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Magneto-optical nanomaterials: a SPIO–phthalocyanine scaffold built *step-by-step* towards bimodal imaging[†]

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A SPIO-phthalocyanine nanohybrid is developed as a bimodal contrast agent for Optical and Magnetic Resonance Imaging. The organic coating was covalently attached onto SPIO in a *step-by-step* approach. Each coated-SPIO was thoroughly characterized. The hydrodynamic size of the SPIO-Pc is *ca*. 60 nm with a coverage of *ca*. 690 Pc/SPIO.

Bimodal Medical Imaging relying on Magnetic Resonance Imaging (MRI),¹ coupled to Optical Imaging (OI),² may improve the accuracy of a diagnosis³ by combining the sensitivity of OI⁴ and the resolution of MRI.5 Reported hybrid MRI-OI probes bear MRI and OI imaging probes, but their design does not usually address either of the following issues: (a) the difference in sensitivity between the two methods (mM-µM for MRI compared with nM-pM for OI) is not addressed when a fluorophore is bound to one MRI-active metal complex (1:1 ratio),⁴ but is more relevant when the probe (Cy5.5, fluorescein, rhodamine) is immobilized on nanoparticles (NPs) (CLIO, SPIO, Gd-doped NPs), because it contains multiple paramagnetic centers;^{4–6} (b) the *photostability* of fluorophores; (c) a step-by-step construction of reported magneto-optical nanohybrids was not carried out, hence the precise composition of the coating on the nanoparticle may not be accurate enough. Conjugate 1 may overcome these drawbacks: (a) combining Super Paramagnetic Iron Oxide (SPIO; T_2 -weighted MRI contrast agents)^{6,7} and the zinc phthalocyanine fluorophore (ZnPc)⁸ together in a single bimodal contrast agent may better address the difference in sensitivity between MRI and OI (a favorable ratio of one optical probe per a large number of magnetic probes). ZnPc has appealing optical properties:⁸ (i) an absorption band in the 650-800 nm region where light penetration in tissues is the greatest

(*i.e.* the so-called imaging window);² (ii) a fluorescence quantum yield ($\Phi_{\rm F}$ (ZnPc) = 0.3) in the range of known fluorophores,⁹ and a high molar extinction coefficient ($\varepsilon = 10^5 \, {\rm M}^{-1} \, {\rm cm}^{-1}$), resulting in high brightness ($\varepsilon \times \Phi_{\rm F}$), making ZnPc very competitive compared to other near-IR emitting fluorophores.⁹ (b) Moreover, ZnPc is one of the most photostable fluorophores reported to date (degradation quantum yield of 10^{-6} , *i.e.* about 30 times more robust than rhodamine or Cy5).^{6,10} † (c) Our experience in SPIO^{6,11} and in the step-by-step immobilization of functional porphyrin/porphyrinoid models on gold¹² prompted us to investigate a similar strategy for the immobilization of ZnPc onto SPIO (Fig. 1).‡ Each coated-SPIO was thoroughly characterized by an array of analytical techniques, which allowed us to keep track of the precise composition of the coated-SPIO at a given step. We stuck to the highest standards of characterization of hybrid materials.



SPIO nanoparticles were prepared according to the classical Massart method.⁶⁺ The NPs obtained were crystallized in the spinel phase $a = (8.381 \pm 0.002)$ Å. The NPs crystallite size was obtained using X-Ray Diffraction $d_{\text{XRD}} = (9.1 \pm 0.2)$ nm and the mean diameter using Transmission Electron Microscopy $d_{\text{TEM}} = (9 \pm 3)$ nm (on 100 spinel crystallites at least). These NPs are superparamagnetic at room temperature: a blocking temperature of 170 K is measured for the applied field of 100 Oe (Field Cooling, FC, and Zero Field Cooling, ZFC, Fig. 2A).[†] The agglomerate size $d_{\text{TEM}} = (25 \pm 2)$ nm correlates with the hydrodynamic diameter determined using Dynamic Light Scattering (DLS) $d_{\text{DLS}} = (44 \pm 1)$ nm. The BET specific surface area of the powder is $S = (103 \pm 1)$ m² g⁻¹. ζ -Potential measurements (Fig. 2B–A) indicated an Isoelectric Point (IEP) at pH 7.4 and maximum potentials reached 30 mV. FTIR data[†] are consistent with those of previous studies.¹³

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Fig. 1 Step-by-step synthesis. Top and right: SPIO–NH₂, SPIO–N₃; bottom left: Pc 2, and SPIO–Pc conjugate 1.

Free hydroxyl groups (TGA gave 7 OH⁻ nm⁻², 12 μ mol m⁻²) on the surface of bare SPIO NPs were reacted with 3-aminopropyltriethoxysilane (APTES) to afford amine-functionalized SPIO NPs. Both the average size and size distribution of agglomerates are increased when compared to bare SPIO NPs (d_{TEM} = (35 ± 8) nm and $d_{\text{DLS}} = (91 \pm 2)$ nm). The BET specific surface area of the SPIO–NH₂ powder increased to $S = (148 \pm 1) \text{ m}^2 \text{ g}^{-1}$. ζ -Potential measurements (Fig. 2B-B) indicated an IEP at pH 9.8, which shifted higher because of the presence of amine functions at the NP surface and preserving maximum potential values. The elemental analysis suggests about 5 NH₂ per nm² (8.3 μ mol m⁻²) on SPIO-NH₂. XPS measurements showed that the C 1s peak decomposes into C-C/C-H, C-N and O-C=O contributions and that the N 1s peak only corresponds to the N-C contribution.[†] The latter are correlated by the FTIR measurements[†] that exhibit the characteristic band of secondary amines and protonated amine bands. SPIO-NH₂ nanohybrids were subsequently coupled with azide/carboxyl terminated-PEG chains by EDC coupling, to afford azido-functionalized SPIO (SPIO-N₃). The observed size and dispersion state are given by TEM observations: the agglomerate sizes are $d_{\text{TEM}} = (73 \pm 25) \text{ nm.}^{\dagger}$

The SPIO-N₃ NPs were characterized using FTIR spectroscopy, and the spectra exhibited a characteristic azide stretch at 2100 cm⁻¹ (Fig. 2C–C). ζ -Potential measurements (Fig. 2B–C) indicated an IEP shifted down to pH 8.4, along with a 10 mV decrease in the maximum potential. These observations are consistent with the modification of the NP surface by introducing azide groups (IEP variation) at the end of short ethylene oxide chains (slight shielding effect). The XPS measurements are in correlation with other characterizations: the N 1s peak corresponds to the N–C contribution; the C 1s peak decomposes into three different contributions, C–N and C–C/C–H components are in equal proportions meaning that the C–N contribution increased because of the presence of the azide groups on the particle surface.

Phthalocyanine 2 was synthesized according to the synthetic pathway described in Fig. 1 and ESI.[†] Briefly, the mixed condensation of compound 3 with phthalonitrile in the presence of a zinc salt led to a statistical mixture containing the alkynebearing A_3B -ZnPc 2 and the click-unreactive A_4 -ZnPc counterpart. This mixture was purified by a series of washings, leading to the removal of the non-phthalocyanine material.[‡]

As for SPIO-NH₂ and SPIO-N₃, the working hypothesis was to ensure a successful coupling and to supply a proof of concept, i.e. to achieve a significant coverage in Pc to reliably characterize SPIO-Pc 1. Cu-catalyzed Azide-Alkyne Coupling (CuAAC)¹⁴ between the alkyne-containing species 2 and the azide-containing SPIO-N₃ was achieved in the presence of a Cu salt and sodium ascorbate to afford conjugate 1.[‡] To ensure that no material was adsorbed a series of thorough washing-filtration was performed by resuspending the SPIO-Pc material in THF, magnetically precipitating down the adsorbents, removing the supernatant and ultrafiltering the mixture. The observed agglomerate mean size is about d_{TEM} = (56 ± 5) nm (Fig. 2D).[‡] The agglomerate size of conjugate **1** is in agreement with the DLS agglomerate size which is known to be less than 100 nm according to the biomedical application for which it was designed.¹⁵ Commercial contrast agents are indeed a compromise between rapid elimination in kidneys (monodisperse state) or no cell incorporation (very large agglomerates). ζ-Potential measurements indicated (a) an IEP around pH 8.1 close to that of the previous functionalization stage (SPIO-N₃), and (b) a distinct shielding effect due to the high proportion of grafted ZnPc. This is consistent with the Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) analysis that indicates a significant Zn: Fe ratio of about 1:24. However, all azide groups were not reacted with alkynylphthalocyanine 2. Given the characterization data, our calculation indicates about 2 ZnPc per nm² (3.3 μ mol m⁻²); i.e. ca. 690 ZnPc per NP (ca. 15700 Fe atoms).⁺ This corresponds to about 40% coverage when compared to SPIO-NH₂. The ZnPc steric bulk could be at stake here leading to non-quantitative coverage of the SPIO-NH2 NP surface. XPS analysis showed that the C 1s peak decomposes into three different contributions[†] and the N 1s peak



Fig. 2 Characterization of SPIO–Pc and precursors: (A) FC and ZFC measurements between 5 and 320 K; (B) ζ-potential and (C) FTIR (both labeled A: SPIO–OH, B: SPIO–NH₂; C: SPIO–N₃; D: SPIO–Pc); (D) SPIO–Pc TEM; (E) SPIO–Pc XPS contributions of nitrogen 1s; (F) SPIO–Pc fluorescence.

is shared into two contributions (Fig. 2E). The appearance of a new N 1s contribution at 397 eV is thus attributed to the presence of ZnPc (NznPc-C contribution). The C-C/C-H component also increased because of the presence of the ZnPc aromatics. FTIR (Fig. 2C-D) spectra showed the disappearance of the azide stretch $(\nu_{as} N_3)$ at 2100 cm⁻¹, which indicates that the reaction with the alkyne occurred, leading to the formation of a triazole linker. UV-Visible spectroscopy in ethanol⁺ showed the characteristic ZnPc absorption bands: Q bands (667, 640 and 603 nm) and the Soret band (344 nm). At high surface coverage in 2, the SPIO-Pc spectrum in ethanol is still comparable to that of the monomeric ZnPc with no significant shift indicating that no π -stacking occurred; hence, the optical properties of immobilized (1) and free (2) A₃B-ZnPc are comparable to each other and to that of the parent A₄-ZnPc species.‡ The relevant indicator of the step-by-step nanoparticle-coating achievement is the surface coverage rates that are expressed as a function of the available surface groups in the previous step. At the different stages of conjugate 1 elaboration, coverage rates were determined to be: (a) 7 OH⁻ per nm² (12 μ mol m⁻²) on initial bare SPIO; (b) 5 NH₂ per nm² (8.3 μ mol m⁻²) on SPIO–NH₂ (71% yield); (c) 5 N_3 per nm² (8.3 µmol m⁻²) on SPIO-N₃ (quantitative yield assuming that all amines were acylated);[†] and (d) 2 ZnPc per nm² $(3.3 \ \mu mol \ m^{-2})$ on SPIO-Pc (40% yield). Therefore, the overall yield is ca. 30% grafting on bare SPIO NPs. This was achieved because of the low steric hindrance of neighboring ZnPc and the positioning of the short PEG chains (9 ethylene oxide units) that probably do not imbed the azide function inside the organic layer.

Subsequent spectroscopic studies were performed to address the imaging capabilities of each probe in the well characterized SPIO-Pc conjugate 1: fluorescence (ZnPc probe for OI) and relaxivity (SPIO probe for MRI) measurements. Upon excitation of 1 at 600 nm in ethanol solution, a fluorescence emission (Fig. 2F) at 673 nm was observed, which is comparable to the fluorescence emission of the parent free ZnPc 2. The results seem to indicate that there is no Aggregation Caused Quenching (ACQ) due to π -stacking and Förster Resonance Energy Transfer (FRET) quenching in the organic solvent. The relaxivity measurements led to R_2 and R_1 values of 73 mM_{Fe}⁻¹ s⁻¹ and 3 mM_{Fe}⁻¹ s⁻¹, respectively $(R_2/R_1 = 24)$.⁺ These values are reminiscent of currently available T_2 contrast agents.¹⁶ The thickness of the organic coating on the SPIO core is known to affect the R_2 relaxivity, and the outer "hydrophobic phthalocyanine belt" may prevent, to a certain extent, the access of water molecules to the inner superparamagnetic iron oxide core of 1.17 In addition magnetic measurements showed that SPIO-Pc remain superparamagnetic and their blocking temperature decreases to 105 K indicating the decrease in dipolar magnetic interactions due to organic shells (Fig. 2A). Hence the hydrophilic nature of 1 may be increased upon tuning down the Pc coverage rate (or reacting the remaining azides with alkyne-PEG). Finally, conjugate 1 may be considered as a putative theranostic agent (*i.e.* a species that can be used to perform imaging and therapy simultaneously)^{12,18,19}[†] because it could be used in hyperthermia (SPIO)^{20,21} and photodynamic therapy (ZnPc).^{8,22}

In summary, SPIO NPs were successfully functionalized three consecutive times (a) by setting a higher proportion of magnetic probes with respect to the optical ones to address the difference in sensitivity; (b) by using zinc phthalocyanines as actual photostable fluorophores;[†] and (c) by proceeding in a step-by-step manner using classical reactions of organic synthesis (silanization, acylation, cycloaddition) with the following functional groups or molecules found at the SPIO surface: hydroxyl groups (bare SPIOs), amine groups (first stage coating), azide groups (second stage coating), and phthalocyanines (third stage coating), respectively. These reactions proceeded smoothly and each SPIO end-product was well identified and thoroughly characterized by an extensive number of spectroscopic techniques. Based on preliminary data (fluorescence and relaxivity measurements), we are confident on the relevant nature of **1**.

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Notes and references

[‡] Pc groups have been immobilized on other NPs for other applications in a *direct* (as opposed to *step-by-step*) approach.^{23,24} The remaining A₄-ZnPc coproduct that does not bear the alkyne moiety could not be clicked, hence it was easily washed away after the coupling reaction with SPIO NPs. A mixture of ZnPc and SPIO NPs treated without the Cu catalyst did not lead to the SPIO-Pc conjugate. Hence, this indicates that these spectroscopic observations such as UV-Vis do not reflect an artifact, *i.e.* adsorption of ZnPc onto NP, but a covalent linkage instead.

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