mol cm^{-2}), and this may be the reason for its limited effect. On the other hand,

Table 1 ME values^a

	<i>S. Aureus</i>		<i>E. Coli</i>	
	5 h	24 h	5 h	24 h
SURF-PTS[Cu(13aneN4)] ²⁺	0.98	1.65	1.02	2.46
SURF-PTS[Cu(13aneN4)] ²⁺ -NP	2.33	6.60	5.29	7.06

^a All values are obtained as an average of 5 experiments. ME = log N_C – log N_E (N_C is the number of CFU ml⁻¹ developed on the unmodified control glasses, N_E being the number of CFU ml⁻¹ counted after exposure to modified glasses; CFU = colony forming unit).

the NP-coated glass slides display a very efficient and long-lasting ME (values at 24 h are greater than those at 5 h, and they also are well above the acceptable threshold of 4). It must be pointed out that ME increases 1–2 orders of magnitude with respect to what we obtained with an Ag NP SAM grafted on MPTS-modified glasses (ME were 5.54 and 5.90 after 24 h, for *S. aureus* and *E. coli*, respectively).⁶ The dramatic ME increase is most likely due to the more abundant Ag⁺ release found in this case, as evidenced by the experiments on SURF-PTS[Cu(13aneN4)]²⁺-NP ageing in water.

To conclude, stable monolayers of Ag NP were obtained on glass and quartz surfaces by the LbL approach, exploiting the efficient interactions of SAM of a Cu²⁺ macrocyclic complex with the citrate anionic layer surrounding Ag NP. The coated materials release more efficiently Ag⁺ with respect to similar surfaces where the NP are grafted on a traditional –SH terminated SAM. This allows a dramatically increased and long lasting effect against *S. aureus* and *E. coli*, candidating SURF-PTS[Cu(13aneN4)]²⁺-NP as new and efficient surface-active bactericidal materials.

Experimental

PTS[Cu(13aneN4)](CF₃SO₃)₂ was prepared from [Cu(13aneN4)]-(CF₃SO₃)₂ and (3-isocyanatopropyl) trimethoxysilane in refluxing acetone under nitrogen (2 h). Solvent removal under reduced pressure yielded PTS[Cu(13aneN4)](CF₃SO₃)₂ quantitatively. Pre-cleaned glass and quartz slides were dipped for 4 h in a 0.1% solution of PTS[Cu(13aneN4)](CF₃SO₃)₂ in ethanol thermostated at 40 °C, then washed 3 times with ethanol in a sonic bath and blow-dried with N₂ to obtain SURF-PTS-[Cu(13aneN4)]²⁺. Modified glass or quartz objects were then immersed into a colloidal suspension of Ag NP⁶ and kept at 30 °C for 18 h to obtain SURF-PTS[Cu(13aneN4)]²⁺-NP. After this, the yellow surfaces were placed in water and sonicated for 5 min. This procedure was repeated twice, then the surfaces were dried by a N₂ stream and stored in air (ESI† for further details, characterization and antibacterial tests).

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