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1 **Microgels of silylated HPMC as a multimodal system** 2 **for drug co-encapsulation**

3
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15 **Abstract**

16 Combined therapy is a global strategy developed to prevent drug resistance in cancer and infectious
17 diseases. In this field, there is a need of multifunctional drug delivery systems able to co-encapsulate
18 small drug molecules, peptides, proteins, associated to targeting functions, nanoparticles. Silylated
19 hydrogels are alkoxy silane hybrid polymers that can be engaged in a sol-gel process, providing
20 chemical cross linking in physiological conditions, and functionalized biocompatible hybrid
21 materials.

22 In the present work, microgels were prepared with silylated (hydroxypropyl)methyl cellulose (Si-
23 HPMC) that was chemically cross linked in soft conditions of pH and temperature. They were

24 prepared by an emulsion templating process, water in oil (W/O), as microreactors where the
25 condensation reaction took place.

26 The ability to functionalize the microgels, so-called FMGs, in a one-pot process, was evaluated by
27 grafting a silylated hydrophilic model drug, fluorescein (Si-Fluor), using the same reaction of
28 condensation. Biphasic microgels (BPMGs) were prepared to evaluate their potential to encapsulate
29 lipophilic model drug (Nile red). They were composed of two separate compartments, one oily phase
30 (sesame oil) trapped in the cross linked Si-HPMC hydrophilic phase.

31 The FMGs and BPMGs were characterized by different microscopic techniques (optic, epi-
32 fluorescence, Confocal Laser Scanning Microscopy and scanning electronic microscopy), the
33 mechanical properties were monitored using nano indentation by Atomic Force Microscopy (AFM),
34 and different preliminary tests were performed to evaluate their chemical and physical stability.

35 Finally, it was demonstrated that it is possible to co-encapsulate both hydrophilic and hydrophobic
36 drugs, in silylated microgels, that were physically and chemically stable. They were obtained by
37 chemical cross linking in soft conditions, and without surfactant addition during the emulsification
38 process. The amount of drug loaded was in favor of further biological activity. Mechanical
39 stimulations should be necessary to trigger drug release.

40 **Key words:** Microgels, Sol-Gel, alkoxy silane functionalization, co-encapsulation.

41

42 **1. Introduction**

43

44 Combined therapies gather a wide field of applications where drugs have to be co-encapsulated, to
45 overcome drug resistance in cancer treatments (Borcoman, Le Tourneau, 2016; Bradshaw, Sobel,
46 2016; Teo, *et al.*, 2016), infectious diseases (Le Douce, *et al.*, 2016; Bento da Silva, *et al.*, 2016),
47 and surgical diseases (Morgan *et al.*, 2016), or to combine growth factor action in tissue regeneration
48 (Subbiah, *et al.*, 2015). Targeted delivery or theranostic approaches could also be combined with
49 drug controlled release providing one dose drug delivery systems (Ulbrich, *et al.*, 2016; Kelkar,

50 Reineke, 2011). So, there is a potential and challenging benefit to design multimodal injectable drug
51 delivery systems embedding complementary activities and/or combining cell surface functions such
52 as ligands, adhesion molecules, cytokines, growth factors. One of the limitations of these approaches
53 is to take into account all together the therapeutic goals as well as the formulation constraints.
54 Another limitation is the multi-step building chemistry process used to functionalize nano/
55 microparticles. Such designs need multi-process and multi-functional approaches. In this context,
56 there is a need of new functionalized polymers to embed different complementary activities, which
57 respects biocompatibility, but also reduces the time, the cost, and the environmental impact of the
58 manufacturing process.

59

60 Microgels are defined as microparticles of chemical (covalent links) or physical (weak interactions)
61 cross linked hydrogels. They consist of three-dimensional polymer network, able to swell or shrink
62 under large amount of water exchanges. They could be responsive to the changes in pH, temperature
63 or other physiological stimuli, suitable for drug controlled release. They are basically obtained and
64 stabilized by hydrogen-bond or electrostatic interactions in alginate (Matalanis, *et al.*, 2011),
65 chitosan (Dang, *et al.*, 2016), and pectine. (Marquis, *et al.*, 2014) Nevertheless for some applications,
66 chemical cross linking could be recommended to improve the mechanical properties, stabilize the
67 drug interactions and prevent burst release effects. (Zhao, 2013)

68

69 For decades hydrogels have been investigated to design delivery systems for Active Pharmaceutical
70 Ingredients (APIs). They are composed of natural or synthetic polymers that could be cross linked by
71 chemical covalent bonds, or physically *via* non-covalent links, in order to form a network able to
72 control the API delivery. (Pagels, 2015) Physical cross linked hydrogels are usually obtained by
73 weak ionic interactions, in ionisable polymers such as hyaluronic acid, pectin, chitosan and alginate.
74 (Hamidi, *et al.*, 2008) Chemical hydrogels are obtained by covalent cross linking most of the time
75 using polymers modified with methacrylate derivatives (Hamidi, *et al.*, 2008; Jagadeesan, *et al.*,
76 2011; Nguyen, *et al.*, 2015; Cha, *et al.*, 2014; Bachman, *et al.*, 2015; Glaubitz, *et al.*, 2014), or using
77 cross linking reagents such as gluteraldehyde, carbodiimides, or di-halo species (Pagels,

78 Prud'homme, 2015). The presence of such species and/or the formation of bypass products during the
79 cross linking reaction, could interact with biological environment and disturb the cell metabolism.

80

81 Recently, a new family of hydrogels were functionalized with alkoxysilane groups, allowing
82 chemical cross linking, based on the hydrolysis condensation reaction of $-\text{Si}(\text{OR})_3$ into stable Si-O-Si
83 covalent links. Thus, silylated (hydroxypropyl)methyl cellulose (Si-HMPC) were developed as
84 biocompatible and injectable silylated hydrogels (Bourges, *et al.*, 2002a, 2002b; Echalier, *et al.*,
85 2016; Vinatier, *et al.*, 2009; Vinatier, *et al.*, 2007; Vinatier, *et al.*, 2009; Liu, *et al.*, 2014).
86 Interestingly, bisilylated poly(ethylene glycol) (Si-PEG) was also synthesized and exhibits relevant
87 cytotoxicity results (Echalier, *et al.*, 2016). Then, Si-hydrogels open up a wide range of applications
88 including bone repair (Zhang, *et al.*, 2016; Struillou, *et al.*, 2013), cartilage tissue engineering
89 (Buchtova, *et al.*, 2013), and treatment of chronic colonic diseases (Moussa, *et al.*, 2017). Echalier *et*
90 *al.* has developed Si-peptides hydrogels leading to biological responsive hydrogels such as adhesive
91 peptide sequence RGD, peptide sequence having antibacterial activity (Echalier, *et al.*, 2016) or
92 collagen inspired (Echalier, *et al.*, 2017).

93

94 To the best of our knowledge, there is no example of microgels obtained with silylated hydrogels,
95 and little examples of chemical microgels bearing lipidic compartments for co-encapsulation of
96 complementary APIs. Jagadeesan *et al.* have trapped hexadecane droplets in an aqueous phase
97 containing a monomer n-isopropylacrylamide, a cross linker N,N'-methylenebis(acrylamide) a
98 surfactant Brij 35), and a photoinitiator 2,2'-azobis (2-methylpropionamide) dihydrochloride.
99 (Jagadeesan, *et al.*, 2011) Recently Busatto *et al.* proposed oil in hyaluronic acid microgels for
100 enzymatic trigger release of an hydrophobic drug (progesterone). (Busatto, *et al.*, 2017) Whatever the
101 emulsification strategies, encapsulation of lipophilic components remains a complex task because of
102 the stabilization of the O/W primary emulsion (Matalanis, *et al.*, 2013) and may require approaches
103 such as Pickering emulsions. (Marquis, *et al.*, 2016)

104

105 The aim of this work was to (i) demonstrate the ability to produce microgels using silylated HPMC
106 by the way of the Sol-Gel reaction of condensation of the -SiOR functions, (ii) to functionalize them,
107 with a model function, using the same Sol-Gel reaction in a one-pot process (FMGs), and (iii) to
108 generate biphasic microgels (BPMGs) containing lipophilic compartments intended to formulate
109 poorly soluble drugs. Model drugs were used in the present work, Nile Red (NR) as lipophilic and
110 silylated Fluorescein (Si-Fluor) as chemically linked function.

111

112 **2. Materials and methods**

113

114 **2.1. Materials**

115

116 Silylated (hydroxypropyl)methyl cellulose (Si-HPMC) ($MM = 290\ 000\ \text{g}\cdot\text{mol}^{-1}$) was obtained as
117 previously described (Bourges, *et al.*, 2002b) by grafting 0.6 wt% of 3-glycidoxy-
118 propyltrimethoxysilane on E4M® grade cellulose in heterogeneous medium. It was used in its
119 hydrolyzed form at 3 wt.% in 0.2M NaOH that was done under constant stirring for 48 h and
120 sterilized by steam (121°C, 30 min). (Vinatier, *et al.*, 2007) Si-Fluor was obtained as previously
121 described (Ciccione, *et al.*, 2016) using 25 mg of fluorescein isothiocyanate stirred with 500 μL of
122 dimethyl sulfoxide (DMSO) and 3-aminopropyltriethoxysilane for 2 h in the dark at room
123 temperature and used without further purification. A mother solution was used at 170 mM in ethanol
124 and stored at -20°C for months. Refined sesame oil was purchased from Cooper (Melun, Franc. Nile
125 red (NR) was obtained from Sigma Aldrich chimie GmbH (France), $MM = 318.3\ \text{g}/\text{mol}$, and Lutrol
126 F-127 from BASF (Ludwigshaffen, Germany).

127

128 **2.2. Preparation of the functionalized microgels (FMGs)**

129

130 The W/O emulsion was obtained after the addition of the Si-HPMC solution into the oily phase. It
131 was added to the sesame oil with a controlled feeding rate of 0.1 mL/min, using a syringe pump
132 apparatus (HARVARD APPARATUS pump 33), at a ratio of 1/10 v:v. The dispersion was done

133 under high shearing stress using a disperser (Disperser T18 digital ULTRA-TURRAX), with a 10 mm
134 rotor-stator, IKA®-WERK GmbH & Co. KG (Staufen, Germany) at a speed of 5000 rpm in ice for
135 20 min. Then, the temperature was increased to 45°C and maintained for 10 min. The dispersion was
136 left to stand overnight at room temperature under mild stirring (500 rpm) for aging. Finally, FMGs
137 were washed with H₂O/ethanol (1/1 v:v) and stabilized with 0.05% Lutrol F-127, an injectable
138 surfactant, to facilitate the suspension handling.

139 The aqueous phase was composed of 1 mL of Si-HPMC solution 3 wt.%, that was neutralized with 2-
140 [4-(2-hydroxyethyl)piperazin-1-yl]ethane sulfonic acid buffer (HEPES, PM = 238,3 g/mol) at pH
141 3.5, with a volume ratio of 1:2 (Vinatier, *et al.*, 2007). The Si-HPMC hydrogel was functionalized by
142 Si-Fluor ethanolic solution that was added to the Si-HPMC phase, and kept in ice. The mixture was
143 diluted two times with fresh sesame oil to reduce the aggregation of the microgels.

144

145 **2.3. Preparation of the biphasic microgels (BPMGs)**

146

147 A O/W primary emulsion was obtained by dispersing the sesame oil solution of NR (Ultra Turrax,
148 5000 rpm for 1 min) in the Si-HPMC solution with a volumic ratio of 5%. The Si-HPMC continuous
149 phase was then neutralized and maintained in ice. To prepare the BPMGs, the O/W primary emulsion
150 was dispersed in the sesame oil continuous phase (O/W/O) with a syringe pumps apparatus
151 (HARVARD APPARATUS pump 33). The feeding rate was optimized in the range of 0.1 to 1
152 mL/min. The final volume ratio was 1/10. After this step, the process was the same as for FMGs.

153

154 **2.4. Interfacial tension and rheological properties**

155

156 The surface and interfacial tension were measured with a Krüss tensiometer K100 using the
157 Wilhelmy plate method. The experiments were performed at room temperature (20-25°C) in aqueous
158 solution of Si-HPMC in the range 0.03 wt.% and 3.75×10^{-6} wt.%.

159 The dynamic viscosity of the continuous phase was measured with a coaxial cylinder rheometer,
160 Rheomat RM 200 (Lamy, France) at 7.7°C, 21.0°C, 38.0°C and 48.0 °C. The velocity, ω , was
161 continuously increased from 64.6 to 1290.0 s⁻¹. The force exerted on the cylinder (diameter 30 mm)
162 was measured and converted to a shear stress.

163

164 **2.5. FMGs and BPMGs Characterizations**

165 The optical characterizations were performed by Epi-fluorescence microscopy on an inverted EVOS
166 ® FL, Life Technologies, laser scanning confocal microscope (LSCM) was done on a Confocal Leica
167 SP8. The scanning electronic microscopy (SEM) was done on a MEB FEI Quanta 200 environmental,
168 for the observation of the as-synthesized microgels, as well as the freeze-dried samples.

169

170 The size distribution of the FMGs, BPMGs and oil droplets were achieved with the Image J program.
171 The size was measured for at least 100 items as possible, with $n \geq 20$, and by using at least two
172 different photos. The size distribution was graphically expressed in percentage number of particles
173 and fitted by the Log Normal function where x_c gave the mean size and w the standard deviation
174 (OriginPro 8.1).

175 **2.6. Nano indentation and topographic analysis by AFM**

176 Study of the nanomechanical properties of the FMGs and BPMGs and the scanning
177 topographically of its surface was done by atomic force microscope (AFM D3100 Bruker
178 Instruments), with a Nanoscope 3A Quadrex (electronics device) in which the mode
179 contact was used without scanning (fixed point to perform approach retract curve). The tip
180 has a platinumium coverage (around 10 nm) and on the apex of a lever there are point probe
181 nanosensors with a nominal spring constant 0,4N/m (measured with thermal tune). The tip
182 is mounted on a tip-holder suitable for measurement on liquid environment. The
183 measurement of the Force, F (N), to indentation distance, δ (m), were done on hydrated
184 microgels, whose surface was totally dried before applying the AFM to prevent attachment

185 of the microgels to the tip of the cantilever. The Young's elastic modulus, E (Pa), was
186 calculated after linearization of the equation of the Sneddon model:

$$F = \frac{2}{\pi} \frac{E}{(1 - \nu^2)} \tan(\alpha) \delta^2$$

187 with the Poisson's ratio, $\nu = 0.5$, the half angle of the indenter, $\alpha = 35^\circ$. The topographic
188 observations were done after drying the samples under air overnight.

189 **2.7. Determination of the amount of Si-Fluor linked to cross linked Si-HPMC**

190

191 The amount of Si-FITC which was covalently linked, C_L , was evaluated in bulk Si-HPMC gels, at
192 different initial concentrations (C_i). The bulk Si-HPMC gels were with 0.100 mL of a solution of Si-
193 HPMC 3 wt.% that was neutralized by HEPES pH 3.5. After 2 days of aging, the bulk gels were
194 washed consequently with two saline solutions. Firstly, the bulk gels were washed with Phosphate
195 Buffer Solution (PBS) at pH 7.4 and room temperature, to remove the free Si-FITC not chemically
196 linked or weakly adsorbed to the Si-HPMC (C_{Ads}). Secondly, the gels were soaked in NaOH 0.2 M
197 pH 10. These hydrolytic conditions led to the cleavage of the Si-O-Si bonds, and allowed to extract
198 the chemically linked species or the one less adsorbed but not extracted in PBS (C_H). Then, a full
199 extraction was done by Dimethyl Sulfoxide (C_{DMSO}). The amount of FITC extracted were determined
200 by spectrofluorimetry (spectrofluorimeter RF 5000, Shimadzu) at $\lambda_{ex} = 490$ nm and $\lambda_{em} = 515$ nm. The
201 concentration of strongly linked species (C_L) was calculated according to the following equation:

$$202 \quad C_L = C_i - (C_{Ads} + C_{DMSO} + C_H)$$

203

204 **2.8. Chemical and physical stability of FMGs and BPMGs**

205 The stability of FMGs and BPMGs was evaluated in different media and followed by means of
206 dosage of Nile Red (NR) and fluorescein, microscopic observations and their mechanical properties.
207 20 mg of FMGs or BPMGs were introduced into 50 mL of each medium, namely (i) in physiological
208 simulated media, that is in Phosphate Buffer Saline (PBS) 66 mM at pH 7.4 and in HCl medium at

209 pH 1.5 for 40 days at 37°C, (ii) in alkaline hydrolytic conditions in 0.2 M NaOH at pH 10 for 1 hour
210 at room temperature, and (iii) in Dimethyl Sulfoxide (DMSO) for full extraction.

211 Samples were taken at different times and measured on the spectrofluorimeter (RF 5000, Shimadzu),
212 using $\lambda_{\text{ex}}=551$ nm and $\lambda_{\text{em}} = 609$ nm to detect the NR, and $\lambda_{\text{ex}} = 490$ nm and $\lambda_{\text{em}} = 515$ nm for
213 fluorescein.

214 3. Results and discussion

215

216 3.1. Preparation of the microgels

217

218 The microgels were obtained using an emulsion-templating method that produces water in oil
219 droplets, acting as microreactors, where the cross linking reaction could take place. As previously
220 shown by P. Weiss and co-workers, the cross linking of Si-HPMC was done in soft conditions, with a
221 maximum rate of condensation of the silanolate functions into siloxane bridges at pH 7.4 and a
222 temperature of 45°C (Fatimi, Tassin, 2009). As suggested by the authors, the gelation time increased
223 as a function of temperature which has a catalytic action on the condensation reaction. They suggest
224 also that, at high temperature (45°C or higher), additional mechanisms, such as the association of the
225 hydrophobic zones in the polysaccharide chain, can reinforce the essential network formation
226 process.

227

228 This reaction was adapted to occur in the microreactors, containing the Si-HPMC hydrogel solution,
229 in its alkaline form, $-\text{SiO}^-\text{Na}^+$ and the buffer for the neutralization. To obtain the narrower size
230 distribution and the more spherical microparticles, different parameters were carefully tuned in order
231 to separate in time the dispersion of the water phase and the reaction of condensation. The whole
232 optimizations were done on the preparation of the BPMGs. Scheme 1 summarizes the principal of the
233 formation of the functionalized microgels with the silylated fluorescein (FMGs) and the biphasic
234 microgels (BPMGs), thanks to the critical physico-chemical parameters involved in the process.

235

236 First of all, the W/O emulsion formed a microenvironment at which the condensation reaction could
237 take place. This step was common to both the preparation of the FMGs and the BPMGs, and was the
238 critical step to obtain the microgels. Indeed, it required the emulsion stability and the kinetic control
239 of the condensation reaction. The first point was insured by the unique intrinsic interfacial properties
240 of HPMC to stabilize the W/O emulsion. Indeed, aqueous solutions of Si-HPMC and HPMC
241 exhibited similar interfacial liquid/air activities, Γ , critical aggregation concentrations, CAC, and
242 Gibbs surface areas, A_{Gibbs} (Table 1) (Wollenweber, *et al.*, 2000). The mean interfacial tensions, $\gamma_{\text{o/w}}$,
243 between water and sesame oil were measured at 35.2 mN/m and was significantly decreased in
244 presence of Si-HPMC at 14.8 mN/m, allowing to stabilize the emulsion interface. Then, it was
245 possible to obtain stable emulsions without addition of extra surfactant or other chemical
246 modification to decrease the interfacial tension (Figure 1A, D).

247 Secondly, because of the kinetic aspect of the condensation reaction, the emulsification step has to be
248 carefully separated from the cross linking initiation during the preparation process. Indeed, as the pH
249 neutralization lead to the condensation of silanolate groups, even at room temperature, and form the
250 three dimensional network in about 15 min (Fatimi, Tassin, 2009), the W/O preparations were done
251 below 10°C, to delay the condensation reaction. Moreover, high shearing rate dispersion was applied
252 to favor the formation of the microreactors during the water phase dispersion. Hence, the
253 condensation reaction led to a strong increase of the dynamic viscosity, η_{disp} , of dispersed aqueous
254 phase, from 2000 Pa.s (pH 12-13, 20°C) to 20000 Pa.s (pH 7, 45°C), while it tend to slightly
255 decrease in the continuous phase from $\eta_{\text{cont}} = 69$ Pa.s to 30 Pa.s, respectively. In these conditions the
256 ratio $\eta_{\text{disp}}/\eta_{\text{cont}} \gg 1$, so that a high shearing stress was necessary to split the dispersed aqueous
257 phase into microdroplets before the cross linking occurred. When the temperature was increased up
258 to 45°C, the condensation reaction was induced.

259

260 Figure 1B shows that the formation of the microgels was only done in presence of the siloxane
261 functions, after induction of the condensation by tuning both the pH and the temperature. Si-HPMC
262 precipitations (Figure 1C) may appear even without these stimulations, but with no control of the

263 morphology. Furthermore, the formation of the Si-O-Si bridges contributed to the stability of the
264 W/O emulsion, as no droplets could be found at the end of the process when native HPMC was used
265 (Figure1E).

266 Finally, the feeding rate of the water phase was optimized because it was found to be a limiting
267 factor to obtain spherical, non-aggregated microgels with controlled size distribution. The optimized
268 microparticles were obtained for a feeding rate of 0.1 mL/min of O/W emulsion added to the oily
269 phase as shown in Figure 2. The size distribution of the microgels was centered at 46.0 μm (STD =
270 0.2; $r^2 = 0.95$). When they are filled of oil droplets, BPMGs, it was centered at 72.6 μm (STD = 0.3;
271 $r^2 = 0.84$). Here a compromise had to be done between the mean size and the distribution width.

272

273 **3.2. Evaluation of the functionalization potential of the microgels (FMGs)**

274 Fluorescein isothiocyanate modified by silanization (Si-Fluor) (Echalier, *et al.*, 2016) was chemically
275 linked to the free siloxanes functions of the Si-HPMC polymer chains of the microgels during the
276 cross linking reaction, in a one-pot process. It could be noticed that no toxic catalyzer such as
277 sodium fluoride (usually applied to catalyze sol-gel reactions) was used in this process as it could be
278 necessary for the linking of Si-Fluor in Si-PEG hydrogels even at low concentration respecting the
279 cell viability (Echalier, *et al.*, 2016). That represents a great advantage against cytotoxicity and
280 purification steps.

281 Different consecutive extraction steps were applied to the bulk Si-HPMC gels in order to estimate the
282 maximum amount of Si-FITC, C_L , which could be strongly linked to the silylated polymer (Table 2). It was
283 calculated depending on the equation $C_L = C_i - (C_{Ads} + C_{DMSO} + C_H)$. The amount of covalently linked
284 Si-FITC was obtained by subtracting from the initial concentration of Si-FITC (C_i), the sum of the
285 free Si-FITC weakly adsorbed to the Si-HPMC (C_{Ads}), the chemically linked less stable species (C_H)
286 and the amount extracted by DMSO (C_{DMSO}). DMSO is an organic solvent that could solubilize all
287 the species present, without any effect on the Si-O-Si covalent link.

288 A maximum of 1.12 μg of Si-FITC per gram of Si-HPMC was covalently linked, as no Si-FITC could be
289 detected in the PBS medium, meaning that the whole Si-FITC species added were totally linked. This
290 represents about 70 μmole of FITC per 1 mole of HPMC, taking into account a total of siloxanes functions
291 available in the hydrogel estimated to 0.6 wt%. (Fatimi, *et al.*, 2008)

292 These amounts are in good agreement with biological activities usually expected or observed, and comprises
293 in the pM to μM range of concentrations in interactions (Popp, *et al.*, 2017), cells adhesion (Wu *et al.*, 2017),
294 cancer therapy (Rui, *et al.*, 2017), or antibody recognition. (Colombo *et al.*, 2016)

295

296 **3.3. Biphasic microgels (BPMGs) preparation and Nile Red loading efficiency**

297 BPMGs were produced by following a two steps O/W/O emulsion templating process. The W/O
298 emulsion templating step was the same as for the production of the FMGs. Here we describe the
299 formation of the primary emulsion O/W that was used as a reservoir for the lipophilic model drug.
300 Figure 3 shows a microscopic description of a homogeneous primary O/W emulsion. The size
301 distribution of the oil droplets was sharpened and centered to 4.0 μm with a STD of 0.15 determined
302 by the log normal fitting curve. After freeze drying, the oil droplets were still trapped in the Si-
303 HPMC matrix (Figure 3D) in the same size range observed in primary emulsion O/W. These results
304 gave an evidence of the emulsion stability using the interfacial activity of the HPMC, that simplify
305 the chemical composition of continuous phase, usually requiring hydrophilic surfactant (Jagadeesan,
306 *et al.*, 2011) or nanoparticles in Pickering O/W emulsions. (Marquis, *et al.*, 2016)

307 SEM observations of the as-synthesized and freeze-dried BPMGs gave the proof that the oil droplets
308 were still trapped inside the Si-HPMC network (Figure 4) as it was observed in the freeze dried O/W
309 primary emulsion (Figure 3). The oil droplets were uniformly distributed in whole volume of the
310 BPMGs as shown by LSCM observations (Figure 4C). The internal texture of the Si-HPMC matrix
311 exhibited fibers of the cellulosic network, that had a diameter in the range of 10 nm. It formed a
312 porous network of a mean ladder distance of 20 nm determined on the topographic picture obtained
313 by AFM (Figure 4D).

314 Nile red (NR) was chosen as a model drug because its log P value comprises between 3.0 and 5.0
315 (Küchler, *et al.*, 2009; Merian, *et al.*, 2012; Weber, *et al.*, 2014) which is the value of 30 to 40 % of
316 the commercialized APIs (lipophilic to low lipophilic) covering a wide range of applications. (Pyka,
317 *et al.*, 2006; Pallicer, *et al.*, 2014) Sesame oil was selected because of its good solubilization power
318 for poorly soluble drugs (Ahmed, Hassan, 2007). It is injectable for subcutaneous or intramuscular
319 administration and it is used as a constituent for the preparation of multiple-emulsion formulation.
320 (Rowe, *et al.*, 2009) NR was directly loaded in the primary O/W emulsion up to saturation in the
321 sesame oil, with a maximum of 25.0 µg/mL (Table 3). Over this concentration NR diffused in the Si-
322 HPMC water phase and BPMGs formation was not possible anymore. This was ascribed to an
323 increase of the microviscosity in the cellulosic hydrogel, due to the interactions with NR that disrupt
324 the first layer of hydration (Katzhändler, *et al.*, 2000). The size distribution of the oil droplets was
325 also affected by the NR loading.

326 **3.4. Mechanical properties of the FMGs and the BPMGs**

327 The cross linking efficiency was evaluated by measuring the nanomechanical properties of the FMGs
328 and the BPMGs by the means of atomic force microscopic nano indentation (Figure 5). In addition to
329 the measurement of the hardness value, the depth-sensing indentation has been routinely used to
330 extract the elastic properties of the specimen, with known indenter geometry and material properties.
331 (Poon, *et al.*, 2008) According to Sneddon's equation, the elastic modulus E (kPa) was calculated to
332 give an indication of the hardness of the samples.

333 The elastic modulus of the FMGs was found to be 5.9 kPa which is two times higher than the value
334 previously observed for cylindrical gels Si-HPMC-Si ($\varnothing \approx 2.4$ cm) that was determined at 2.96 kPa
335 by measuring their compressive modulus. (Rederstorff, *et al.*, 2011) This confirmed the cross linking
336 efficiency inside the microdroplets of the Si-HPMC W/O emulsion. The elastic modulus of the
337 BPMGs was significantly increased up to 144 kPa due to the presence of the oily phase trapped
338 inside the cross linked Si-HPMC hydrogel. There is a great increase in the elastic modulus which is
339 an indication of its hardness. Sekine *et al.* reported that the hardness and stability of o/w/o multiple

340 emulsion was enhanced by increasing the weight ratio of the inner O/W emulsion. (Sekine, *et al.*,
341 2000)

342 These values match with elastic modulus of 2 to 204 kPa, that can be recorded on gelatin methacrylic
343 microgels ≤ 10 wt% with sizes in the same range (< 5 - $150 \mu\text{m}$) and depending on the formulation
344 parameters. (Cha, *et al.*, 2014; Nguyen, *et al.*, 2015) As comparison, living cells exhibit elastic
345 modulus comprised in a range of 0.2 - 200 kPa that depends on the differentiation stage, growth,
346 adhesion. (Kuznetsova, *et al.*, 2007) Both the size and elastic modulus of the FMGs and the BPMGs
347 were comparable to those of eukaryotic living cells. This allows good expectations for their
348 coexistence in confined physiological environment, such as injectable or implantable scaffolds.

349 **3.5. Chemical and physical stability of FMGs and BPMGs**

350 Polysaccharide-based microgels with high water content, controllable particle size and high stability
351 have been widely studied as candidates for drug release and delivery. Different release mechanisms
352 of drugs could be observed from hydrogels. (Hamidi, *et al.*, 2008) The most common mechanism is
353 passive diffusion, meaning free diffusion into/out of the hydrogel network. It depends mainly on the molecular
354 size and the mesh size of the hydrogel network, and follows the Fick's first law of diffusion. Typical porosities
355 expressed in mesh size are reported for biomedical hydrogels in the range of 5 to 100 nm (in their swollen
356 state). After comparison, it could be related to the dimensions observed on the ladder size of the Si-HPMC
357 network (Figure 4D), which are much larger than most of small molecule drugs.

358 The release of the model drugs from BPMGs was evaluated in a phosphate buffer saline (PBS) at pH 7.4 and
359 37°C to mimic standard physiological conditions. After a period of 40 days, no diffusion of fluorescein was
360 observed indicating a stable linkage to the Si-HPMC (Figure 6). No diffusion in the external medium was
361 either observed for the Nile Red. The detection threshold was found to be in the range of 0.5 pM both in
362 the PBS buffer and in DMSO. The intensities of fluorescence were in the same range from 1 μM and 25 μM
363 in both solvents. The maximum concentration of NR that could be released in the PBS was about 20 μM . So,
364 there was not a problem of detection of the NR in the aqueous medium. Moreover, some accumulation of NR
365 was observed in the Si-HPMC phase, as it can be seen in Figure 7 (B, D, F).

366 This was ascribed to a partition with the oil droplets, as described by Busatto *et al.* with progesterone
367 encapsulated in oil droplets trapped in microgels based on methacrylated hyaluronic acid. (Busatto, *et al.*,
368 2017) The NR behavior was hypothesized to adsorbed to the hydrophobic isopropyl and methyl parts of
369 HPMC. It is important to notice that this NR retention is not due to its poor water solubility but rather due to
370 the physical properties of the microgels. In fact, NR encapsulated in lipid nanoparticles (Delmas *et al.*,2012)
371 and either in nanoemulsions dispersed in chitosan hydrogel (Delmar *et al.*, 2016) showed sustained but
372 complete releases.

373 After 17 days of incubation, however, microscopic observations exhibited a loss of the spherical structure and
374 a release of the oil droplets, accompanied by a loss of the mechanical properties, oil droplets were released
375 leaving empty skin structures of cross linked microgels (Figure 7C).

376 A full extraction with an organic solvent, DMSO, allowed the extraction of the fractions of fluorescein and
377 NR supposed to be adsorbed by weak interactions in the hydrogel. DMSO did not have hydrolytic action on
378 the Si-O-Si chemical bonds, but is able to solubilize all the components of the BPMGs namely sesame
379 oil, Nile Red, fluorescein and HPMC (Cao, *et al.*, 2015). This resulted in the extraction of 85 ± 7 wt.%
380 of the total initial amount of fluorescein. This means that about 15% of the fluorescein was still
381 linked to Si-HPMC matrix. About 70 ± 7 wt.% of the initial amount of NR were also extracted (Figure
382 6).

383 Extreme values of pH (acidic pH 1.5 and alkaline pH 10.0) were applied to the BPMGs in order to evaluate
384 the chemical stability of the Si-O-Si bridges (Brinker, Scherer, 1990). The acidic conditions did not affect
385 the release. It was correlated to the strengthening of the mechanical properties of both BPMGs and
386 FMGs (Figure 7). This behavior could be related to self hardening process due to the condensation of
387 silanolates into siloxane bridges, which is promoted at low pH values (Bourges, *et al.*, 2002b)
388 (Brinker & Scherer, 1990).

389 However, in alkaline conditions, 25 ± 5 wt.% of the total amount of Si-Fluor and 15 ± 3 wt.% of the
390 total amount of NR could be extracted. The release of these fractions was then subjected to the
391 degradation of the Si-O-Si bonds. A fraction of the Si-Fluor species were released by hydrolysis of

392 the Si-O-Si bond. The oil droplets trapping the NR could be released by the hydrolytic degradation of
393 the Si-O-Si bonds involved in the Si-HPMC network. Actually, a good stability of the linkage of Si-
394 Fluor to the Si-HPMC hydrogel was observed, which will be suitable for further biological
395 functionalization of the FMGs and the BPMGs.

396 These results showed a strong retention of the model drugs, Si-Fluor and NR, in simulated
397 physiological conditions. Therefore, the hydrophobic drug control release should be obtained under
398 mechanical stimulation.

399 **Conclusion**

400 The preparation and the functionalization of microgels made of Si-HPMC were obtained by the
401 unique condensation reaction of alkoxy silane functions in physiological conditions of temperature
402 and pH. An emulsion templating process was applied using only the interfacial properties of HPMC
403 without the use of any surfactant.

404 The size distributions of the microgels are relevant with injections in implant sites for intra-articular, sub-
405 cutaneous.

406 The model function Si-Fluor was chemically linked with a good stability and in the range of amounts
407 compatible with a further biological activity.

408 Biphasic microgels were obtained with a double emulsion process for the loading of a lipophilic
409 model drug in oil droplets trapped in the hydrogel core. The oil droplets could be released after
410 incubation in physiological conditions for 17 days. Mechanical stimulation will be needed to trigger
411 further drug release.

412 Finally, it was shown that the alkoxy silane modified hydrogels represents a promising modular
413 strategy that open a new way to design and to multi-functionalize drug delivery systems in limited
414 preparation steps.

415 **Authors contribution**

416 M. Zayed, PhD fellowship, did the full preparation and characterizations of the materials. Prof. P. Weiss
417 synthesized and provided the silylated HPMC. Prof. G. Subra and Prof. A. Mehdi synthesized and provided
418 the silylated fluorescein. M. Ramonda performed the AFM measurements and Elastic modulus calculations.
419 Prof. P. Legrand, Prof. J.-M. Devoisselle and Dr C. Tourne-Peteilh supervised the processing and
420 characterizations of the elaboration of the microgels.

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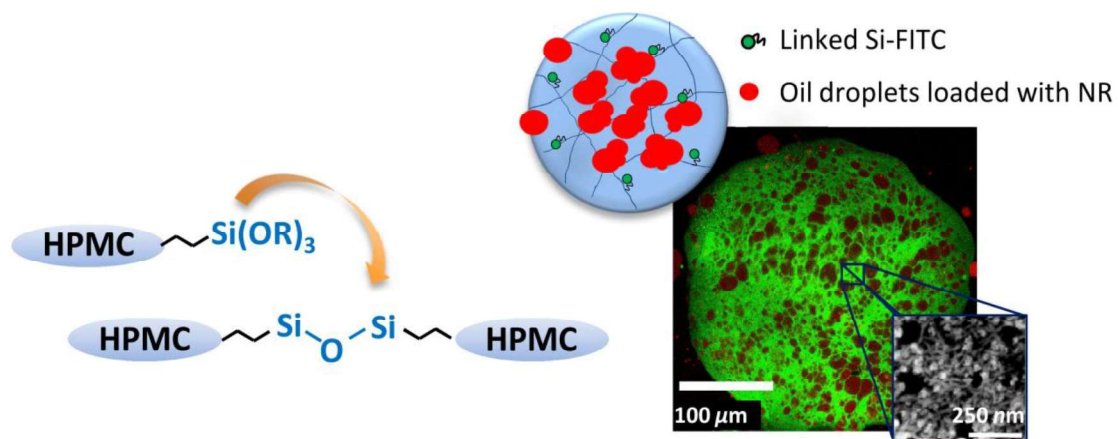
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Scheme 1: This scheme represents the protocol of preparation of FMGs and BPMGs based on the sol-gel reaction and a templating emulsification process. The pH and the temperature are the main parameters that control the chemical cross-linking reaction, leading to formation of Si-O-Si bonds. The Si-HPMC network could be functionalized with a model molecule, such as Si-FITC in FMGs. Oil compartments for lipophilic molecules encapsulation, were trapped in the BPMGs.

Table 1: Interfacial behavior of Si-HPMC versus HPMC.

Figure 1: Emulsions W/O of Si-HPMC (A) leads to the formation of the microgels only after neutralization and increasing the temperature to 45°C (B), while no spherical morphologies were obtained at pH 12 and room temperature (C). HPMC W/O emulsion (D) did not give any structures after pH and temperature treatment (pH neutralization, T°C = 45°C) (E). (bar = 400 μm)

Figure 2: Optimization of the size distribution of the microgels by adjusting the water phase in oil feeding rate, for the microgels W/O (A) and BPMGs O/W/O (B, C, D). The red curve is the Log Normal fit. (bar = 400 μm)

Table 2: Loading efficiency of Si-Fluor determined in bulk hydrogel (C_I = initial weight concentration; C_L = linked weight concentration).

Figure 3: (A) Optical microscopy of the first dispersion O/W (O = sesame oil; W = Si-HPMC 3wt%), (B) Size distribution of the oil droplets (log normal fitting curve), (C) Phase separation between the oil droplets phase (Nile Red labeling) dispersed in Si-HPMC 3 wt% sol labeled with Si-Fluor by LCSM, (D) SEM of the freeze-dried O/W emulsion.

Table 3: Loading efficiency of Nile red in the oily phase, effect on the size distribution of the oil droplets and of the BPMGs

Figure 4: SEM observations of (A) hydrated and (B) freeze-dried BPMGs, their internal phase separation (C) between the hydrophilic and the lipophilic phases by LCSM, and the topography (D) of the Si-HPMC network of dried BPMGs determined by AFM.

Figure 5: AFM nanoindentation on FMGs (black) and BPMGs (red) reporting the Force (N) to indentation distance fitted by the Sneddon model.

Figure 6: Effect of physiological and extreme pH on the extraction and release evaluation of the model drugs from the BPMGs.

Figure 7: Mechanical properties evolution of BPMGs with their optical microscopy (A, C, E) and epifluorescence (B, D, F) observations at different times of incubation : (A, B) BPMGs at D_0 , (C, D) after 17 days in PBS 60 mM pH 7.4, (E, F) after 17 days in HCl pH 1.5. bar = 200 μm

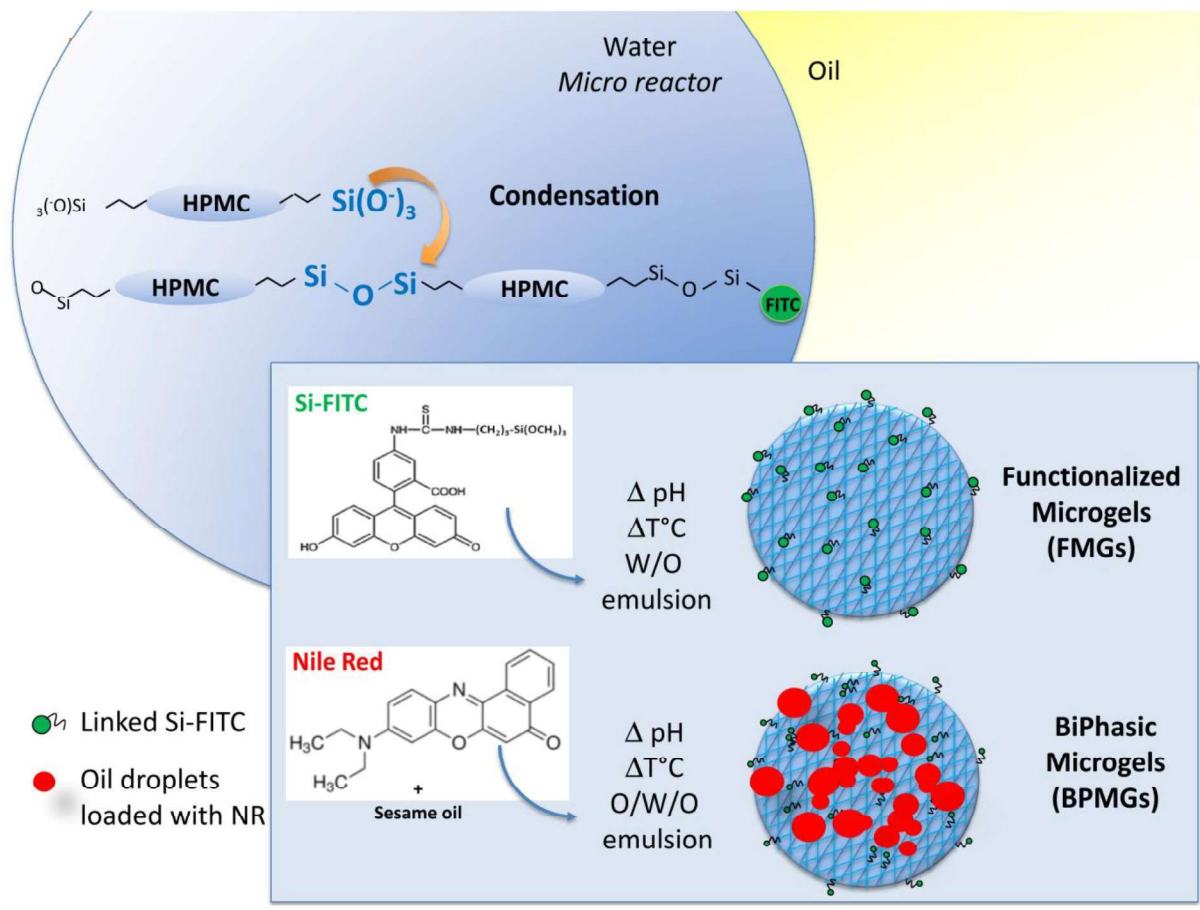


Table 1

	Γ (mol/m ²)	CAC (M)	A _{Gibbs} (nm ²)
HPMC 2910 [38]	6.9 10 ⁻⁶	1.82 10 ⁻⁷	0.24
Si-HPMC	5.4 10 ⁻⁶	5.40 10 ⁻⁸	0.31

Figure 1

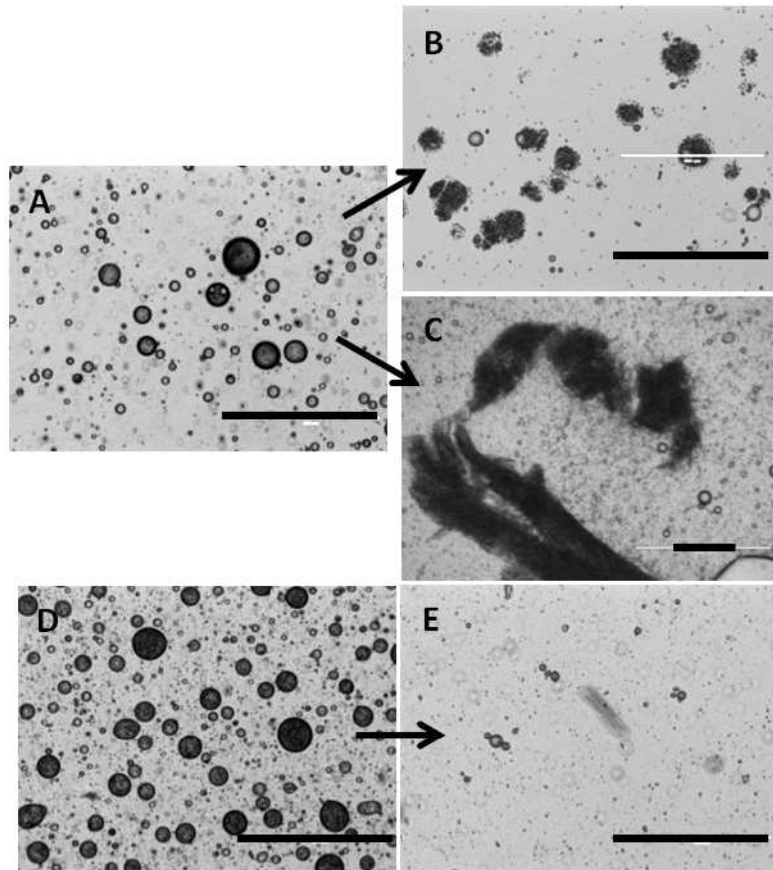


Figure 2

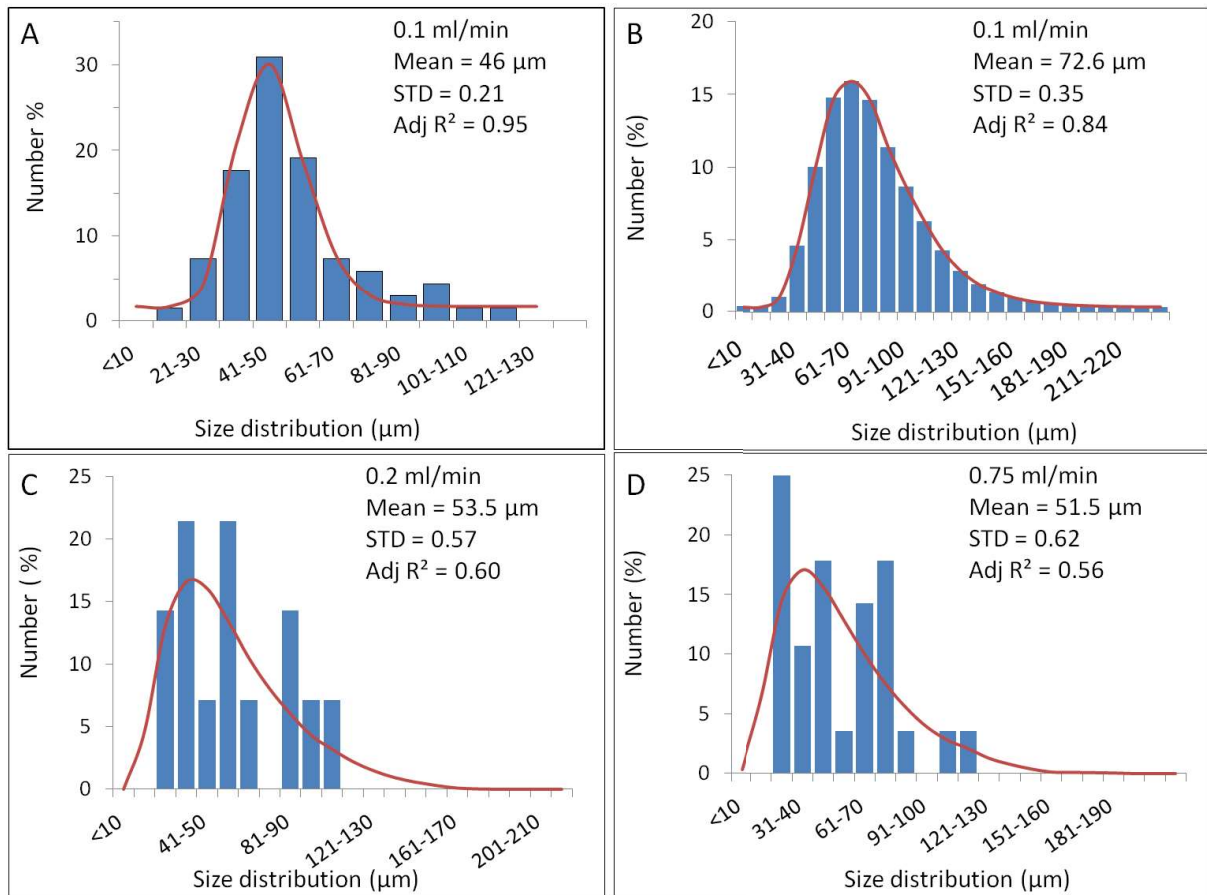


Table 2

C_i	C_L
(μg /g HPMC-Si)	(μg/g HPMC-Si)
0.09	0.09
0.45	0.44
1.12	1.12
2.23	0.73
11.12	0.80

Figure 3

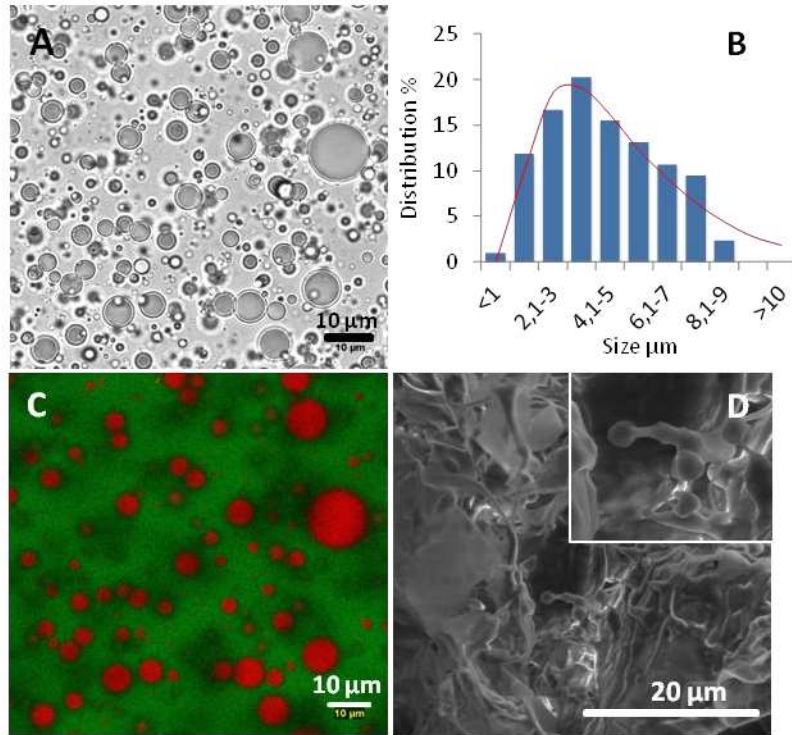


Table 3

Concentration in the oil phase (µg/ml)	O/W oil droplets		BPMGs	
	Size (µm)	STD (%)	Size (µm)	STD (%)
7.0	9.4	0.40	84.2	25
25.0	4.1	0.15	81.1	24
62.0	7.3	0.20	127.7	35
≥ 124.0	5.7	0.10	No BPMGs	No BPMGs

Figure 4

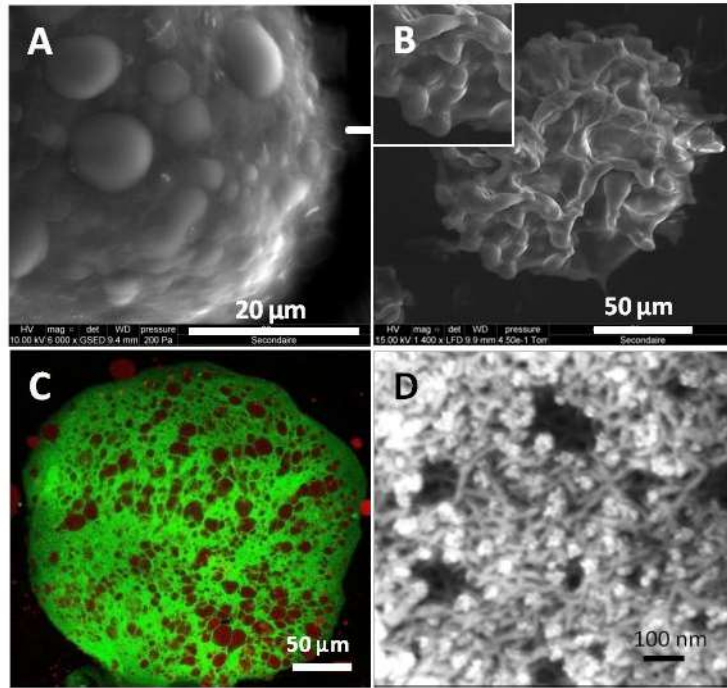


Figure 5

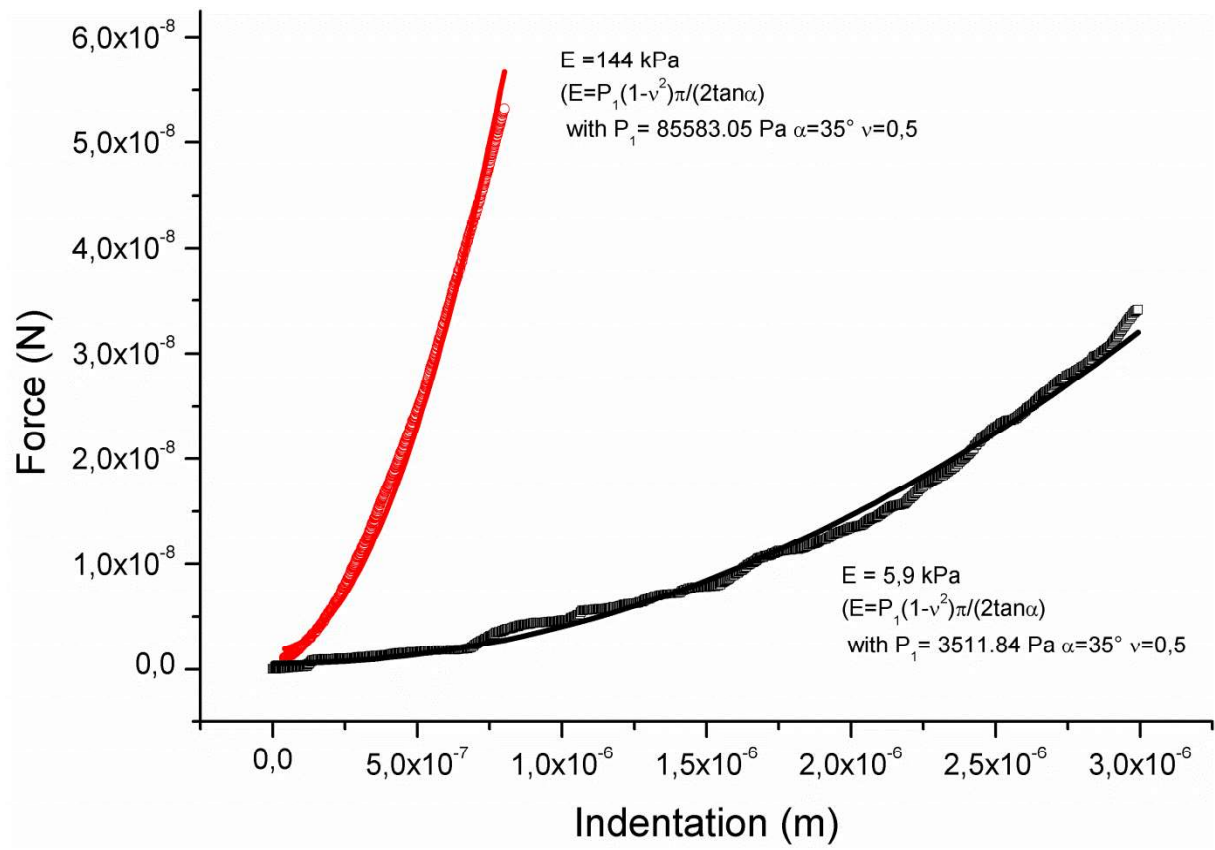


Figure 6

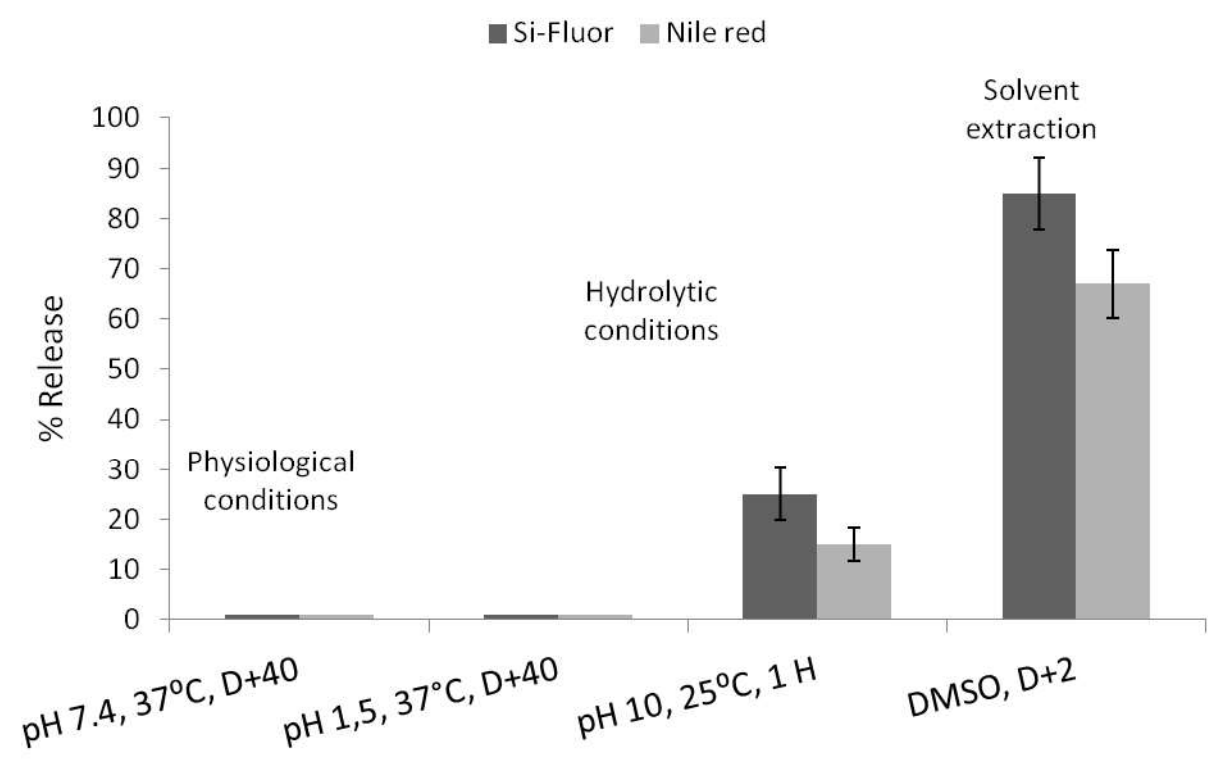


Figure 7

