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1 Porphyrin-xylan-coated silica nanoparticles for anticancer photodynamic therapy

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12 Highlights

- Xylan-porphyrin-coated silica nanoparticles (PX SNPs) have been synthesized.
- Valorization of hemicellulose as a drug carrier for photodynamic therapy.
- Formed nanoparticles show high anticancer activity against colorectal cancer cell
 lines.

17 Abstract

Porphyrins are widely used in anticancer photodynamic therapy (PDT). However, low 18 19 physiological solubility and lack of selectivity towards cancer cells are the main limitations of 20 their clinical use. Nanoparticles are being intensively explored as photosensitizer carriers for 21 PDT to overcome these limitations. The aims of this work are to synthesize core-shell hybrid 22 nanoparticles formed by a silica core and xylan carrying a 5-(4-hydroxyphenyl)-10,15,20-23 triphenylporphyrin (TPPOH) shell, and evaluate their anticancer activity. To afford drug-24 controlled incorporation and enhance blood circulation, TPPOH was covalently linked to 25 xylan. Different xylans with degrees of substitution in TPPOH ranging from 0.034 to 1.11, 26 were obtained and characterized. Then, the xylan-TPPOH conjugate (PX) was used to coat 27 the silica nanoparticles (PX SNPs). The obtained nano-objects were characterized and their 28 therapeutic potential for photodynamic therapy evaluated against colorectal cancer cell lines.

- 29 In vitro analysis showed that PX SNPs were 40-fold and 10-fold more effective against
- 30 HCT116 cells and HT-29 cells respectively compared to free TPPOH.
- Keywords : xylan; polysaccharides; silica nanoparticles; photodynamic therapy; colorectal
 cancer.
- 33

1. Introduction

Being the second leading cause of death in the world, after cardiovascular pathologies, cancer is the most dreaded disease of our time (Nagai & Kim, 2017). It consists of the anarchic proliferation of abnormal cells which escape from normal control mechanisms. Several options are available for treating cancer; the most classic are surgery, chemotherapy and radiotherapy (Saini, Chouhan, Bagri, & Bajpai, 2012). These treatments are invasive and lack selectivity toward tumor cells. To better target cancer cells and minimize adverse effects, new or complementary treatments have emerged over the past few years.

42 Photodynamic therapy (PDT) is an alternative and non-invasive cancer treatment requiring the 43 simultaneous presence of three elements: a photosensitive molecule, a light source and 44 molecular oxygen (Kwiatkowski et al., 2018). PDT usually involves intravenous 45 administration of a photosensitizer (PS) and subsequent specific-wavelength irradiation of the 46 tumor tissue. When irradiated, the PS passes from the ground state to the excited singlet state. 47 The excited photosensitizer is very unstable and loses its excess energy by, either non-48 radiative (heat emission) or radiative (fluorescence emission) pathways. Another process can 49 occur, namely intersystem crossing, to form a more stable excited triplet state with parallel 50 spins. In this case, return to the ground state, theoretically forbidden transition, is very slow. 51 PS can be deactivated by phosphorescence or by reacting with its environment according to 52 two types of mechanisms. The Type I mechanism involves electron transfer from 53 photosensitizer to biological substrates to form radicals and radical ions which, after 54 interaction with oxygen, provide reactive oxygen species (ROS) such as the superoxide radical anion $(O_2^{\bullet-})$. A Type II photochemical process consists of a triplet-triplet energy 55 transfer from PS to molecular oxygen in its ground state (triplet state). This energy transfer 56 leads to the formation of singlet oxygen ${}^{1}O_{2}$ and thus regenerates the ground-state 57 58 photosensitizer. All these species are highly reactive and are very powerful oxidants that can 59 damage cancer cells and lead to their death (Bonnett, 1995; Henderson & Dougherty, 1992). 60 Tetrapyrrole compounds such as porphyrins, chlorins, and bacteriochlorins are the most 61 utilized photosensitizers in PDT (Abrahamse & Hamblin, 2016; Sternberg, Dolphin, & 62 Brückner, 1998). However, these hydrophobic molecules are sparingly soluble in biological 63 fluids and they suffer from poor selectivity towards tumor cells which restricts their use in 64 clinical protocols.

65 Several approaches have been developed to improve PS bioavailability and efficacy in PDT.
66 One of the most promising is the attachment of PS to nanoparticles (NPs) (Kydd et al.,2017)

which not only enhances the solubility of hydrophobic photosensitizers but also promotes their deposition inside tumors, due to so-called passive targeting. Indeed, leaky vasculature and the resulting aberrant architecture of solid tumors leads to preferential nanoparticle accumulation in tumors which remain there because of poor lymphatic drainage. This phenomenon, known as Enhanced Permeability and Retention (EPR) effect, was first described by Matsumura and Maeda (Matsumura & Maeda, 1986; Maeda & Matsumura, 1989; Maeda, 2001)[.]

74 Various NPs have been used as delivery systems for water-insoluble drugs (Debele, Peng, & 75 Tsai, 2015), and especially silica nanoparticles (SNPs), which have been recognized as 76 promising vectors for PDT applications (Bharathiraja et al., 2017; Brezániová et al., 2018; 77 Roy et al., 2003; Yan & Kopelman, 2003; Yan et al., 2018;). In the last two decades, SNPs 78 have gained an ever-increasing interest for medical applications because of a large number of 79 qualities, including biocompatibility, stability, high specific surface area, ease of synthesis 80 with controllable size, shape and surface charge, along with their ease of functionalization 81 with a number of varied materials (Tamba et al., 2015).

82 A major obstacle to controlled drug delivery is the mononuclear phagocytic system (MPS) 83 which is responsible for the premature removal of drug carriers from the body through 84 opsonization (Frank & Fries, 1991; Gref et al., 1994). One solution proposed to slow 85 opsonization and increase nanoparticle half-lives in the bloodstream by coating them with 86 hydrophilic groups via adsorption or covalent grafting (Lemarchand, Gref, & Couvreur, 87 2004). These groups generally consist of long chain polymers such as polyethylene glycol 88 (PEG) (Mozar & Chowdhury, 2018; Peracchia et al., 1999), or polysaccharides such as 89 chitosan (Zhu et al., 2009) and dextran (Coombes et al., 1997; Mbakidi et al., 2013). These 90 polymers create a hydrophilic protective layer around the nanoparticles which prevents the 91 binding of opsonins via steric repulsion forces, thereby delaying opsonization and 92 phagocytosis (Owens & Peppas, 2006).

Xylan is a natural, biodegradable, and non-toxic biomaterial. It is the main hemicellulose component of secondary cell walls constituting approximately 20–30% of the biomass of dicotyl plants (hardwoods and herbaceous plants). In hardwood, 4-*O*-methylglucuronoxylan (MGX) is the most abundant hemicellulose. Its structure consists of a β (1-4)-D-xylopyranosyl backbone with 4-*O*-methyl-D-glucuronic acid (MeGlcA) side chains attached at the 2 position of the xylose units (Ebringerová & Hromádková, 2005). The degree of substitution in MeGlcA depends on the forest species and varies between 3.7 and 12 xylose units for one 4-

100 O-methylglucuronic acid unit in hardwoods. Xylan obtained from corn cobs has been 101 reported to exhibit antioxidant, anticoagulant, antimicrobial and antiproliferative properties. 102 Corn cob xylan has been shown to exert an antiproliferative effect against HeLa cell lines in a 103 dose-dependent manner (Melo-Silveira et al., 2012). In another study, chestnut tree MGX was 104 found to inhibit proliferation of A431 human squamous carcinoma cells, as well as migration 105 and invasion by inhibiting the expression of metalloproteinases MMP2 and MMP9, which are 106 involved in the degradation of the cell basement membrane and subsequent migration through 107 extracellular matrices (Moine et al., 2007). In drug delivery, esterification of beech wood 108 xylan via activation of the carboxylic acid with N, N-carbonyldiimidazole has been performed 109 to produce prodrugs for the controlled release of ibuprofen (Daus & Heinze, 2010). The 110 surface modification of magnetic nanoparticles (MNPs) with xylan has also been reported to 111 stabilize MNPs in biological media and been shown to improve their biocompatibility and 112 biodistribution (Ma et al., 2018).

The functionalization of xylan by porphyrins has never been reported in the literature. We hypothesize that it should be possible to condense porphyrins on xylan by esterification and to control the degree of substitution through the implementation of a well-chosen experimental design. We assume that it must be possible to obtain nano-objects with high porphyrin content through surface coating of SNPs with PX conjugate. We also assume that the anticancer activity of these nanoparticles will be higher than that of free TPPOH.

119 In adequation with our research program on polysaccharide modifications with 120 photosensitizers for PDT applications (Drogat et al., 2012; Ringot et al., 2018), and in order to 121 develop new photosensitizer carriers for PDT applications, we prepared and characterized 122 core-shell hybrid nanoparticles based on xylan for the targeted delivery of 5-(4-123 hydroxyphenyl)-10,15,20-triphenylporphyrin, a second-generation photosensitizer that is 124 relatively easy to synthesize and exhibits a singlet oxygen quantum yield of $\Phi\Delta$ =0.57 125 (Managa et al., 2016). TPPOH was covalently linked to xylan via an esterification reaction 126 and then this conjugate was attached to SNPs through ionic bonds. The *in vitro* cytotoxicity 127 and photodynamic efficiency of these nanomaterials were determined against HCT116 and 128 HT-29 colorectal cancer cell lines.

129 **2.** Materials and methods

130 2.1. Materials

131 For a list of materials, see Supplementary Information (SI).

132 2.2. Analytical methods

133 FTIR analyses were performed on a Perkin Elmer FT-IR Spectrum 1000 spectrometer using 134 KBr pellets (1-2 wt%). NMR analyses were carried out on a Bruker DPX 500 NMR 135 spectrometer, operating at 500 MHz. UV-vis spectra were recorded on a double beam 136 spectrophotometer AnalytikaJena SPECORD 210, using 10 mm quartz cells. High resolution 137 electrospray ionization mass spectrometry (HR ESI-MS) was performed at the ICOA/CBM 138 platform (Orléans University) on a Bruker Q-TOF maXis mass spectrometer, coupled to an 139 Ultimate 3000 RSLC chain (Dionex). Purifications were performed with Combiflash Rf 100® 140 from Teledyne Isco. The stationary phase consisted of an 80 g silica column. The products to 141 be purified were solubilized in a minimum amount of solvent and fixed on Florisil (60-100 142 mesh, VWR). For Transmission Electron Microscopy (TEM) studies, one droplet of 143 nanoparticle suspension was lyophilized on a copper grid. Images were taken with a JEOL 144 2010 UHR instrument. Particle size distribution and zeta potential were analyzed through 145 dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern, UK). SNPs suspension 146 was diluted in absolute ethanol and analyzed at 20 °C at a scattering angle of 173°. The mean 147 diameter of SNPs was expressed as the average value of two measurements, each one 148 comprising 15 runs. Zeta potential data was collected through electrophoretic light scattering 149 at 20 °C, 150 V using this same instrument. Each zeta potential value is the average of two 150 measurements, each one comprised of 100 single runs. Red light (630-660nm, 75 J/cm2) was 151 delivered from the light source PhotoCureTM Lamp CURElight (PhotoCure ASA, Oslo, 152 Norway). Cell morphology was analyzed by an electron microscope JEOL JEM-1011 153 operated at 80 KeV (JEOL, Croissy-sur-Seine, France).

154 2.3. Porphyrin synthesis

155

2.3.1. [3-ethoxycarbonylpropyloxy] phenyl) -10,15,20-triphenylporphyrin (2)

156 5-(4-hydroxyphenyl)-10,15,20-triphenylporphyrin (1) was synthesized according to the Little 157 method (Little, Anton, Loach, & Ibers, 1975; Boscencu, Licsandru, Socoteanu, Oliveira, & 158 Ferreira, 2007). Compound 1 (0.2 g, 1 equiv, 0.32 mmol) was dissolved in 15 mL of DMF 159 with excess K_2CO_3 (25 equiv, 1.09 g, 7.92 mmol) and stirred for 5 min at room temperature. 160 Then 0.43 g of ethyl 4-bromobutyrate (7 equiv, 2.22 mmol) were added. The reaction was 161 carried out using microwave irradiation at 120 °C and 300 W for 7 min and monitored by 162 thin-layer chromatography (TLC). After evaporation of DMF under vacuum, the crude 163 product was dissolved in chloroform (15 mL). The organic layer was washed with water (3 \times

164 30 mL), dried (MgSO₄) and then evaporated to give, after purification by flash 165 chromatography on a silica column, (eluent: petroleum ether/CHCl₃ from 80 to 100%) a pure porphyrin 2 with 91% yield. Rf= 0.44 (CHCl₃), ¹H NMR (CDCl₃, 500.15 MHz) δ H, ppm : -166 167 2.74 (s, 2H, NH pyrrole), 1.32 (t, 3H, J = 7.5 Hz, CH₃ ethyl), 2.27 (q, 2H, J= 6.5 Hz, H β -168 CH₂-), 2.66 (t, 2H, J = 7.5 Hz, -CH₂-C=O), 4.22 (q, 2H, J= 7 Hz, CH₂ ethyl), 4.25 (t, 2H, J= 169 6 Hz, -O-CH₂-), 7.22 (d, 2H, J= 8.5 Hz, H3,5 aryl), 7.72 (m, 9H, H3,4,5 phenyl), 8.10 (d, 2H, 170 J = 8.5 Hz, H2,6 aryl), 8.20 (d, 6H, J= 7.5 Hz, H2,6 phenyl), 8.83 (s, 8H, H β pyrrole). ¹³C 171 NMR (CDCl₃, 500.15 MHz) δc, ppm : 14.31 (CH₃ ethyl) ; 24.87 (-CH₂-) ; 30.99 (CH₂-C=O) ; 172 60.52 (CH₂ ethyl); 67.03 (O-CH₂); 112.72 (C-3.5 aryl); 119.96-120.07 (C meso); 126.68-173 127.67 (C phenyl); 130.88-131.13 (Cβ pyrrole); 134.54-134.59 (C-2,6 aryl); 135.59 (C-1 aryl) 174 ; 142.19-142.22(Cα pyrrole); 158.70 (C-4 aryl); 173.30 (C=O). UV-Visible : UV-Vis $(CHCl_3) \lambda \max \min (\epsilon, 10^3 \text{ L.mol}^{-1}, \text{cm}^{-1}) : 420 (609), 519 (21), 553 (10), 591 (6), 649 (5). \text{ MS}$ 175 176 (ESI): $m/z = 745.3173 [M+H]^+$.

177

2.3.2. 5-(2-[3-carboxypropyloxy] phenyl) -10,15,20-triphenylporphyrin (3)

178 Compound 2 (0.3 g, 1 equiv, 0.4 mmol) was dissolved in 15 mL DMF and then 0.16 g (10 179 equiv, 4 mmol) NaOH previously dissolved in 2 mL of distilled water, was added. The 180 reaction was allowed to proceed under stirring for 2 h at 100 °C. After solvent evaporation, 181 the crude product was dissolved in chloroform (15 mL) and neutralized with 287.5 µL 182 hydrochloric acid (HCl) (37%, 12 M), washed with distilled water (3x30 mL), dried over 183 $MgSO_4$, and evaporated. Then, the crude product was purified by flash chromatography to 184 give TPPOH (compound 3) as a purple solid (yield 96%) (eluent: CHCl₃/Ethanol from 100 to 185 90%). Rf= 0.6 (EtOH/CHCl₃ 1/9), ¹H NMR (CDCl₃+10% TFA, 500.15 MHz) δ H, ppm: 2.39 186 (q, 2H, -CH₂-, J= 8Hz), 2.86 (t, 2H, CH₂-C=O, J = 6.5Hz), 2.48 (t, 2H, CH₂-O, J= 6Hz), 7.56 187 (d, 2H, H3,5 aryl, J= 7.7Hz), 8.02 (m, 9H, H3,4,5 phenyl), 8.53 (m, 8H, H2,6 phenyl, H2,6 aryl), 8.7 (s, 8H, Hβ pyrrole). ¹³C NMR (CDCl₃, 500.15 MHz) δc, ppm : 24.6 (-CH₂-) ; 30.9 188 189 (CH₂-C=O); 67.1 (O-CH₂); 115.09 (C-3,5 aryl); 122.44-123.98 (C meso); 128.79-129.55 (C 190 phenyl); 130.79-133.02 (C βpyrrole); 139.2-138.3 (C-2,6 aryl); 140.3 (C1 aryl); 146.46-191 145.17(C apyrrole) ; 161.1 (C-4 aryl) ; 181 (C=O). UV-Vis (CHCl₃) λ max nm (ϵ , 10³ 192 L.mol⁻¹.cm⁻¹): 420 (451), 516 (21), 553 (10), 591 (6), 647 (5). MS (ESI): m/z = 717.286193 $[M+H]^{+}$.

194

196 2.4. Synthesis of PX

197 3.4.1. Solubilization of xylan

198 Xylan (1g) was dissolved in 20 mL DMSO (60 °C) and filtered to remove the insoluble 199 fraction. To determine the concentration of this solution, 1 mL was precipitated with 3 mL 200 absolute ethanol, filtered and dried under vacuum. The mass of precipitated xylan was 44.5 201 mg, therefore the xylan solution in DMSO had a concentration of 44.5 mg/mL (absence of 202 polysaccharide in the filtrate was verified by TLC.

203 3.4.2. TPPOH grafting to xylan

All syntheses were carried out following the same protocol. TPPOH was added to 1 equivalent of N, N-carbonyldiimidazole (CDI) and stirred in DMSO at room temperature for 24 h. This solution was then added to a solution of 100 mg (0.76 mmol anhydroxylose unit) xylan in DMSO and allowed to react under stirring. Reaction parameters are summarized in Table S1 (SI). The product was precipitated out in absolute ethanol and was washed three times with ethanol and three times with chloroform to remove unreacted starting material and then dried under vacuum.

211 2.5. Synthesis and surface modification of SNPs

3 mL aqueous ammonia (28%) and 1.5 mL tetraethoxysilane (TEOS) were successively added dropwise to 45 mL absolute ethanol. This solution was stirred for 4 h at 48 °C. Resulting SNPs were collected by centrifugation (8000 rpm, 30 min), washed three times with ethanol and then dispersed in 25 mL 96% ethanol. Then, 4 mL (3-aminopropyl) triethoxysilane (APTES) was added and stirred overnight. After three cycles of centrifugation/redispersion in ethanol, SNPs were obtained as a white solid and were stored in 25 mL of absolute ethanol.

219 2.6. Grafting of PX onto surface-modified SNPs

PX (10 mg) was dissolved in 20 mL distilled water. Then 100 mg SNPs in ethanol were added dropwise and ultrasonicated during the addition. This mixture was then stirred for 20 min. The resulting PX SNPs were centrifuged and washed three times with water and absolute ethanol.

224

226 2.7. Determination of TPPOH concentration in SNPs

The amount of TPPOH attached to the SNPs was determined by UV-visible assay. PX SNPs were dispersed in absolute ethanol at a concentration of 10 mg/mL and diluted to 0.5 mg/mL. Absorbance of this sample was measured at 418 nm, and absorbance of SNPs was subtracted. The PX content of SNPs was calculated from a calibration curve constructed with different concentrations of free PX in distilled water. The concentration of TPPOH in mol/L was calculated according to Eq. (1):

$$C = \frac{DSxM(PX)}{M(repeat unit of xylan) + DSx(M(TPPOH) - 18)}$$
(1)

233 M(PX) = concentration of PX in g/L attached to SNPs, calculated from the standard 234 calibration curve

- 235 M (repeat unit of xylan: xyl/MeGlcA 10:1) = 1511 g/mol
- 236 M (TPPOH)= 717.286 g/mol

237 2.8. In vitro experiments

238 2.8.1. Cell lines, cell culture, and PX SNPs

For all experiments, cells were seeded at 1.2×10^4 cells/cm² and 2.08×10^4 cells/cm² for HCT116 and HT-29 cells, respectively and were grown for 24 h in culture medium prior to exposure, or lack of, to TPPOH or PX SNPs at indicated concentrations. Stock solutions of TPPOH (5 mg/mL) and PX SNPs 80 nm (20 mg/mL) were prepared in ethanol.

243 2.8.2. PX SNPs toxicity tests

244 determined **PX-SNPs** cytotoxicity was using 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) assays. Human colorectal cancer cells were seeded at 245 $4x10^3$ cells/well and $7x10^3$ cells/well for HCT116 and HT-29 cells, respectively, in 96-well 246 247 culture plates and grown for 24 h in culture medium prior to exposure, or lack of, to TPPOH 248 or PX SNPs. Stock solutions of TPPOH and PX SNPs were diluted in culture medium to 249 obtain the appropriate final concentrations. The same amount of vehicle (percentage of 250 ethanol did not exceed 0.6%) was added to control cells. After 24 h, culture medium was 251 replaced by phenol red-free culture medium and cells were irradiated, or not, by red light 252 (630-660 nm, 75 J/cm²). MTT assays were done 48 h after irradiation and experiments were 253 performed in triplicate.

254 2.8.3. Transmission electron microscopy (TEM)

HCT116 cells $(3x10^5)$ were seeded in 25 cm² tissue culture flasks and grown for 24 h in 255 culture medium prior to exposure or not to PX SNPs. After 24 h, culture medium was 256 257 replaced by phenol red-free culture medium and cells were irradiated, or not, by red light (75 258 J/cm²). Then, cells were fixed in 2.5% glutaraldehyde for 30 min at room temperature and 259 washed in phosphate buffer for 15 min. They were then incubated in 1% osmium tetroxide 260 solution for 30 min at room temperature, dehydrated with increasing ethanol concentrations, 261 and embedded in epon. They were polymerized over 48 h at 60 °C and ultrathin sections (80-262 100 nm) were prepared. Grids were stained with uranyl acetate and lead citrate and examined 263 with TEM operated at 80 KeV.

264 2.8.4. Statistical analysis

265 Data are expressed as the arithmetic means \pm standard error of the mean (SEM) of separate 266 experiments. The statistical significance of results obtained from *in vitro* studies was 267 evaluated by the two tailed unpaired Student's t-test, with p<0.05 being considered as 268 significant.

269 **3. Results and discussion**

Preparation of PX SNPs is summarized in Fig.1. TPPOH was conjugated with xylan via an esterification reaction forming PX which was used in the surface modification of SNPs. The presence of glucuronic acid groups on xylan allows the formation of ionic bonds on the surface of SNPs which is made cationic by APTES.



275 Fig. 1. General procedure for the synthesis of PX SNPs.

277 3.1. Characterization of xylan

278 The xylan used in this study was characterized by the classical methods already described in 279 the literature (Kerrouche et al. 2018). It had an average polymerization degree of 208 280 calculated as the ratio of amount of total sugars to the quantity of reducing sugars. The 281 composition was characterized by colorimetric assay and contained 92.5% xylose and 7.5% 282 glucuronic acids. ¹H NMR spectrum of this xylan (Fig. S1, SI) showed the signal 283 corresponding to anomeric protons of substituted xylose units at 4.5 ppm, non-substituted 284 xylose units at 4.6 ppm and anomeric protons of uronic acid at 5.3 ppm. The degree of 285 substitution of xylan by 4-O-methylglucuronic acid, which was about 1/10, was calculated 286 according to Eq. (A.1, SI).

287

288 3.2. PX synthesis

289 The synthesis route of PX is summarized in Fig. 2. First, 5-(4-hydroxyphenyl)-10,15,20-290 triphenylporphyrin (1) was synthesized according to the Little method and obtained with a 291 3.7% yield in agreement with the literature. It was then functionalized with ethyl 4-292 bromobutyrate through nucleophilic substitution, activated by microwave irradiation 293 according to Chaleix et al. (2009). Product 2 was obtained with 91% yield. After saponification with sodium hydroxide, porphyrin derivative 3 was obtained with 96% yield. 294 The structures of all compounds were confirmed by ¹H, ¹³C NMR and mass spectrometry. 295 296 Compound 3 was then covalently attached to xylan by esterification using CDI as the 297 coupling reagent.



298

- Fig. 2. Synthesis of PX. *i*: ethyl 4-bromobutyrate, K₂CO₃, DMF, MW; *ii*: NaOH, DMF/Water, HCl (0.1M); *iii*:
- 300 Xylan, DMSO, CDI, t(h), $T(^{\circ}C)$.

301 The covalent binding between xylan and TPPOH was confirmed by FTIR. The corresponding

302 FTIR spectrum of PX is displayed in Fig. 3 (c). This compound and native xylan (Fig. 3 b),

303 shared a characteristic absorption band around 3400 cm⁻¹, which has been assigned to the stretching vibrations of hydroxyl groups of xylan. There were two other bands at 2920 cm⁻¹ 304 and 1441 cm⁻¹ that represent the stretching and deformation vibrations of C-H bond. PX also 305 displayed new signals that correspond to the TPPOH moiety (1245, 1178, 796, 730 cm⁻¹) and 306 a peak at 1739 cm⁻¹ that corresponds to the carbonyl functionality of TPPOH. The ¹H NMR 307 spectrum of modified xylan (Fig. 4) identified protons of the xylose units between 3 and 5.5 308 309 ppm, and the peripheral protons of TPPOH between 7 and 9 ppm. The broad singlet at -2.9 310 ppm was assigned to the NH internal protons of the porphyrin pyrrole units, which is in agreement with classical ¹H NMR spectra of porphyrins. 311



312

313 Fig. 3. FTIR Spectra of TPPOH (a), native xylan (b), and PX (c).

- 314
- 315



317 Fig. 4. ¹H NMR spectra of native xylan (a), and PX (b) in DMSO (DS=0.2).

318 3.3. Experimental design

319 Esterification reactions are mainly influenced by reaction time, temperature and molar ratio of 320 reagents. The efficiency of this esterification reaction was expressed by the degree of 321 substitution and weight yield. In order to determine optimal conditions and study the 322 variations of the degree of substitution (DS) and weight yield as a function of temperature, 323 reaction time and TPPOH equivalents, an experimental design was built. The choice of 324 factors and their modes are listed in Table S2 (SI). For 3 factors, 27 experiments needed to be 325 performed to explore the entire experimental field. In order to reduce the number of tests and 326 still keep the possibility of studying all factors, a latin square design of experiments was 327 constructed. In this way only nine experiments were performed, applying the experimental 328 conditions summarized in Table S1 (SI). For the nine samples, DS and weight yield were 329 measured and the obtained values listed in Table S3 (SI).

331 DS was expressed as the number of TPPOH molecules per xylan repeat unit. The DS value
 332 was calculated from ¹H NMR data according to Eq. (2):

$$DS = \frac{H(TPPOH)/27}{H1(xylan)/10}$$
(2)

333 Where H(TPPOH) is the integral area of TPPOH protons at δ 7.26-9.12 ppm, and H1(xylan) 334 is the integral area of anomeric protons of substituted and non-substituted xylose units at δ 335 4.12-4.7 ppm. To determine the effect of each factor on the degree of substitution in TPPOH, 336 independently from the other factors, an average DS for each modality was calculated. Then, 337 variation of the mean DS according to the modalities of the three factors was examined. As 338 shown in Fig. 5, at all temperature and reaction times, an increase in TPPOH equivalents led 339 to an increase in DS. Reaction time had no significant effect on DS beyond 24 hours. 340 Furthermore, temperature increases over 50°C resulted in a drop in DS.



341

342 Fig. 5. Average effect of temperature, reaction time and TPPOH equivalent on DS.

343 The influence of these factors on weight gain was also studied. The variation of the average

344 weight yield according to each factor is illustrated in Fig. 6.





346 Fig. 6. Effects of temperature, reaction time and TPPOH equivalent on weight yield.

Increases in the amount of TPPOH led to increased weight yields, and a slight increase in
weight was observed as a function of reaction time beyond 24 h. Temperatures above 50 °C
induced a slight decrease in DS.

Different degrees of substitution ranging from 0.034 to 1.11 were obtained. Among the nine samples we chose S9 sample showed the highest DS in TPPOH (DS = 0.2) while remaining water soluble.

- 353 3.4. Synthesis of core-shell hybrid nanoparticles
- 354 3.4.1. Synthesis of SNPs

To prepare core-shell hybrid nanoparticles, SNPs were first synthesized by the sol-gel process based on the modified Stöber method (Stöber, Fink, & Bohn, 1968), using TEOS as starting material. Monodispersed colloidal silica particles were produced by hydrolysis and polycondensation of TEOS, in the presence of ammonia as a catalyst in alcoholic media.

359 TEM images showed spherical shape nanoparticles with an average diameter of 80 nm (Fig. 7

360 A). Dynamic light scattering (DLS) confirmed TEM results and the hydrodynamic diameter

361 was measured as 78.43±19.92 nm with a 0.062 polydispersity index (PDI). Zeta potential

- 362 measurements showed the presence of negative charges on the surface of nanoparticles due to
- 363 hydroxyl groups, as reported in Table S4 (SI).





Fig. 7. TEM images of (A) SNPs, (B) SNPs-APTES, and (C) PX SNPs.

These nanoparticles were then functionalized by APTES according to the same sol-gel mechanism. The surface modification of SNPs was confirmed by zeta potential measurements. Results showed the presence of positive charges on the surface of nanoparticles due to the ammonium groups supplied by APTES. TEM measurements (Fig. 7 B) showed no significant changes in size compared to starting nanoparticles. DLS size

distribution and zeta potential curves are reported in Fig. S2 (SI).

372 3.4.2. Functionalization of SNPs with PX

373 Beech xylan exhibits a specific xylose to methyl-glucuronic acid ratio estimated to be 10:1. 374 Furthermore, it has been reported that the pKa value for the most common types of uronic 375 acids found in xylan is 3.2–3.7 (Österberg, Laine, Stenius, Kumpulainen, & Claesson, 2001). 376 Therefore, in ultra-pure water ($pH\sim6.5$) glucuronic acid groups will allow the attachment of 377 xylan to the SNPs through ionic bonds with the previously introduced ammonium groups. In 378 practice, a suspension of SNPs in ethanol was added dropwise to a solution of PX in water. 379 The resulted PX SNPs were collected by centrifugation and the unattached polymer was 380 removed by washing several times with water. Color change of SNPs after coating with PX 381 can be seen in Fig. S3 (SI). TEM measurements (Fig. 7 C) showed no significant changes in 382 size and shape of SNPs.

The functionalization of SNPs with PX was confirmed by UV-visible analysis (Fig. S4, SI). The amount of PX bound to the nanoparticles was evaluated using a standard calibration curve obtained from different concentrations of free PX diluted in water. We used a PX characterized by a 0.2 degree of substitution which associated 9.77x10⁻⁶ mol TPPOH per gram of silica (Table S5, SI).

388 3.5. In vitro cytotoxicity of PX SNPs

389 Phototoxicity of TPPOH and PX SNPs was studied in two human colorectal cancer cell lines 390 with different grade: HCT116 (grade I) and HT-29 (grade II). Toxicity was determined 48 h 391 post-irradiation by MTT assay. Both compounds proved to be non toxic against cells kept in 392 the dark (data not shown). When the PS was irradiated by 630-660 nm red light, strong 393 toxicity was exerted on the cell lines. IC_{50} values were determined (Table 1) in order to 394 compare PS effects. We observed that TPPOH was less effective against the two human 395 colorectal cancer cell lines. In fact, compared to TPPOH, PX SNPs were 40-fold and 10-fold 396 more effective against HCT116 cells against HT-29 cells respectively, the former being 397 significantly less resistant than the latter. These compounds were used at their respective IC_{50} 398 values in the next experiments.

399 Table 1. *In vitro* phototoxicity of PX SNPs in human colorectal cancer cells.

	IC ₅₀ (nM)	
	HCT116	HT-29
TPPOH free	2943 ± 102	5959 ± 430
PX SNPs ^a	72.6 ± 2.8	550.2 ± 7.5

400 ^a IC50 nM concentrations are directly corresponding to the amount of TPPOH on the SNPs.

The morphology of HCT116 cells was characterized by TEM analysis (Fig. 8). After 630-660 nm irradiation, untreated cells exhibited abundant microvilli, the sign of normal morphology. Cells treated with PX SNPs kept in the dark showed NPs uptake without morphological changes. However, cells treated with PX SNPs and then irradiated showed complete breakdown of intracellular structures. These cells exhibited morphological features such as cell membrane shrinkage, nuclear condensation and formation of phagocytic vesicles or apoptotic bodies, hallmark events of apoptotsis (Elmore, 2007).



409 Fig. 8. TEM analysis of HCT116 cells (a) control cell (irradiated) (b) PX SNPs (dark), (c) PX SNPs (irradiated);
410 White arrows indicate SNPs inside cytoplasm.

411

412 **4. Conclusion**

413 Xylan was successfully modified with TPPOH and nine samples were obtained with DS 414 ranging from 0.034 to 1.11. The sample showing the highest DS in TPPOH while remaining 415 soluble in water was used to coat SNPs through ionic bonds. The color change of SNPs even 416 after several washes with distilled water indicated efficient coating of SNPs with PX. Thus, 417 xylan proved effective for the surface incorporation of active molecules in controlled 418 quantities. At the cellular level, the conjugation of TPPOH to SNPs significantly enhanced its 419 solubility and consequently its uptake by cancer cells. Therefore, the cell-killing efficiency

- 420 was higher with PX SNPs compared to free TPPOH. Furthermore, cell death occurred by
- 421 apoptosis as confirmed by TEM morphological characterization.
- 422

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