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Toward a molecular engineering approach

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1 **Solid silica nanoparticles as carriers of fluorescent squaraine dyes in**
2 **aqueous media: toward a molecular engineering approach**

3

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15

16 *Keywords: squaraines in water; hybrid dye-silica solid nanoparticles; fluorescence; tissue optical*
17 *window.*

18

19 *Highlights*

- 20 • Water insoluble and soluble squaraine dyes were encapsulated inside solid silica nanoparticles
21 50±2 nm in size by exploiting the reverse microemulsion method
- 22 • Hybrid dye-SiO₂ nanoparticles with photoluminescent intensity per dye molecule similar to that
23 of squaraines in organic solvent were prepared
- 24 • The hydrophilicity of squaraine-3-aminopropyltriethoxysilane (APTS) adducts is a key
25 parameters ruling the dispersion of the fluorophors within the nascent silica matrix
- 26 • The administration of the squaraine-APTS adducts together with the co-surfactant plays also a
27 relevant role

28

29 **Abstract**

30 **Hypothesis.** The relative hydrophilicity of dye-3-aminopropyltriethoxysilane (APTS) adducts and
31 tetraethylorthosilicate (TEOS) plays a key role in determining the distribution of fluorophores when
32 preparing hybrid dye-SiO₂ fluorescent nanoparticles, intended to be used in aqueous media. Here,
33 this hypothesis is applied to the case of high quantum-yield squaraine dyes, otherwise insoluble in
34 such media, and suffering a severe decrease of quantum yield if derivatised with polar groups and
35 dissolved in water.

36

37 **Experiments.** Hybrid squaraine-SiO₂ nanoparticles extremely homogeneous in size (50±2 nm) were
38 prepared by using the reverse microemulsion technique. Three different squaraine dyes were used and
39 the photophysical behavior of the prepared samples were qualitatively and quantitatively studied, by
40 steady state and time-resolved photoluminescence, in order to establish structure-property
41 relationships useful for the optimization of the preparation method.

42

43 **Findings.** Hybrid squaraine-SiO₂ nanoparticles with fluorescent intensity per dye molecule similar
44 to that of parent squaraines in organic solvent were successfully prepared. The role of the
45 hydrophilicity of the dye-APTS adduct in ruling the dispersion of such adduct in the nascent silica
46 matrix was confirmed, and the possibility to improve the tuning of the process by properly
47 administration of the adduct with the co-surfactant was demonstrated.

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53 1. Introduction

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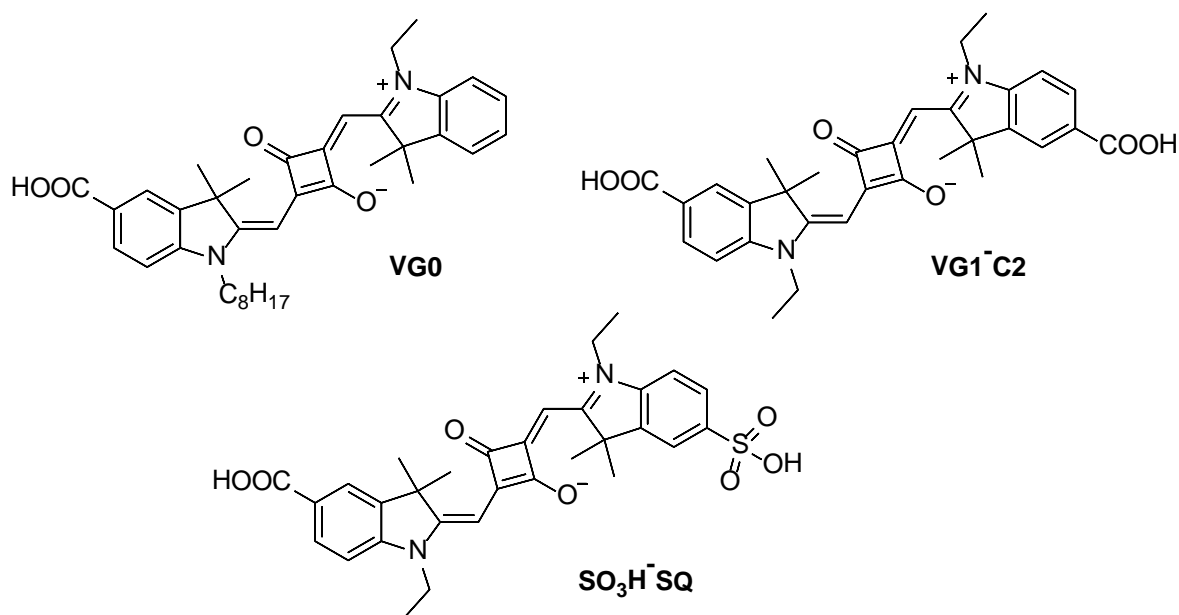
55 Organic dyes with absorption in the optical tissue window from 600 to 850 nm (where the self-
56 fluorescence of biological molecules is negligible) have emerged as a promising tool for bioanalytical
57 or biologically related applications and for *in vivo* fluorescence imaging. Polymethine dyes[1], such
58 as cyanines and squaraines, offer numerous advantages such as their easiness in designing new
59 molecules with the desired photochemical properties simply by elongating the central bridge and/or
60 tuning the lateral functional groups[3]. In this manner, dyes with absorption and emission spectra
61 properly located in the optical tissue window are obtained[4]. Cyanines and squaraines have both
62 their own pros and cons, however squaraines, resulting from the dicondensation between squaric acid
63 and electron rich molecules, are typically characterized by a quantum yield and a photostability
64 significantly higher than those found for cyanines[5]. Unfortunately, squaraines are poorly soluble in
65 aqueous media, and the consequent easy formation of non-fluorescent aggregates heavily limited their
66 adoption as fluorescent markers for biological applications. Moreover, the usual strategy used in
67 organic chemistry targeting, i.e. the introduction of a group ionizable in water, such as a sulfonic
68 moiety, is detrimental, because once dissolved in water, such derivatized squaraine dyes show a very
69 low quantum yield with respect to pristine fluorophores dissolved in less polar media[6]. In order to
70 overcome these problems, the incorporation of these hydrophobic dyes in carriers appeared to be a
71 mandatory step for their dispersion in physiological conditions. As far as molecular carriers are
72 concerned, a proposed approach is the encapsulation of the dye inside a permanently interlocked
73 rotaxane molecule, also increasing the resistance to chemical and photochemical degradation[7], or
74 in micelles[8]. Moreover, SQ-based self-assembly in hydrophobic phospholipid bilayers of liposomes
75 were successfully used for *in vivo* imaging[9]. In the domain of inorganic carriers, a possibility is the
76 intercalation of photoactive compounds into hydrotalcite (layered double hydroxides; LDH)[10], or
77 they can be loaded inside amorphous silica nanoparticles, depending on the specific application.
78 When multifunctional nanoplatfoms are pursued, the materials of interest are mesoporous silica

79 nanoparticles, used as such, for instance for photodynamic therapy[11], or wrapped with graphene
80 oxide sheets, to protect the squaric ring from possible nucleophilic attack of molecular components
81 of *in-vitro* and *in-vivo* biological media[12]. When imaging is the intended application, a relevant
82 nanocarrier is constituted by solid non-porous silica nanoparticles (NPs), highly protecting
83 encapsulated dye molecules[13–15]. Hybrid dye doped solid silica NPs can be prepared by hydrolysis
84 and polycondensation of a silicon alkoxide, such as tetraethylorthosilicate (TEOS), and fluorophores
85 derivatized with an alkoxy silane moiety, allowing the encapsulation of dye molecules in the nascent
86 silica matrix. The process can be carried out in homogeneous solution[16], i.e. exploiting the so-
87 called Stöber method, or in reverse microemulsion[17]. In both cases, the condensation reaction
88 among molecular precursors of silica typically occurs by addition of a basic agent, and this can
89 prevent the production of silica NPs hybridized with dyes as penta/hepta-methine cyanines, which do
90 not resist a high pH. Conversely, squaraines do not suffer this limitation, and then appear as relevant
91 organic dyes to be vehiculated in aqueous media by entrapment in silica NPs. Despite the complexity
92 of the synthesis procedure, the microemulsion method can be preferred to the Stöber one, owing to
93 the possibility to obtain particles with a very narrow size distribution, thus simplifying at least one
94 aspect of the complex combination of parameters affecting the behavior of nanomaterials in
95 biological media. Moreover, previous research works devoted to the encapsulation of three methine
96 cyanines[18,19] allowed to highlight that the dispersion of these visible dye molecules throughout
97 the nascent silica matrix is ruled by the relative hydrophilicity of TEOS and the dye-APTS adduct,
98 kinetically competing for the water pool inside reverse micelles[13]. Avoiding aggregation of dyes
99 within the nanocarrier is mandatory to preserve photoemission performances. To the best of our
100 knowledge, no examples of squaraine dyes encapsulated in amorphous silica NPs have been reported
101 so far.

102 This was the basis of the present work, devoted to the elucidation of molecular aspects and preparation
103 procedures relevant for the production of photoluminescent hybrid squaraines-SiO₂NPs. To this aim,
104 three squaraine dyes with different chemical structures and degree of hydrophilicity (figure 1) were

105 used to synthesize silica NPs by the reverse microemulsion method. Each sample was characterized
106 in terms of size and morphology by electronic transmission microscopy and their photophysical
107 properties were investigated by UV-Vis absorption and steady-state/time-resolved photoemission
108 spectroscopy in order to define structure-property relationships useful for the optimization of the
109 photoemission performances.

110



111

112

113

Figure 1. Structures of VG0, VG1-C2 and SO₃H-SQ.

114

115

116 2. Experimental details

117

118 2.1 Syntheses: chemicals and procedures

119 All reagents and solvents, of a highly pure grade, were purchased from Sigma Aldrich, Fluka, Merck
120 or Riedel de Haen and were used without any further purification.

121

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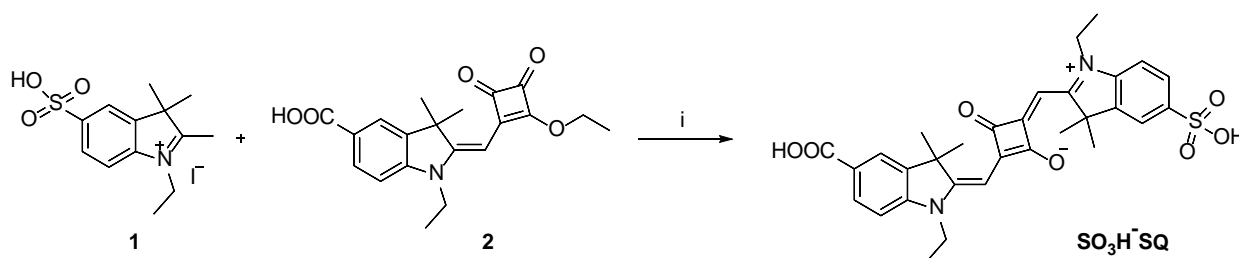
123 **2.1.1 Synthesis of squaraines**

124 **VG0** and **VG1-C2** were prepared as previously described[3]. For the synthesis of **SO₃H-SQ** a
125 modified procedure as reported in the literature [3,20] was followed. An equimolar mixture (0.84
126 mmol) of compound **1** and **2** (scheme 1) were introduced into a 20 ml microwave reaction vial with
127 toluene and butanol (18 ml, 1:1), sealed with a crimp cap and heated in the microwave system (single-
128 mode Biotage Initiator 2.5) at 160°C for 25 min. The green precipitate which is formed is washed
129 with diethyl ether and further purified by a semi-preparative HPLC system (by Schimatzu, equipped
130 with SCL 10Avp, SPD 10Avp, a LC8A pump and a Phenomenex column Synergi Fusion-RP 4 μ,
131 150x21.20 mm) using an isocratic flow of methanol with formic acid (1%, v/v) to obtain **SO₃H-SQ**
132 (105 mg, yield = 22%) as a green powder.

133 ¹H NMR (200MHz, D₂O), δ: 8.01-7.72 (m, 6H), 7.15 (s, 1H), 6.91 (s, 1H), 4.42 (m, 2H), 3.79 (m,
134 2H), 1.47 (s, 12H), 1.14 (s, 6H).

135 MS (ESI) [M-H]⁻ 575.60

136



138

139 **Scheme 1.** Synthesis of **SO₃H-SQ**. Experimental conditions: (i) toluene/butanol (1:1), MW, 25 min,
140 160°C.

141

142 **2.1.2 Synthesis of squaraine-silane derivatives**

143 The *N*-Hydroxysuccinimide (NHS) active esters of the two non-symmetrical squaraines **VG0** and
144 **SO₃H-SQ** were synthesized by reacting 0.02 mmol of each fluorophore with NHS (0.08 mmol) and
145 *N,N'*-Dicyclohexylcarbodiimide (DCC, 0.08mmol) in dimethylformamide (DMF, 2.0 ml) and stirring

146 for 4h at 80°C; in the case of the symmetrical VG1-C2, 0.16 mmol of both NHS and DCC were used
147 due to the presence of two carboxyl groups. Reactions were monitored by mass spectrometry until
148 complete conversion and then the product was separated through dilution in diethyl ether and
149 filtration to obtain products as powders.

150 Squaraine-silane derivatives were then prepared by adding 3-aminopropyltriethoxysilane (APTS;
151 46.0 μmol, 10 μL for VG0-NHS and SO₃H-SQ-NHS; 92.0 μmol, 20 μL for VG1-C2-NHS) to
152 squaraine-NHS solutions in 0.5 ml of anhydrous DMF and stirring the mixture for 24h at room
153 temperature; reactions were monitored by thin layer chromatography until complete disappearance
154 of former NHS esters. The obtained squaraine-silane derivatives (hereafter X-APTS, where X is the
155 squaraine code) were then used without further purification in order to avoid the occurrence of self-
156 polymerization.

157 Schemes of the syntheses are shown in the Supporting Information (hereafter SI).

158

159 **2.1.3 Synthesis of squaraine-loaded silica NPs**

160 Hybrid squaraine-silica NPs were prepared by the reverse microemulsion technique already reported
161 in previous works[18,19,21]. Briefly, a water in oil microemulsion was prepared by mixing
162 cyclohexane (75.0 ml), Triton X-100 (18.85 g), *n*-hexanol (18.0 ml) and distilled water (5.4 ml); the
163 mixture was gently stirred for ca. 30 minutes and then 0.05 ml of a 0.01 M squaraine-APTS in DMF
164 was added; after further 15 minutes, TEOS (1.0 ml, 4.5 mmol) and NH₄OH (28-30%, 0.7 ml) were
165 added to start the NPs formation. Reaction was stirred for 16 hours at room temperature and then was
166 stopped by adding 50.0 ml of acetone; particles were extracted from the supernatant by centrifugation
167 (10k rpm, r.t.) and washed twice in ethanol and several times in distilled water by resuspension and
168 centrifugation cycles until complete removal of the surfactant. Finally, squaraine-loaded silica NPs
169 (hereafter X-NPs, where X is the squaraine code) were stored as suspensions in distilled water at
170 room temperature in the dark until needed.

171 In order to attain possible optimization of the preparation technique, in two cases the hybrid NPs

172 preparation protocol was changed (*vide infra*) and the resulting materials were labeled as X-NPs-B.
173 For solvatochromic experiments, NPs stored as water suspensions were first centrifuged at 10k rpm
174 for 20 minutes and then resuspended using acetone. Then, NPs were washed twice by
175 centrifugation/resuspension cycles in order to completely remove water and, finally, the
176 concentration of the final suspensions was adjusted to 1 mg·ml⁻¹.

177

178 **2.2 Methods**

179

180 **2.2.1 Characterization of dyes**

181 Thin-layer chromatography was performed on silica gel 60 F254 plates. ESI-MS spectra were
182 recorded using a LCQ Thermo Advantage Max spectrometer, with electrospray interface and ion trap
183 as mass analyzer. The flow injection effluent was delivered into the ion source using nitrogen as
184 sheath and auxiliary gas. ¹H NMR (200 MHz) spectra were recorded on a Bruker Avance 200 NMR.

185

186 **2.2.2 UV-Vis absorption spectroscopy**

187 UV-VIS electronic absorption spectra of the squaraine in solution and of the supernatants derived
188 from nanoparticle extraction from the microemulsion mixture were measured by a Cary 300 Bio
189 spectrophotometer (Varian, Santa Clara, CA, USA), using quartz cuvettes (1 cm pathway length).

190 For the determination of absorption coefficients, every dye was weighed, (7.0-10.0 mg), and diluted
191 to 10.0 ml in a flask using DMSO. From this solution, 0.25 ml were taken and diluted to 25.0 ml with
192 the proper solvent (mother solution). Three dilutions were prepared by diluting 1.0, 2.5 and 5.0 ml of
193 this solution to 25.0 ml. Those solutions were analyzed by UV-Vis spectroscopy. Absorbance at the
194 λ_{\max} for every diluted solution was plotted vs. dye concentration and a linear fitting was performed.
195 The slope of the plot is the molar absorption coefficient (ϵ). The determination was made, in duplicate,
196 by preparing two separate concentrated dye mother solutions in DMSO. The $\log \epsilon$ obtained from the
197 two separate data sets was compared: if their difference was less or equal to 0.02 respect to their

198 average, the data were considered acceptable and the average of the two values was taken as the
199 official value. Otherwise, a further concentrated dye mother solution in DMSO was prepared, the
200 whole procedure was repeated and the log_e data were compared.

201

202 **2.2.3 UV-Vis photoemission spectroscopy**

203 Photoluminescence and excitation spectra in steady state mode were acquired using a Horiba Jobin
204 Yvon Fluorolog 3 TCSPC fluorimeter equipped with a 450-W Xenon lamp and a Hamamatsu R928
205 photomultiplier.

206 The absolute quantum yield of each dye in solution was determined combining Quanta-φ with
207 Fluorolog 3. The reported values are the average of three measurements using three different dye
208 solutions.

209 Fluorescence lifetimes were measured by the time correlated single photon counting method (Horiba
210 Jobin Yvon) using a 560 nm Horiba Jobin Yvon NanoLED as excitation source and an impulse
211 repetition frequency of 1 MHz positioned at 90° with respect to a TBX-04 detector. Lifetimes were
212 calculated using DAS6 decay analysis software.

213

214 **2.2.4. High resolution transmission electron microscopy (HRTEM)**

215 Size and morphology of NPs were analyzed with a 3010 Jeol microscope operating at 300 kV. A
216 droplet of each nanoparticle water suspension (1.0 mg ml⁻¹) was spread on a copper grid coated with
217 a carbon film and then water was allowed to slowly evaporate in order to limit particle agglomeration.

218 Size distribution was evaluated by measuring at least 300 nanoparticles and the mean diameters were
219 calculated as $d_m = \sum d_i n_i / \sum n_i$ (n_i = number of particles of diameter d_i); results were reported as $d_m \pm$
220 stdv.

221

222

223

224 **3. Results and discussion**

225

226 **3.1 Synthesis of squaraines and squaraine-silane derivatives**

227 **VG0** and **VG1-C2** squaraines were prepared via a microwave condensation reaction based on a
228 previously reported procedure[3] while **SO₃H-SQ** was synthesized by a microwave-assisted
229 condensation of 5-sulfo-indolenine salt **1**[20] and carboxyemisquarate **2**[3] (see Scheme 1).

230 Squaraine-silane derivatives were prepared via a previously reported method [12] through the NHS
231 ester modification of the squaraine carboxylic group and subsequent reaction with APTS (see Scheme
232 S2 in the SI for **SO₃H-SQ** modification).

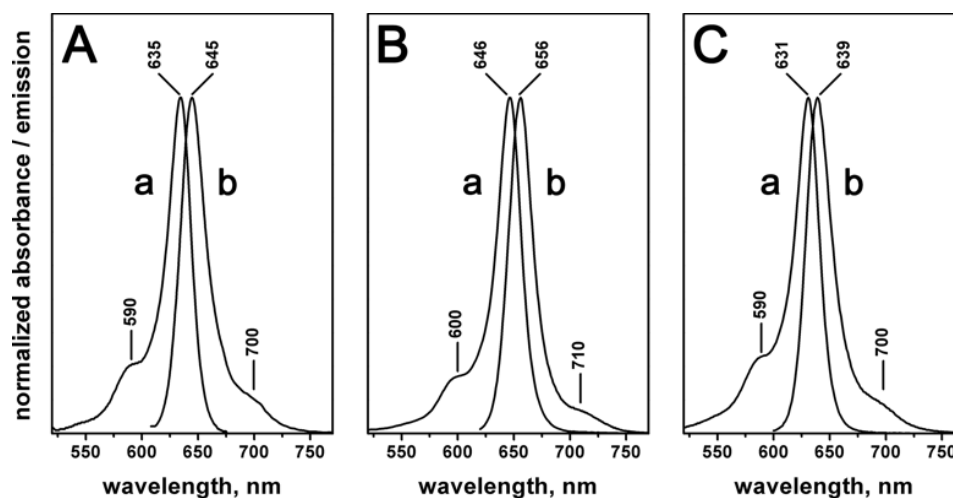
233

234 **3.2 Photophysical properties of squaraines in solution**

235 The photophysical behaviour of each squaraine dye in solution was investigated together with the
236 determination of the correspondent molar decadic absorption coefficient and absolute quantum yield
237 by means of absorption and both steady-state and time-resolved photoemission spectroscopies (see
238 Table 1).

239 The UV-Vis absorption spectra (see Figure 2) of the squaraine dyes show absorption maxima between
240 630 nm and 646 nm with high molar extinction coefficients ($\log\epsilon$ around 5.40 in organic solvents and
241 4.51 in water). The main absorption peak is associated to the $\pi\rightarrow\pi^*$ HOMO-LUMO transitions,
242 mainly localized on the squarainic core[22,23], while the shoulder at higher energy is due to the
243 HOMO-LUMO+1 transition. When excited within the absorption band at room temperature, the three
244 types of squaraines emit a luminescence spectrum almost specular to the absorption one, with maxima
245 ranging from 639 to 656 nm, thus with small Stokes shifts as expected for squaraine dyes[24].

246



247

248 **Figure 2.** Absorption (a) and emission spectra ($\lambda_{\text{ex}} = 590$ nm) (b) of **VG0** in methanol (panel A),
 249 **VG1-C2** in acetone (panel B) and **SO₃H-SQ** in water (panel C).

250

251 Fluorescence lifetime and quantum yield of VG0 in methanol and VG2-C1 in acetone are in the
 252 ranges typical for squaraines in organic media [25]. Also the much lower τ and Φ of SO₃H-SQ is in
 253 agreement with the expected photoemission behaviour in water [26,27].

254

255 **Table 1.** Main optical characteristics of **VG0** in methanol, **VG1-C2** in acetone and **SO₃H-SQ** in
 256 water.

	λ_{abs} (nm)	$\log \epsilon$	λ_{em} (nm)	τ (ns)	ϕ
VG0 [†]	635	5.39	645	0.28 (100%)	0.15
VG1-C2 [*]	646	5.40	656	0.99 (100%)	0.27
SO₃H-SQ [□]	631	4.51	639	0.11 (100%)	0.023

257 [†] in methanol, ^{*} in acetone, [□] in water

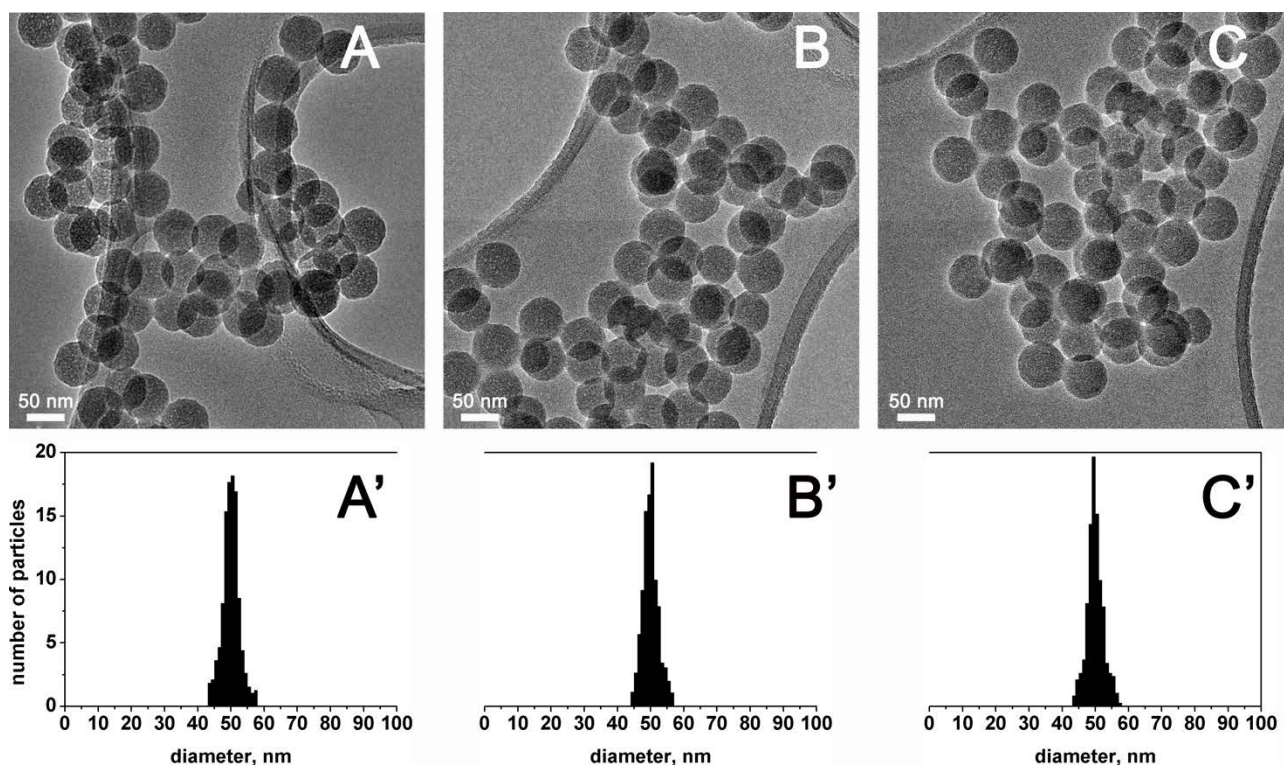
258

259 3.3 Shape, size and dye-content of squaraine loaded silica NPs

260 Representative TEM micrographs of the three squaraine-NPs samples are shown in figure 3, where
 261 the highly regular spherical shape (upper panels) and quite homogeneous size of NPs around a mean
 262 diameter of 50 nm (lower panels) can be appreciated. Hence, on the basis of the TEOS-to-SiO₂
 263 conversion yield, the density of NPs (ca. 2.2 g·cm⁻³)[19] and the calculated average nanoparticle
 264 volume, the total number of NPs obtained by each preparation was estimated.

265 As a second step, for each sample the total amount of squaraine molecules associated to NPs was
266 calculated as the difference between their initial amount and that remained in the reaction media after
267 the accomplishment of the formation of NPs, as determined spectrophotometrically after the
268 separation of NPs by centrifugation. This was possible because no squaraine degradation products
269 were detected in the UV-Vis spectra of the post-reaction liquid medium. Thus, the average number
270 of dye molecules per nanoparticle was calculated (Table 2). Apparently, the VG0-APTS adduct was
271 unable to participate to the formation of NPs, whereas this was the case for ca. 45 % and 65% of
272 VG1-C2-APTS and SO₃H-SQ-APTS species present in the relevant reaction media, resulting in ca.
273 50 and 70 dye molecules-per-NP, respectively.

274



275

276 **Figure 3.** Representative TEM images of VG0-NPs (A), VG1-C2-NPs (B) and SO₃H-SQ-NPs (C)
277 and correspondent size distribution histograms. Original magnification of images: 500000×. Scale
278 bar: 50 nm

279

280

281

282

Table 2. Yields of entrapment of squaraine-APTS molecules in silica NPs

	Yield of Squaraine-APTS molecules entrapment (%)	Squaraine-APTS molecules per NP (n°)
VG0-NPs	0	0
VG1-C2-NPs	46	47
SO₃H-SQ-NPs	66	68
VG1-C2-NPs-B	41	42
SO₃H-SQ-NPs-B	63	65

283

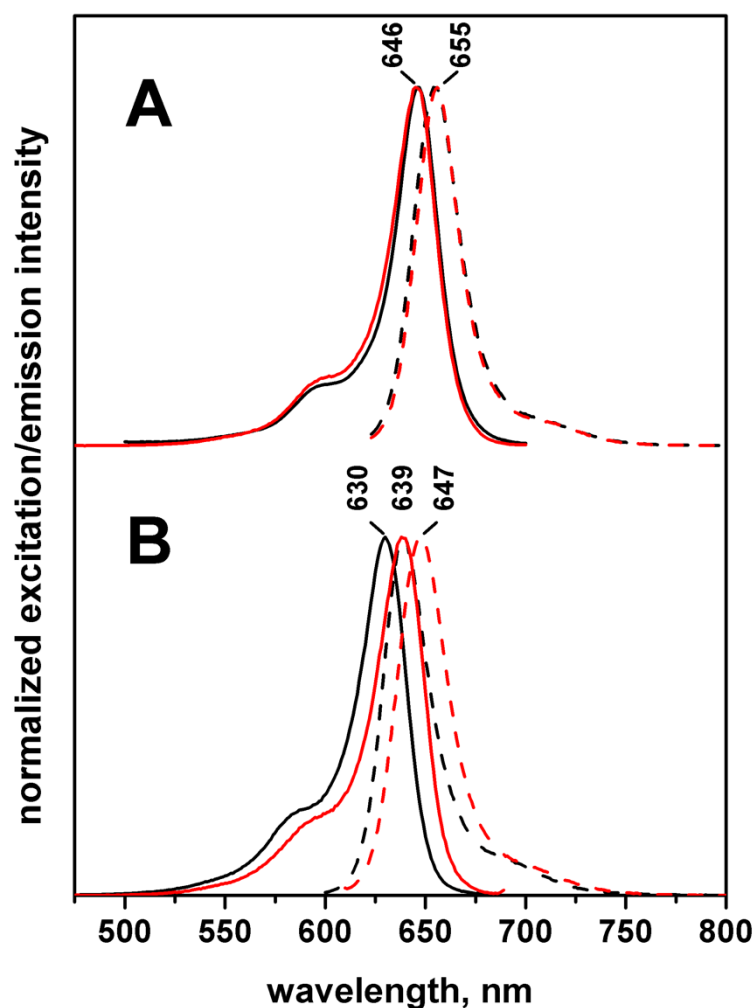
284 The lack of encapsulation of VG0-APTS in the silica matrix should be rationalized in terms of a so
 285 poor hydrophilicity of this squaraine derivative to prevent its transfer into the water pool in the core
 286 of reverse micelles, and thus this squaraine derivative will be no longer considered. Conversely, the
 287 presence of two triethoxysilane moieties in VG1-C2-APTS counterbalance enough the
 288 hydrophobicity of the squaraine motif to allow this derivative to reach the water pools and participate
 289 to hydrolysis and condensation with TEOS during the formation of NPs. As expected, a stronger
 290 effect resulted from the presence of the sulphonic group in SO₃H-SQ-APTS, which was involved in
 291 the formation of NPs in a larger extent. However, the rate of transfer into the micelles of neither VG1-
 292 C2-APTS nor SO₃H-SQ-APTS was high enough to allow all the dye derivative to participate to the
 293 reaction before the accomplishment of the formation of the NPs.

294

295 **3.4 Photoemission properties and location of the entrapped squaraines**

296 In order to evaluate any possible modification of the photophysical behaviour of the entrapped
 297 fluorophores, steady state photoemission and excitation spectra of hybrid VG1-C2-NPs and SO₃H-
 298 SQ-NPs were recorded in suspension and compared to the data obtained for the correspondent dye
 299 derivatives in solution (figure 4). Data obtained for VG1-C2 and VG1-C2-APTS were similar,
 300 indicating that the derivatization did not modify significantly the photophysical behaviour of the dye.

301 $\text{SO}_3\text{H-SQ}$ was only soluble in water, and this prevented a comparison with $\text{SO}_3\text{H-SQ-APTS}$ in the
302 same medium, because of the sensitivity of the derivative to hydrolysis. Thus, the pristine VG1-C2
303 and $\text{SO}_3\text{H-SQ}$ were considered for the comparisons with their derivative associated to NPs.
304 For both types of hybrid NPs, excitation instead of absorption in transmission was recorded because
305 the lower sensitivity of the latter did not allow to record spectra with an acceptable signal-to-noise
306 ratio at suspension concentrations low enough to avoid light scattering.
307 In figure 4 panel A, excitation and photoemission spectra of VG1-C2 in solution and VG1-C2-NPs
308 in suspension are reported; acetone was used as solution and suspension medium due to the low
309 solubility of VG1-C2 in water. It can be clearly observed that both excitation and emission signals of
310 the NPs are identical to the correspondent ones of the fluorophore in solution suggesting that no
311 modifications of the energies and probabilities of the electronic transitions occurred due to the
312 association with the inorganic matrix. On the contrary, in the case of $\text{SO}_3\text{H-SQ}$ (figure 4, panel B),



313 **Figure 4.** Panel A: excitation (solid curves, $\lambda_{em} = 655$ nm) and photoemission (dashed curves, $\lambda_{exc} =$
 314 590 nm) spectra of VG1-C2 in acetone solution (black curve, $5.0 \cdot 10^{-6}$ M) and VG1-C2-NPs in acetone
 315 suspension (red curve, $3.0 \cdot 10^{-8}$ mg · ml⁻¹); Panel B: excitation (solid curves, $\lambda_{em} = 640$ nm) and
 316 photoemission (dashed curves, $\lambda_{exc} = 590$ nm) spectra of SO₃H-SQ in water solution (black curve,
 317 $5.0 \cdot 10^{-6}$ M) and SO₃H-SQ-NPs in water suspension (red curve, $3.0 \cdot 10^{-8}$ mg · ml⁻¹). All spectra were
 318 normalized with respect to the maximum for the sake of clarity.

319

320 where the hydrophilicity of the dye allowed to use water as medium for both the molecule in solution
 321 and the hybrid NP suspension, the association of dye derivatives with silica produced a decrease of
 322 the energies of absorption and fluorescence transitions as indicated by the 9 nm red shift of both
 323 signals. The coincidence of the shape of the spectra profiles exhibited by both dye/hybrid NPs pairs
 324 allowed to exclude the occurrence of intra-particle autoabsorption effects. Hence, as the framework
 325 of amorphous silica is apolar[28] the red-shift observed for SO₃H-SQ-NPs should be reasonably

326 assigned to a solvatochromic effect due to the decrease of the polarity of the environment experienced
327 by entrapped squaraine derivatives with respect to the ones in water solution. The solvathocromic
328 behavior of the SO₃H-SQ in solution were reported in figure S1 in the SI for the sake of comparison.
329 Time-resolved photoemission measurements were also carried out, and the results are listed in table
330 3, compared with relevant cases of squaraine derivatives in solution. The targeted suspension medium
331 for hybrid NPs is water, but also acetone was considered because of its lower polarity, thus useful to
332 reveal possible solvatochromic effects due to dye molecules not entrapped in the bulk of NPs. For all
333 suspensions of hybrid NPs, bi-functional equations were necessary to fit the decay curves, indicating
334 that for both VG1-C2-APTS and SO₃H-SQ-APTS derivatives two different scenarios of interaction
335 with the silica host are present. For VG1-C2-APTS, independently on the suspension medium, one
336 fluorescence lifetime (τ^0_F) is slightly longer than what found for the molecular form in acetone
337 solution, whereas the other (τ^1_F) appears increased of ca. 2.5 times with respect to that comparative
338 form. In the case of SO₃H-SQ-APTS, the association with the silica matrix resulted in two lifetimes
339 both significantly longer than for the molecular form in water solution, with an increase of ca. 7-10
340 and ca. 20 times, for τ^0_F and τ^1_F , respectively. For both types of hybrid dye-SiO₂ NPs, the relative
341 population of fluorescent molecules showing the longer lifetime is the more abundant by far.
342 For hybrid dye-NPs, the increase of fluorescence lifetime is the result of the decrease in rotational
343 degrees of freedom, and in possible detrimental effects due to the interaction with highly polar media,
344 such as water. Thus, data obtained for SO₃H-SQ-NPs indicate that SO₃H-SQ derivatives should be
345 not simply anchored on the surface of NPs. Because of the similarity of τ^1_F values, significantly
346 longer than the fluorescence lifetime of VG1-C2-APTS in acetone solution, the same should occur at
347 least for the most abundant fraction of fluorescent dyes in VG1-C2-NPs. Conversely, the other
348 fraction should experience an interaction with its environment with more limited differences with
349 respect of acetone as solvent.

350 Noticeably, the time resolved photoemission behavior of both VG1-C2-NPs and SO₃H-SQ-NPs
 351 appeared sensitive to the suspension medium, with a slight increase of both τ^0_F and τ^1_F and, more
 352 significantly, with the transfer of ca. a 10% of fluorescent molecules between the relative abundances
 353 of dyes showing different fluorescence lifetime.

354

355 **Table 3.** Emission decay times ($\lambda_{exc} = 560$ nm) of: VG1-C2 in acetone solution ($5 \cdot 10^{-7}$ M);
 356 VG1-C2-NPs and VG1-C2-NPs-B in acetone and water suspension ($0.1 \text{ mg} \cdot \text{ml}^{-1}$); SO₃H-SQ in water
 357 solution ($1 \cdot 10^{-6}$ M); SO₃H-SQ-NPs and SO₃H-SQ-NPs-B in water and acetone suspension (0.1
 358 $\text{mg} \cdot \text{ml}^{-1}$)

359

	Medium	τ^0_F (ns)	% τ^0_F (ns)	τ^1_F (ns)	% τ^1_F (ns)	χ^2
VG1-C2	acetone	0.99	100	---	---	1.01
VG1-C2-NPs	acetone	1.19	21	2.42	79	1.12
	water	1.06	31	2.37	69	1.04
SO₃H-SQ-NPs	acetone	1.10	15	2.38	85	1.08
	water	0.80	21	2.31	79	1.02
SO₃H-SQ	water	0.11	100	---	---	1.03
VG1-C2-NPs-B	acetone	1.35	25	2.44	75	1.04
	water	1.02	43	2.19	57	1.13
SO₃H-SQ-NPs-B	acetone	1.21	35	2.62	65	1.07
	water	0.99	46	2.38	54	1.02

360

361 For both VG1-C2-NPs and SO₃H-SQ-NPs, fluorescent dyes showing the longer lifetime are
 362 candidate to be depicted as well entrapped in the silica matrix; however, their sensitivity to the
 363 suspension medium, and in particular their change in relative abundance, pose a problem.

364 In this respect, it is useful to remind that silica NPs prepared by the microemulsion or Stöber methods
 365 are characterized by the presence of domains, extended from the surface towards the interior, of
 366 non-completely condensed silica, resulting in a mass fractal structure sensitive to changes in the
 367 external environment [29,30]. Moreover, H/D isotopic exchange indicated that such domains can be
 368 infiltrated by water molecules[21]. Therefore, although the sensitivity of the measurement technique

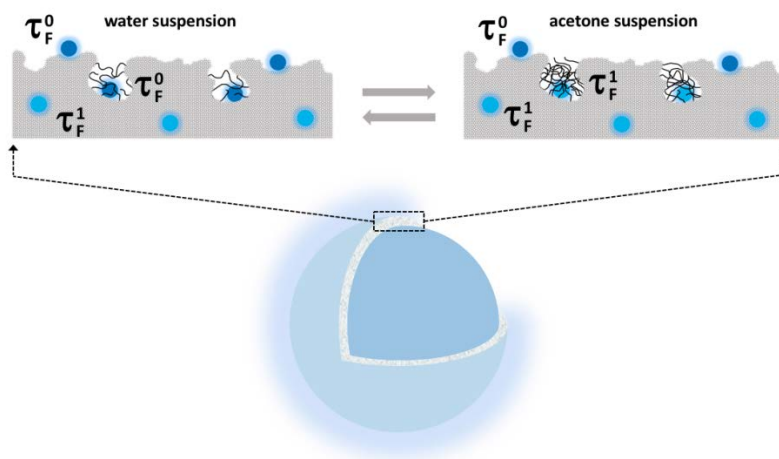
369 did not allow to distinguish more than two relative abundances of fluorescent dyes for each type of
370 hybrid NPs, the obtained data could be explained by assuming the presence of three ensembles of
371 fluorescent dyes in the NPs (scheme 2):

372 i) a most abundant fraction, characterized by the longer fluorescent lifetime, τ^1_F (ca.70% and 80%
373 for VG1-C2-NPs and SO₃H-SQ-NPs, respectively), well constrained by the silica matrix, likely
374 in its fully condensed part, insensitive to changes in suspension medium

375 ii) a fraction of ca. 20% for VG1-C2-NPs and 15% for SO₃H-SQ-NP, showing the shorter
376 fluorescent lifetime τ^0_F , located at/near the nanoparticle surface and less constrained by the silica
377 matrix independently on the type of suspension medium

378 iii) a fraction, ca. 10% and 6% for VG1-C2-NPs and SO₃H-SQ-NPs, respectively, located in not
379 fully condensed parts of silica matrix more sensitive to the nature of the suspension medium,
380 where fluorescent dyes are less constrained when these parts are infiltrated by water.

381



382

383 **Scheme 2.** Distribution of dye molecules in the NPs explaining the different sensitivity to the
384 suspension medium

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389 **3.5 Dyes in solution vs hybrid NPs: comparison of fluorescence intensity**

390 Because of the direct proportionality between fluorescence lifetime and quantum yield[31], the
391 measured increase of fluorescence lifetime for all dye fractions in SO₃H-SQ-NPs and the most
392 abundant one for VG1-C2-NPs appears the basis for expecting an increase in fluorescence intensity
393 of hybrid NPs suspensions with respect to the squaraine derivatives in solutions, for equivalent dye
394 molar concentrations. Hence, the following experimental procedure was used for VG1-C2 and
395 SO₃H-SQ-based systems, differing only for the suspension medium, as described in the previous
396 section. On the basis of the calculated number of squaraine per nanoparticle (table 2), a series of
397 suspensions at known dye concentration was prepared for both VG1-C2-NPs and SO₃H-SQ-NPs, as
398 well as a series of solutions of VG1-C2 and SO₃H-SQ in the same concentration range. The
399 photoemission spectra were recorded, strictly using the same excitation conditions, and the integrated
400 intensities of photoemission spectra were plotted with respect to the correspondent concentration
401 values (figure 5). A straight linear dependence was obtained, clearly indicating that the measurements
402 were affected by neither light scattering nor inter-particle auto-absorption effects.

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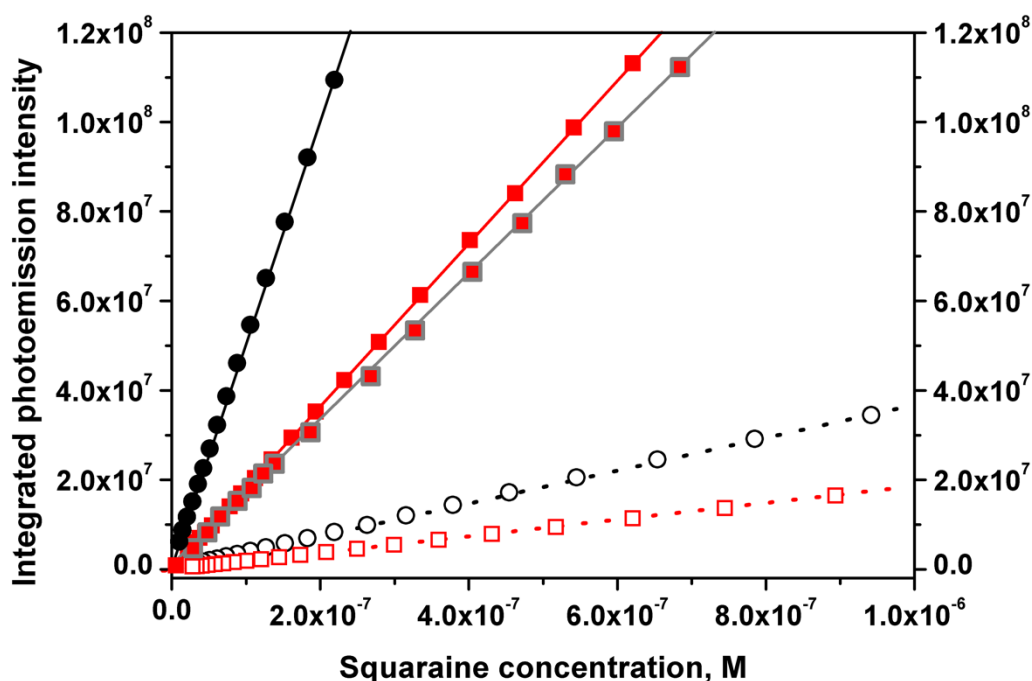
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415 **Figure 5.** Comparison of the integrated intensities of photoemission spectra ($\lambda_{\text{exc}} = 590$ nm) of
 416 VG1-C2 in acetone solution (black solid dots), VG1-C2-NPs in acetone and water suspension (red
 417 solid squares and grey bordered red squares, respectively), SO₃H-SQ in water solution (black empty
 418 dots) and SO₃H-SQ-NPs in water suspension (red empty squares)

419

420 In agreement with the absolute quantum yields (table 1), solutions of the two squaraines produces
 421 significantly different fluorescence outputs, with VG1-C2 (black solid dots) showing integrated
 422 intensities of photoemission spectra ca. 13 times higher than SO₃H-SQ (black empty dots).
 423 Conversely, data obtained for the hybrid NPs were not in agreement with expectations: the integrated
 424 intensities of photoemission spectra of VG1-C2-NPs in both acetone (red solid squares) and water
 425 (grey bordered red squares) suspensions are ca. 35% and 30%, respectively, of what obtained for
 426 VG1-C2 solutions (black solid dots), and the relative decrease is limited to ca. 50% when comparing
 427 the SO₃H-SQ-NPs/SO₃H-SQ pair (red empty squares and black empty dots, respectively).

428 Such lower than expected fluorescence intensity should be due to a decrease of the decadic absorption
 429 coefficient of squaraines and/or a quenching of a part of them when linked to/entrapped in the silica

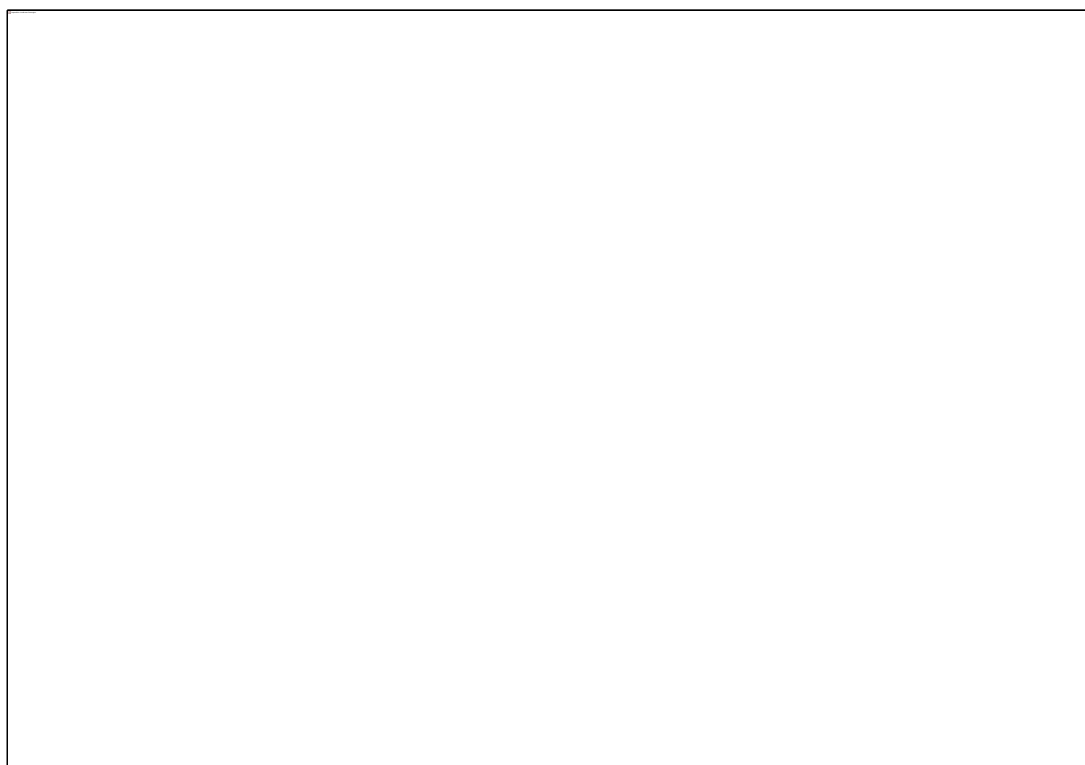
430 matrix. Non-light-scattering suspensions of hybrid NPs were too diluted to allow the recording of
431 absorbance spectra, and then no insights were obtained for the first possibility.

432 Focusing on fluorescence quenching, if occurred it might be of the concentration type, resulting from
433 the formation of aggregates of squaraine derivatives when involved in the growth of NPs. In
434 particular, SO₃H-SQ-APTS, highly hydrophilic and then easily transferred from the oil phase to the
435 inner water pool of reverse micelles, could have formed aggregates in the first steps of the reaction,
436 whereas the contrary might have occurred for VG1-C2-APTS, definitely less hydrophilic and then
437 involved in the reaction when the nanoparticle formation was almost accomplished.

438 Based on these hypotheses, two additional samples were prepared by modifying the synthesis
439 procedure with an opposite target, depending on the squaraine derivative considered. Thus, to dilute
440 in time the entering of SO₃H-SQ-APTS in the micelles, it was dosed in the reaction medium in small
441 aliquots at regular time intervals (0.01 ml every 10 min) during the first hour of the NPs synthesis.
442 Conversely, VG1-C2-APTS was added to the microemulsion as solution in *n*-hexanol, i.e. the
443 co-surfactant of micelles, in order to facilitate its partition at the boundary between the oil and water
444 phases. In this way, the shortening of the diffusion path should allow this squaraine derivative to be
445 involved not only in late steps of the formation of NPs. These two samples were labeled as
446 SO₃H-SQ-NPs-B and VG1-C2-NPs-B.

447 As for the previous cases, the amount of dyes effectively associated with NPs was calculated, and
448 values very close to the ones already found for the previous corresponding hybrid NPs were obtained
449 (table 2). The same occurred for steady-state and time resolved photoemission data (Figure S2 in the
450 SI and table 3, respectively). Conversely, the integrated intensity of the photoemission spectra of dye
451 equimolar SO₃H-SQ solutions/hybrid NPs suspensions in water appear almost coincident, and the
452 values obtained for VG1-C2-NPs-B in acetone and water suspensions are ca. 90% and 70%,
453 respectively of those measured with VG1-C2 in acetone solutions (figure 6). Thus, the second set of
454 hybrid NPs shows a definitely better performance as photoemitters with respect to the first one (see
455 figure 5).

456 Apparently, the strategies adopted to affect the distribution of squaraine derivatives on/in the silica
457 NPs were successful, at least partly. In fact, if all dye molecules in hybrid NPs were fluorescent,
458 based on the measured increase in photoemission lifetime, the fluorescence intensity of their
459 suspensions should exceed that of equimolar dye solutions by far. A specific investigation in this
460 respect will be the object of a future investigation.



461

462 **Figure 6.** Comparison of the integrated photoemission intensities ($\lambda_{exc} = 590$ nm) of VG1-C2 in
463 acetone solution (black solid dots), VG1-C2-NPs-B in acetone and water suspension (red solid
464 squares and grey bordered red squares, respectively), SO₃H-SQ in water solution (black empty dots)
465 and SO₃H-SQ-NPs-B in water suspension (red empty squares).

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473 **4. Conclusion**

474

475 The preparation of hybrid squaraines-silica nanoparticles showing, in aqueous suspension, a
476 photoluminescence emission intensity per dye molecule equivalent to parent water insoluble
477 squaraine molecules, when in organic solution, has been achieved successfully. Such achievement
478 stems from the adopted molecular engineering approach, based on the hypothesis that the relative
479 hydrophobicity of dye-3-aminopropyltriethoxysilane (APTS) adducts and tetraethylorthosilicate
480 (TEOS) is a key parameter ruling the distribution of fluorophores within the nascent silica matrix
481 when the reverse microemulsion method is used for producing hybrid dye-SiO₂ nanoparticles. Such
482 hypothesis, developed in previous studies on the preparation of hybrid cyanine- SiO₂
483 nanoparticles[21], and recently assumed also by other researchers[32] has been here confirmed also
484 for squaraines, and in addition, the possibility to improve the tuning of the process by administering
485 the squaraine-APTS adduct with the co-surfactant has been demonstrated. This is the first report, at
486 the best of our knowledge, on molecular factors ruling the dispersion of squaraines in solid silica
487 nanoparticles, but the number of dye molecules per nanoparticles and likely their photoluminescent
488 fraction are significantly lower with respect what obtained with cyanine dyes [19, 21]. Thus, the
489 future steps of this research will be aimed to overcome this gap, targeting more bright hybrid
490 squaraine-SiO₂ nanoparticles.

491

492

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