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Johanne Teychené, Dalina Didacus-Prins, Nadia Chouini-Lalanne, Christophe Dejugnat, Valérie Sartor. Formulation induces direct DNA UV-A photooxidation. Part II. Pro-oxidant effect of formulated Vitamin E via generation of singlet oxygen. *Journal of Molecular Liquids*, 2020, 316, pp.113818. 10.1016/j.molliq.2020.113818 . hal-02916908

HAL Id: hal-02916908

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Formulation induces Direct DNA UV-A photooxidation. Part II. Pro-oxidant effect of formulated Vitamin E via generation of singlet oxygen

Johanne Teychené, Dalina Didacus-Prins, Nadia Chouini-Lalanne, Christophe Déjugnat, Valérie Sartor**

HIGHLIGHTS

- Cetyltriethylammonium bromide (CTEAB) and Vitamin E self-assemble in TRIS/HCl buffer solutions into nanoemulsion composed of CTEAB micelles (that can incorporate Vitamin E) as well as larger Vitamin E droplets.
- CTEAB-Vitamin E nanoemulsion interacts with plasmid DNA pBR322 and the gradual DNA compaction followed by gradual DNA decompaction for increasing CTEAB-Vitamin E amounts have been highlighted.
- UVA irradiation induces photooxidation of DNA on the formulated CTEAB-Vitamin E-DNA lipoplex and underlines the pro-oxidant role of the Vitamin E in this self-assembled system.

1 **Formulation induces Direct DNA**
2 **UV-A photooxidation. Part II. Pro-**
3 **oxidant effect of formulated Vitamin E**
4 ***via* generation of singlet oxygen**

5
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12
13 **Abstract**

14 The study focuses on the photosensitization of DNA in nanoemulsion-type systems
15 containing Vitamin E under UVA radiation. Cetyltriethylammonium bromide (CTEAB)
16 surfactant was first combined with Vitamin E to form a CTEAB-Vitamin E nanoemulsion
17 to complex the plasmid pBR322, in TRIS/HCl buffer. The self-organization properties
18 of the CTEAB-Vitamin E system were studied by complementary physico-chemical
19 characterizations (tensiometry, pyrene fluorimetry and scattering experiments). It
20 formed a nanoemulsion composed of CTEAB micelles (that can incorporate Vitamin

21 E) as well as larger Vitamin E droplets. This nanoemulsion was then contacted with
22 DNA and the resulting formulated CTEAB-Vitamin E-DNA lipoplex was irradiated under
23 UVA radiation ($\lambda \geq 335$ nm). The presence of Vitamin E generated a significant
24 enhancement of the DNA photodamage as shown by agarose gel electrophoresis and
25 quantified by photodensitometry. It underlines the pro-oxidant role of the Vitamin E in
26 this self-assembled system. A mechanistic study based on the use of specific
27 scavengers reveals that the singlet oxygen is responsible for the UVA DNA
28 photooxidation in the CTEAB-Vitamin E-DNA lipoplex.

29

30 **Keywords**

31 Surfactant; self-assembly; DNA; UVA photosensitization; Vitamin E

32

33 **1. Introduction**

34 The development of chemical models capable of preventing highly mutagenic
35 lesions induced by UV radiation is a major therapeutic challenge in the field of oncology
36 and more particularly in the field of prevention. The design of alternative systems to
37 protect people from these lesions is therefore a real challenge.

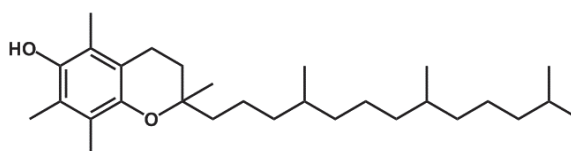
38 Reactive oxygen species (ROS) are permanently generated, transformed, and
39 consumed in all living organisms due to metabolic and biochemical reactions as well
40 as external factors. These species are responsible, directly or indirectly, for many
41 oxidative damage at the molecular level that threaten the stability and integrity of DNA,
42 cell membranes, and proteins. In the cell, antioxidant systems prevent damage caused
43 by free radicals, including alpha-tocopherol, which commonly refers to Vitamin E (VitE)
44 (Fig 1). Thanks to its antioxidant properties against ROS, VitE offers a protective effect
45 to the skin against cell membrane lipid peroxidation and DNA oxidative damage,
46 safeguarding the stability and the integrity of the cell. Therefore, the administration of
47 VitE in many pharmaceutical, cosmetic, and food products^{1,2} has been a strategy to
48 decrease DNA damage and ultimately to prevent skin disorders.^{3,4} However,
49 prooxidant and antioxidant properties of VitE are still not clearly understood. For
50 instance, Dad and al.⁵ evidenced that alpha-tocopherol is able to generate singlet
51 oxygen, a highly reactive oxygen species responsible in particular for oxidative
52 damage to DNA, when excited in the UVB region at 308 nm. Another work reports the
53 production of endogenous photosensitizer for UVA radiation after exposure of VitE to
54 UVB.⁶ Nocentiny and al.⁷ provided evidence of singlet oxygen production during the
55 photolysis of VitE under UVB. These works call into question the VitE functional
56 efficacy as antioxidant.

57

58 VitE is a lipid-soluble biological organic compound present in mammalian skin
59 and located in cell membranes (Fig.1). Its use is still limited because of its poor water
60 solubility. To overcome this problem, new stable formulations of Vit E have been
61 developed to ensure its encapsulation and transport.^{4,8} Among them, micellar solutions
62 are used as delivery systems for active ingredients. Indeed, the solubilization of the
63 active substances into micro- and nanoemulsion-type systems is an efficient way to
64 obtain small sized self-assembled aggregates, allowing the transport of the active
65 compound.^{9,10} However, the efficacy of formulated antioxidants depends on the
66 properties of the system: type of emulsion, type of oil, type of surfactant.⁸ These
67 various studies raise the question of the stability of VitE in nanoemulsion-type systems
68 under UVA radiation.

69 In this context, the purpose of this work is to extend the study of the photosensitization
70 of singlet oxygen by VitE under UVA radiation in self-assembled systems, and to study
71 the impact it could have on DNA duplex.

72



VitE

73

74 **Fig.1. Molecular structure of α -tocopherol (VitE)**

75 The self-organization properties of cetyltriethylammonium bromide (CTEAB)
76 and the CTEAB-DNA lipoplex in TRIS/HCl buffer have been described in our previous
77 paper.¹¹ Results showed that CTEAB can be used to formulate DNA and pointed out
78 also the effects of compaction on DNA photodamage under UVA radiation which
79 induce the self-photosensibilisation of DNA.

80 In the present study, we developed a cationic nanoemulsion system including
81 CTEAB to deliver the active principle VitE as an integral part of the emulsion. More
82 precisely, we used VitE simultaneously as oil phase and as active ingredient, a kind of
83 2-in-1 self-formulated VitE.

84 In a first part, we present the self-organization properties of CTEAB and VitE in
85 the buffer solution used as solvent for DNA. The determination of the critical
86 aggregation concentration (CAC) (by tensiometry and pyrene fluorimetry) and the
87 depiction of the self-assembled aggregates (by scattering experiments) will be shown.
88 Then, the interactions between the CTEAB-VitE formulation – later called as
89 {CTEAB@VitE} – and DNA will be characterized to check that {CTEAB@VitE} can be
90 used to formulate DNA (by tensiometry and pyrene fluorimetry). Finally, the effects of
91 UVA irradiation on these formulations will be investigated by agarose gel
92 electrophoresis. Results will show that the formulations can affect the integrity of DNA
93 due to DNA photooxidative damage. The mechanistic interpretation of the results will
94 be highlighted by the use of peculiar scavengers.

95 **2. Materials and methods**

96 **2.1 Materials**

97 Cetyltriethylammonium bromide (CTEAB) was synthesized as described in our
98 previous article.¹¹ α -tocopherol (Vitamine E or VitE) in racemic form, heparin sodium
99 salt, and sodium azide (NaN_3) were purchased from Alfa Aesar. Agarose and tert-
100 butanol ($t\text{BuOH}$) were obtained from Sigma-Aldrich. Pyrene was purchased from Acros
101 Organics and Ethidium bromide (EB) from Pharmacia Biotech PLUSONE as a 10
102 $\text{mg}\cdot\text{mL}^{-1}$ aqueous solution. Eurisotop furnished D_2O and BioLabs the T4 endonuclease
103 V (pyrimidine dimer glycosylase) enzyme. The reagents were used as received.
104 Tris(hydroxymethyl)aminomethane (TRIS) and supercoiled plasmid DNA (Form I)
105 pBR322 (4361 base pairs) were purchased from Thermo Fisher Scientific. The DNA
106 was used after dilution in a 20 mM TRIS/HCl buffer ($\text{pH} = 7.4$) to obtain a stock solution
107 at $[\text{DNA-PO}_4^-] = 4.5\times 10^{-4} \text{ mol}\cdot\text{L}^{-1}$. All DNA concentrations indicated in the manuscript
108 are in $\text{mol phosphate}\cdot\text{L}^{-1}$.

109 Water used in all experiments was produced by a two-stage Milli-Q filtration
110 system from Millipore and had a resistivity higher than $18.2 \text{ M}\Omega\cdot\text{cm}$.

111 **2.2 {CTEAB-VitE} preparation**

112 Colloidal dispersions {CTEAB-VitE} were formulated using a technique based
113 on the preparation of liposomes by the thin film hydration method.^{12,13} Preparations
114 were obtained by mixing CTEAB and VitE, both dissolved in ethanol. The solution was
115 then evaporated to dryness using a rotary evaporator and the film thus obtained was
116 re-dispersed in a 20 mM TRIS/HCl solution ($\text{pH} = 7.4$). The final CTEAB concentration
117 was $8.33\times 10^{-4} \text{ mol}\cdot\text{L}^{-1}$, corresponding to more than 5 times the CMC value of CTEAB
118 in TRIS/HCl ($1.5\times 10^{-4} \text{ mol}\cdot\text{L}^{-1}$).¹¹ The final concentration of VitE was $3.58\times 10^{-4} \text{ mol}\cdot\text{L}^{-1}$

119 corresponding to a VitE / CTEAB concentration ratio of 0.43. This ratio corresponded
120 to the maximal solubility of formulated VitE in the aqueous solution.

121 **2.3 Surface tension measurements**

122 Surface tension measurements were performed using a Krüss EasyDyne
123 tensiometer using the Wilhelmy plate method. Temperatures were maintained at 25 °C
124 (± 0.1 °C) by circulating thermostated water through a jacketed vessel containing the
125 solution. For each experiment, surface tension was measured three times (each time
126 ten readings were performed and averaged) after an equilibration time of 30 minutes.

127 **2.4 Fluorescence measurements**

128 A stock solution of pyrene (10^{-4} mol·L⁻¹), was prepared in methanol and stored
129 at 4 °C. The solutions for fluorescence probing studies were prepared by adding 1 μ L
130 of this pyrene stock solution to 399 μ L of the sample solution. Final concentration of
131 pyrene was 2.5×10^{-7} mol·L⁻¹, while the amount of methanol remained very low (0.25 %
132 vol.). Fluorescence measurements were performed using a Fluorolog Horiba
133 spectrofluorimeter, with a thermostated cuvette holder at 25 ± 0.1 °C. Excitation
134 wavelength was 335 nm, and the fluorescence emission was recorded from 350 to 500
135 nm.

136 **2.5 Multi-angle light scattering experiments**

137 The measurements were performed on a 3D LS Spectrometer from LS
138 Instruments (Fribourg, Switzerland) equipped with a 100 mW high performance DPPS
139 Laser (Cobolt) operating at $\lambda = 660$ nm, a two-channel multiple tau correlator (auto and
140 cross correlation, 1088 real time channels), a 3D cross-correlation module, a variable-
141 angle detection system, two high sensitivity APD detectors, an automated laser
142 attenuation system combined with on-line laser intensity measurement, a temperature

143 controllable sample chamber with index matching vat, and an external circulator for
144 temperature control (Julabo CF31).

145 Before analysis, samples (1.1 mL) were placed into cylindrical scattering cells
146 (10 mm diameter), either directly or after filtration through hydrophilic PTFE filters (450
147 or 200 nm porosity), as specified in the text.

148 Measurements were performed at 25°C for scattering angles θ ranging from 12°
149 to 150°, corresponding to scattering wave vectors q in the range 2.7×10^{-4} - 2.4×10^{-3} Å⁻¹.
150 As samples produced multiple scattering, the 3D cross-correlation configuration was
151 required.¹⁴

152 At each angle, 3 to 10 measurements were carried out and later averaged after
153 having discarded eventual meaningless correlograms. Analysis of both dynamic- and
154 static light scattering measurements were performed using the home-made MULTI-
155 STORMS software developed by Dr. C. Mingotaud (see *Supporting Information*).

156 **2.6 Small-Angle x-ray Scattering (SAXS) experiments**

157 SAXS measurements were conducted on a Xeus 2.0 SAXS beamline for
158 laboratory from Xenocs (FERMAT federation, Toulouse), with an internal copper
159 source producing 8 keV x-rays (wavelength = 1.54 Å). Signals were recorded using a
160 Pilatus 1M pixel detector from Dectris. The sample-to-detector distance was varied
161 from 387.5 mm to 1687.5 mm so that the covered q range was $0.0042 < q < 0.7$ Å⁻¹.
162 Two scans of 120 min were recorded at each distance and averaged. The observed
163 intensity $I(q)$ was corrected for transmission, solvent, and detector response. Absolute
164 intensity $I(q)$ (cm⁻¹) was obtained after standard normalization. The analysis of the $I(q)$
165 profile was realized using the SasView software (<http://www.sasview.org/>), version
166 4.2.2.

167 **2.7 Irradiation experiments**

168 DNA irradiation was made with a Mercury Xenon lamp (Oriel instruments,
169 500W) equipped with a long pass filter $\lambda \geq 335\text{nm}$ (Newport 20CGA-335 GC505) at
170 20°C . The irradiated and non-irradiated sample volumes were $100\ \mu\text{L}$. The final
171 concentrations in TRIS/HCl buffer (20 mM, pH=7.4) were $9 \times 10^{-5}\ \text{mol phosphate} \cdot \text{L}^{-1}$ for
172 the plasmid DNA pBR322, $5 \times 10^{-4}\ \text{mol} \cdot \text{L}^{-1}$ for CTEAB, $2.15 \times 10^{-4}\ \text{mol} \cdot \text{L}^{-1}$ for VitE,
173 $200 \times 10^{-3}\ \text{mol} \cdot \text{L}^{-1}$ for NaN_3 , and 4% v/v for $t\text{BuOH}$. Each experiment was at least
174 triplicated.

175 **2.8 Agarose gel electrophoresis**

176 **2.8.1 Sample preparation**

177 $50\ \mu\text{L}$ of the sample solution were used in the T4 endonuclease V experiments.
178 It consists to add $10\ \mu\text{L}$ of T4 endonuclease V (10000 U/ml) to $50\ \mu\text{L}$ of DNA containing
179 sample and to incubate the solution 30 min at 37°C . $5\ \mu\text{L}$ of bromophenol blue solution
180 and $10\ \mu\text{L}$ of Heparin 20% (w/w in TRIS/ HCl buffer (20 mM, pH= 7.4) are then further
181 added.

182 For the samples without T4 endonuclease V treatment, $5\ \mu\text{L}$ of bromophenol
183 blue solution, $10\ \mu\text{L}$ of Heparin 20% (w/w in TRIS/ HCl buffer), and $10\ \mu\text{L}$ of 20 mM
184 TRIS/HCl (pH= 7.4) were added to $50\ \mu\text{L}$ of formulated DNA.

185

186 **2.8.2 Electrophoresis and photodensitometry**

187 An aliquot of $40\ \mu\text{L}$ of each sample was deposited into 0.8% agarose gel
188 containing 0.02 % of Ethidium bromide. Electrophoresis migration was carried out for
189 4 hours under a constant electric field of 80 V.

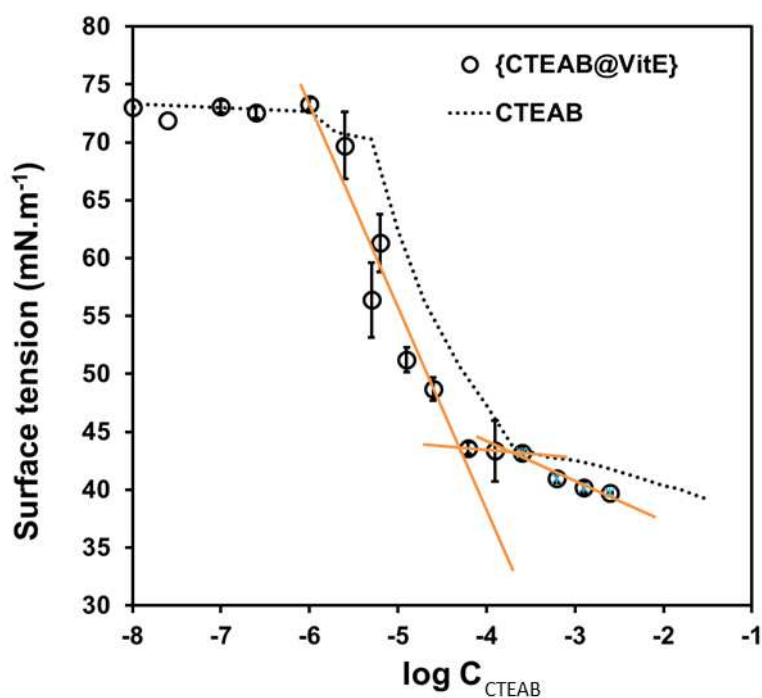
190 Single strand breaks (SSB) were quantified by photodensitometry using the
191 ImageJ software. To correct the lower efficiency of Ethidium bromide binding to Form
192 I DNA with respect to Form II, a coefficient of 1.66 was used.¹⁵

193 3. Results and discussion

194 3.1. Self-organization of CTEAB and VitE in TRIS/HCl buffer

195 The critical aggregation concentration (CAC) measurement has been carried
196 out by two complementary methods (tensiometry and fluorimetry) in {CTEAB@VitE}
197 mixtures.

198 Surface tension as a function of CTEAB concentration in presence of Vitamin E
199 in TRIS/HCl buffer solution is shown in Fig. 2, along with the curve of pure CTEAB for
200 comparison.



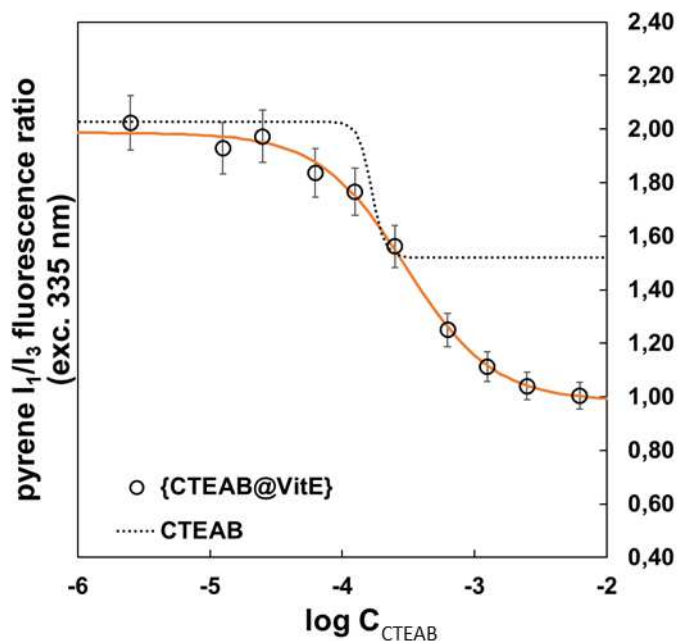
201
202 **Fig. 2.** Surface tension as a function of surfactant concentration in 20 mM TRIS/HCl
203 buffer, pH = 7.4, 25°C. Open circles: {CTEAB@VitE} mixture. Dotted line: pure CTEAB.

204 11

205 In the dilute regime, the surface tension value is equal to the one of water and then it
206 decreases as the concentration of surfactant increases to finally reach a plateau. The
207 surface tension curve of {CTEAB@VitE} mixture exhibits two break points. The first
208 one corresponds to the CAC, from which the {CTEAB@VitE} complex is formed. The
209 second one is the CMC of free remaining CTEAB. By this technique, the CAC of
210 {CTEAB@VitE} and the CMC of CTEAB in {CTEAB@VitE} mixture are found to be
211 $4.8 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$ and $2 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$ respectively. After the {CTEAB@VitE} complex is
212 formed, at a CTEAB concentration of $4.8 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$, the micellization of free
213 remaining CTEAB in the mixture occurs at a CTEAB concentration of $2 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$.
214 Hence, $4.8 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$ corresponds to the difference between $2 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$ and
215 $1.5 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$. That means that the CMC of CTEAB in the {CTEAB@VitE} mixture
216 or in pure form remains the same ($1.5 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$).

217 The surface tension in the mixture is lower than that of the pure CTEAB,
218 indicating the formation of a cooperative complex between CTEAB and VitE. The later
219 attracts more surfactant at the air/solution interface.

220 The aggregation properties studies of {CTEAB@VitE} in TRIS/HCl buffer have
221 also been carried out by pyrene fluorescence measurements. The I_1/I_3 pyrene
222 fluorescence ratio (I_1 at 383 nm and I_3 at 393 nm respectively) are reported in Fig. 3
223 as a function of CTEAB concentration for {CTEAB@VitE} in TRIS/HCl buffer. For
224 comparison, the I_1/I_3 pyrene fluorescence variation is also represented for CTEAB in
225 buffer solution. To determinate the CAC, results have been adjusted by a sigmoidal
226 Boltzmann function.¹⁶



227
 228 **Fig. 3.** Pyrene I₁/I₃ fluorescence ratio as a function of surfactant concentration in 20
 229 mM TRIS/HCl buffer, pH = 7.4, 25°C (excitation 335 nm). Open circles: {CTEAB@VitE}
 230 mixture. Dotted line: CTEAB.¹¹

231
 232 The I₁/I₃ ratio in the {CTEAB@VitE} system changes sharply around the CAC,
 233 *i.e.* about $2.5 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$. This value is quite close to the $2 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$ value obtained
 234 by surface tension measurements. The I₁/I₃ ratio of the pyrene spectra, for high CTEAB
 235 concentrations, is directly related to the apparent dielectric constant of the probe
 236 environment in the micelles; both values are reported in Table 1.¹⁷

237

VitE / CTEAB concentration ratio	I_1/I_3 ratio (for high CTEAB concentrations)	Apparent dielectric constant ϵ
0	1.5	54.5
0.43	1.0	19.2

238

239

240 **Table 1.** Ratio of the first and third vibronic peak of pyrene (I_1/I_3) in micelles as a

241 function of Vitamin E / CTEAB concentration ratio in 20 mM TRIS/HCl buffer, pH = 7.4,

242 25 °C. Apparent dielectric constant ϵ calculated from the correlation $I_1/I_3 = 0.0145 \epsilon +$

243 0.7022 .¹⁷

244

245 The dielectric constant value in {CTEAB@VitE} is 19.2, which is much lower
246 than the value obtained in the pure CTEAB solution (54.5). In {CTEAB@VitE}, the
247 probe microenvironment is less polar than in pure CTEAB solution, meaning that the
248 amount of water in the micelles decreases as the VitE concentration increases.
249 Therefore, the addition of VitE promotes the formation of hydrophobic domains in this
250 system.

251 In summary, close CMC values are obtained whether or not the presence of
252 VitE in solution. This might suggest that VitE has only a slight impact on the self-
253 assembly of CTEAB. However, the presence of VitE creates a less polar environment
254 than in pure surfactant micelles, meaning that water has been ejected from the
255 micelles. Thus, this seems to indicate that the water that hydrated the CTEAB head
256 groups was ejected and replaced by VitE, leading to the formation of mixed
257 CTEAB/VitE micelles.

258 Further characterizations of the aggregates were conducted by the means of
259 scattering experiments (light and x-rays).

260 Multi-angle dynamic- and static light scattering (MADLS and MASLS, respectively)
261 were first performed on the {CTEAB@VitE} system which will be later put in contact
262 with DNA (*i.e.* [CTEAB] = 8.33×10^{-4} mol·L⁻¹ and [VitE]/[CTEAB] = 0.43). The sample
263 was slightly opalescent and preliminary measurements indicated that multiple
264 scattering occurred. For this reason, the 3D cross-correlation technique¹⁴ was used to
265 overcome this problem by discarding the multiple scattering contribution from the total
266 scattered light.
267 In light scattering, the scattering wave vector q is defined as a function of the scattering
268 angle θ as described in (1).

$$q = \frac{4\pi n \sin(\theta/2)}{\lambda} \quad (1)$$

269 where n is the refractive index of the solvent and λ the wavelength of the incident light.

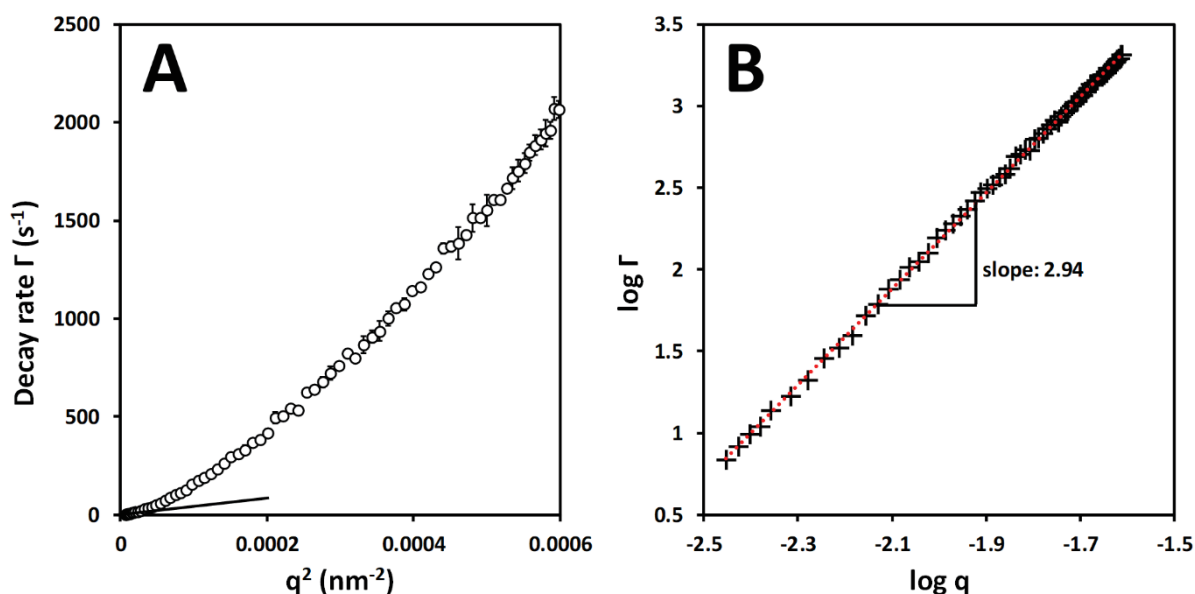
270 MADLS measurements afford the autocorrelation function of the scattered light
271 intensity, normalized over time. A mathematical adjustment then gives access to the
272 decay rate Γ which is related to the translational diffusion coefficient D_0 ($\Gamma = D_0 \cdot q^2$ for a
273 monodisperse system with only translational diffusion). Finally, the hydrodynamic
274 radius R_h is then given by the Stokes-Einstein relationship (2):

$$R_h = \frac{kT}{6\pi\eta D_0} \quad (2)$$

275 where k is the Boltzmann constant, T the absolute temperature, and η the solvent
276 viscosity.

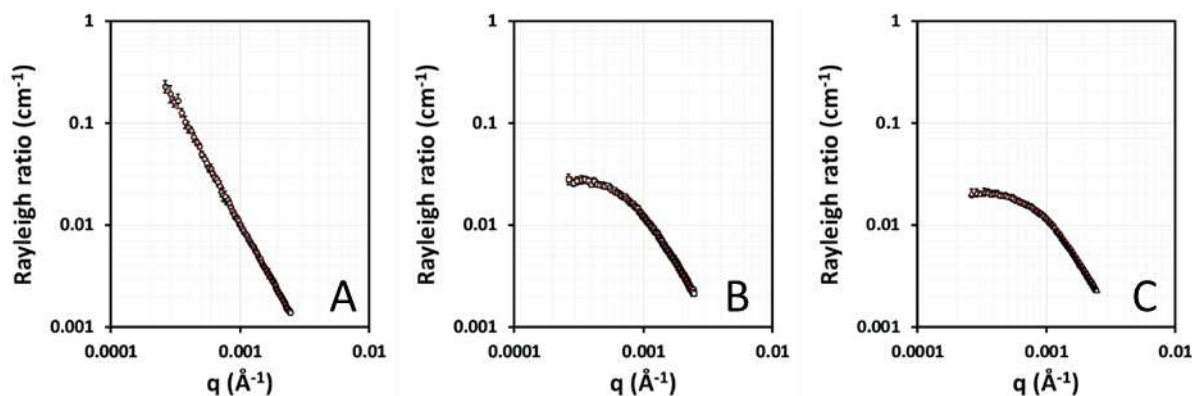
277 The experimental correlation functions (shown in the *Supporting Information, Figure*
278 *S1*) could be well fitted in the whole range of wave vectors by using a 2nd order
279 Cumulant analysis, which afforded the corresponding decay rates. Plot of the first
280 decay rate Γ as a function of q^2 is presented in Fig. 4A. Obviously $\Gamma = f(q^2)$ was not a
281 linear function, indicating that the relaxation motion recorded by DLS was not solely

282 the translational diffusion coefficient of the scattering particles. It could rather include
 283 additional internal motions (such as local rotations for example), probing the particles
 284 dynamics at a length scale smaller than their hydrodynamic radius.¹⁸ Even at very low
 285 q values, data could only roughly be fitted by a line passing through the origin, whose
 286 slope afforded an estimation of an apparent lateral diffusion coefficient $D_{app} \approx 4 \times 10^5$
 287 $\text{nm}^2 \cdot \text{s}^{-1}$, corresponding to an apparent hydrodynamic radius $R_{h,app} \approx 600$ nm. At higher
 288 q values, the q^n dependence of Γ ($n > 2$) could be well described by plotting $\log \Gamma =$
 289 $f(\log q)$, showing a linear dependence with slope $n = 2.94$ (Fig. 4B). This value is very
 290 close to the asymptotic Zimm limit ($n = 3$),¹⁹ which is reached in the case of polymer
 291 chains or fractal aggregates under strong hydrodynamic interactions.^{20–22}
 292



293
 294 **Fig. 4.** (A): decay rate Γ (s^{-1}) as determined from a 2nd order Cumulant analysis, plotted
 295 as a function of q^2 from MADLS measurements of {CTEAB@VitE}; open circles are
 296 the experimental data and the line is the linear fit at very low q values. (B): plot of \log
 297 Γ as a function of $\log q$ showing a linear dependence with a slope very close to 3.
 298

299 As no polymer was present in the {CTEAB@VitE} system, we could hypothesis the
300 presence of a fractal association or a network of strongly (attractive) interacting
301 particles formed by irreversible diffusion-limited aggregation.²³ Therefore, the
302 observed high value of R_h could correspond to long-range assembled structures rather
303 than to elementary aggregates. To get further insights on this self-assembled system,
304 we performed MADLS measurements on samples filtered at 450 nm and 200 nm
305 respectively. Data treatments showed that the slopes in the $\log \Gamma = f(\log q)$ curves
306 decreased and got closer to 2. At very low q values, the $\Gamma = f(q^2)$ variation was even
307 linear, allowing more accurate determination of translational diffusion coefficients and
308 hydrodynamic radii, which were found to tend to $R_h = 120$ nm (Figures S3, S5,
309 *Supporting Information*). Complementary multi-angle static light scattering (MASLS)
310 characterizations were performed on these samples from 12° to 150° ($q = 2.6 \times 10^{-4}$ -
311 $2.4 \times 10^{-3} \text{ \AA}^{-1}$). Absolute scattered intensities $I(q)$ expressed as Rayleigh ratios (see
312 *Supporting Information*) are reported in Figure 5.



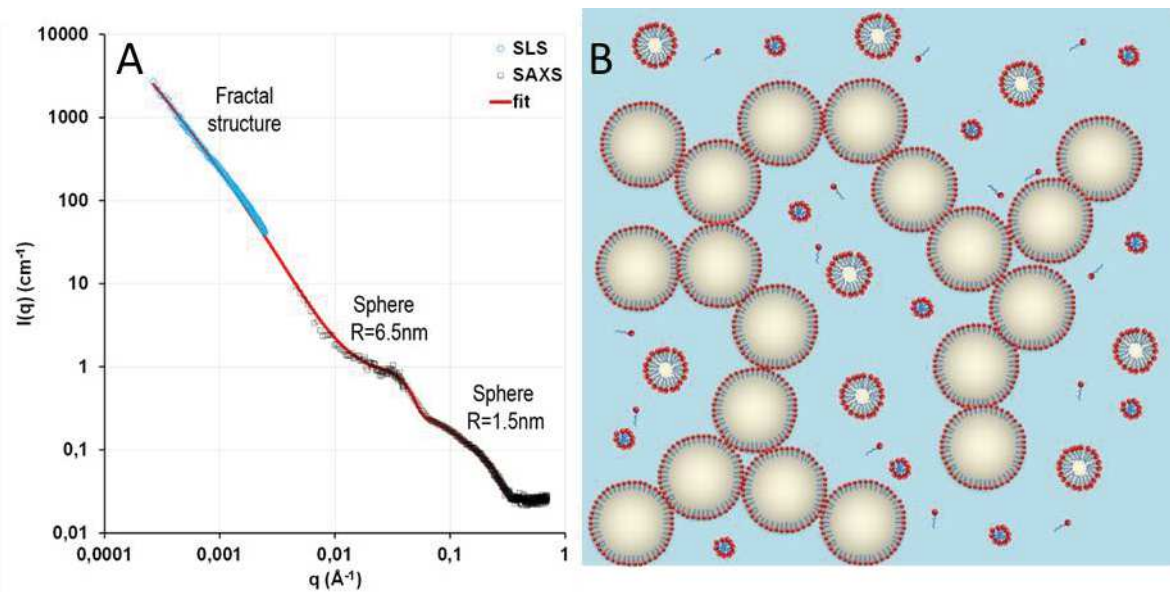
313
314 **Fig. 5.** Absolute scattered light intensities as obtained by MASLS measurements for
315 native {CTEAB@VitE} (A) or after filtrations at 450 nm (B) or 200 nm (C). Red dotted
316 lines are mathematical adjustments.

317

318 Regarding the native sample (unfiltered) (Fig. 5A), the scattering profile showed a $q^{-2.3}$
319 regular variation and could be well adjusted using a fractal model. Indeed, the fractal
320 dimension 2.3 determined from the SLS curve corresponds to a compact fractal
321 system.^{24–26} Upon filtration, the scattering profiles changed and exhibited a plateau at
322 low q values followed by a q^{-2} variation at higher q values (Fig. 5B and 5C). This
323 suggests that these measurements are better describing individual particles (or
324 primary aggregates) forming the fractal network. General Guinier-Porod fits afforded a
325 radius of gyration $R_g = 115$ nm, while the scattering profile of the sample filtered at 200
326 nm could also be fairly well adjusted using a spherical form factor ($R = 143$ nm)
327 associated to a sticky hard sphere structure factor (see models descriptions in the
328 *Supporting Information*). Altogether, these light scattering experiments support the
329 hypothesis of a nanoemulsion system where spherical VitE droplets exhibit strong
330 attractive interactions that lead to a fractal organization at higher scale.

331 Complementary techniques were then used to get further information about this
332 system, namely small-angle x-ray scattering (SAXS). Preliminary experiments
333 indicated that the sample was not concentrated enough to get reliable results. We then
334 had to consider a much more concentrated sample (30 times more concentrated than
335 the initial system). Before SAXS analysis, we first checked by light scattering that the
336 concentrated sample could be representative of the initial one, as not dramatic
337 differences could be evidenced between the two samples by MADLS and MASLS,
338 even upon filtration (Fig. S6, *Supporting Information*). SAXS measurements are
339 reported in Fig. 6A together with MASLS data recorded on the same sample. The
340 overall spectrum could then be adjusted using a multi-contribution function, including
341 a fractal system and two distinct populations of spherical objects with two different
342 sizes (See *Supporting Information*). The overall description of the system then leads

343 to a fractal network of large oil VitE droplets together with very small CTEAB empty
344 micelles (R = 1.5 nm) but also swollen CTEAB micelles containing VitE in their core
345 (R=6.5 nm). This multi-scale description is in accordance to what reported in literature
346 in the case of oil-in-water nanoemulsions obtained by a low-energy method such as
347 the solvent inversion technique,^{9,27} and is depicted in Figure 6B.

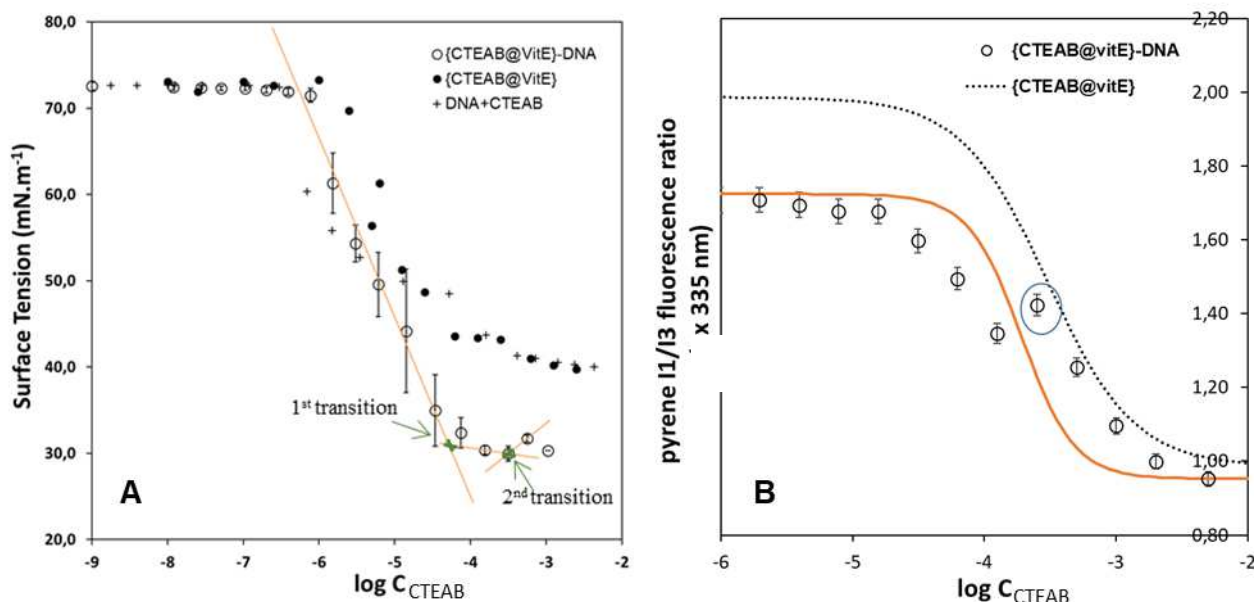


348
349 **Fig. 6.** (A): Combination of MASLS (blue open circles) and SAXS (black open squares)
350 measurements of the {CTEAB@VitE} nanoemulsion, and the corresponding overall fit
351 (red curve) indicating the presence of different aggregates. (B): Schematic
352 representation of the nanoemulsion formed by the {CTEAB@VitE} system, as deduced
353 from scattering experiments.

354 355 3.2. Interaction between DNA and {CTEAB@VitE} aggregates

356 The use of {CTEAB@VitE} aggregates as non-viral vectors for DNA is of
357 concern because of its great potential in gene therapy for the development of protective
358 (antioxidant) formulations. In this context, different techniques were carried out to study
359 the interactions between the {CTEAB@VitE} aggregates and the model plasmid DNA
360 pBR322.

361 The effect of DNA on the {CTEAB@VitE} aggregates was investigated using
 362 tensiometry (Fig. 7A) and fluorimetry (Fig. 7B) for increasing CTEAB concentrations
 363 (VitE / CTEAB concentration ratio = 0.43).



364
 365 **Fig. 7.** (A): Surface tension measurements of DNA solutions as a function of CTEAB
 366 concentration in {CTEAB@VitE}, in 20 mM TRIS/HCl buffer, pH = 7.4, 25 °C
 367 ([VitE]/[CTEAB] = 0.43, [DNA-PO₄⁻] = 9.10⁻⁵ mol·L⁻¹). Open circles: {CTEAB@VitE} –
 368 DNA mixture. Filled circle: {CTEAB@VitE}–DNA mixture. Cross: DNA+CTEAB
 369 mixture.¹¹
 370 (B): Pyrene I₁/I₃ fluorescence ratio in aqueous solution of DNA as a function of CTEAB
 371 concentration in {CTEAB@VitE}, in 20 mM TRIS/HCl buffer, pH = 7.4, 25 °C
 372 ([VitE]/[CTEAB] = 0.43, [DNA-PO₄⁻] = 9.10⁻⁵ mol·L⁻¹). Empty circles: {CTEAB@VitE} –
 373 DNA mixture. Dotted line: {CTEAB@VitE} mixture.

374
 375 The surface tension curve of {CTEAB@VitE}-DNA lipoplex (Fig. 7A) is
 376 underneath the two other curves (DNA + CTEAB¹¹ and {CTEAB@VitE}), suggesting
 377 that the complexation between CTEAB, VitE, and DNA has a strong effect on the

378 surface tension. The surface tension of the {CTEAB@VitE}-DNA solution sharply
379 decreases until a first transition appears at a CTEAB concentration of $5.2 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$
380 1 ($\log C = -4.2$), corresponding to the CAC obtained in {CTEAB@VitE} nanoemulsion
381 ($4.8 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$). Interestingly, further addition {CTEAB@VitE} between CTEAB
382 concentration of $5.2 \times 10^{-5} \text{ mol} \cdot \text{L}^{-1}$ ($\log C = -4.2$) and $3.2 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$ ($\log C = -3.5$),
383 results in a constant surface tension, suggesting a synergistic aggregation between
384 DNA and {CTEAB@VitE}.²⁸ Then, the surface tension seems to increase slightly and
385 shows a second transition at $3.2 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$ ($\log C = -3.5$), that might correspond to
386 the free DNA depletion from the air/water interface.²⁸

387 To complement these results, pyrene I_1/I_3 emission fluorescence ratio as a function of
388 the CTEAB concentration is presented in Fig. 7B in {CTEAB@VitE}-DNA lipoplex. A
389 roughly sigmoidal shape related to a phase transition and a formation of hydrophobic
390 domains is observed. For low CTEAB concentrations, the I_1/I_3 ratio was found to be
391 1.70 instead of 2.00 in the {CTEAB@VitE} system. This decrease means that pyrene
392 has found to locate in more hydrophobic places. Indeed, different works reported
393 pyrene can interact via partial intercalation with DNA.²⁹ Below the phase transition,
394 the increase in {CTEAB@VitE} concentration is accompanied by an increase in
395 turbidity, observed macroscopically. Previous work¹¹ has shown that the increase in
396 turbidity could be associated with strong electrostatic interactions between the DNA
397 and the surfactant, suggesting a gradual compaction of DNA. Progressive compaction
398 refers to a state where individual {CTEAB@vitE} aggregates decorate the DNA and
399 form hydrophobic domains on the DNA strand. The sharp I_1/I_3 ratio decrease can be
400 associated with the critical aggregation concentration that occurs for a CTEAB
401 concentration of $2.5 \times 10^{-4} \text{ mol} \cdot \text{L}^{-1}$ ($\log C = -3.6$) and which corresponded to the 2nd
402 transition observed by tensiometry (Fig. 7A). This CAC evidences again the synergic

403 cooperation between {CTEAB@VitE} and DNA. This remarkable point (Fig.7B, circled
404 point), corresponding to an increase in I_1/I_3 ratio, also matches with a turn from a turbid
405 solution to a limpid solution. This concentration value is in line with the transition
406 observed in surface tension measurements ($3.2 \times 10^{-4} \text{ mol}\cdot\text{L}^{-1}$). The transition from a
407 turbid to a limpid solution could indicate the formation of another type of
408 {CTEAB@VitE}-DNA complex due to a large excess of CTEAB. Notably, it could
409 correspond to a DNA decompaction process, as already reported in the literature for
410 excess amount of cationic surfactant.³⁰⁻³² This transition in {CTEAB@VitE}-DNA
411 system appears at much lower CTEAB concentration than the one reported in CTEAB-
412 DNA system¹¹ (between 20 and $30 \times 10^{-4} \text{ mol}\cdot\text{L}^{-1}$). The presence of vitamin E in the
413 complex seems to promote the DNA decompaction.

414 The I_1/I_3 ratio value of 0.96, obtained for high {CTEAB@VitE} concentrations in
415 {CTEAB@VitE}-DNA system, corresponds to a micellar dielectric constant of 17,
416 meaning that the pyrene environment is apolar. This dielectric constant value is
417 identical as the one obtained for the {CTEAB@VitE} formulation, suggesting that the
418 presence of DNA does not affect the local micropolarity of pyrene environment, which
419 remains in both systems hydrophobic.

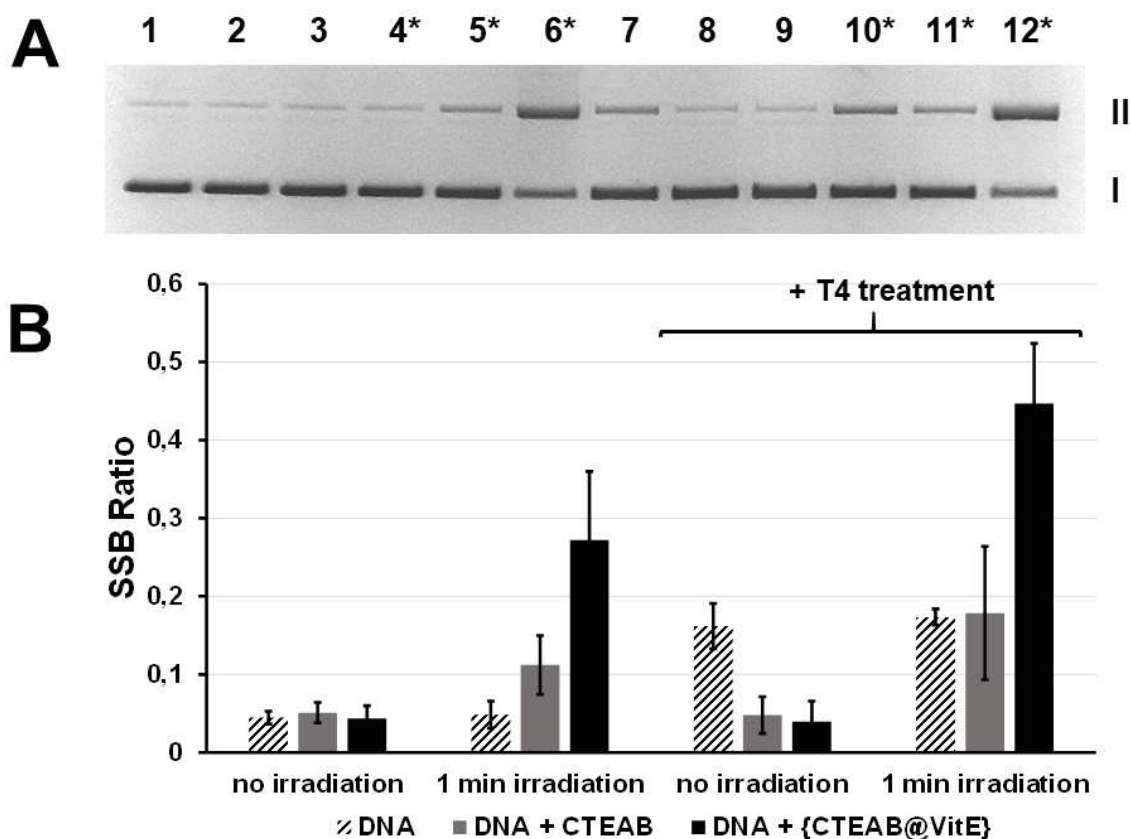
420

421 3.3. Photochemistry

422 Photostability of non-viral vectors is an important concern regarding their topical uses.
423 The {CTEAB@VitE}-DNA lipoplex was formed with a DNA model, the plasmid pBR322,
424 and the nanoemulsion {CTEAB@VitE} described above. Its photostability under UVA
425 irradiation ($\lambda \geq 335$ nm) was studied by agarose gel electrophoresis. Indeed,
426 photodegradation of plasmid pBR322 in supercoiled form (or Form I) induces the
427 formation of single strand breaks (SSB) leading to its relaxed circular form (or Form II).
428 This results in two distinct bands on the agarose gel electrophoresis, noted as I (Form
429 I) and II (Form II) on the gel pictures. In addition, each band can be quantified by
430 photodensitometry.

431 The solutions were irradiated one minute. With this time of irradiation, and in presence
432 of {CTEAB@VitE}-DNA complex, DNA photodamage are well quantified while no
433 overbreaks are observed. (Fig. 8A, lane 6*) UVA irradiation of the {CTEAB@VitE}-DNA
434 lipoplex (Fig. 8A, lane 6*) induces around a threefold increase of the SSB ratio (Fig.
435 8B) compared to CTEAB-DNA complex (Fig. 8A, lane 5*). The presence of Vitamin E
436 on the lipoplex generates a significant enhancement of the DNA photodamage. As the
437 formation of singular oxygen by CTEAB-DNA complex by a Triplet-Triplet energy
438 transfer mechanism has been previously reported,¹¹ we have investigated the
439 formation of damage to DNA that may be caused by such a mechanism, namely the
440 formation of CPD and the formation of singular oxygen to determine the mechanism
441 involved in this photodegradation. First, the samples were treated by a phage T4
442 endonuclease V enzyme (T4), a DNA enzyme repair that cleaves double-strand DNA
443 at cyclobutane pyrimidine dimer (CPD), leading to single strand breaks by a two-step
444 mechanism. In this study, if CPD is formed by photosensitization and the plasmid
445 pBR322 treated with T4, single strand break will be made and the Form II of the

446 plasmid will appear on the electrophoresis agarose gel. T4 treatment on non-irradiated
447 plasmid pBR322 reveals the presence of abasic lesions by AP-lyase activity of the T4
448 (Fig. 8A, lane 7 and Fig. 8B). Without irradiation, in presence of CTEAB or
449 {CTEAB@VitE} and with T4 treatment, the SSB ratio decreases dramatically (lanes 8
450 and 9 respectively), as already shown for CTEAB-DNA lipoplex.¹¹ The hypothesis was
451 an enzyme quenching in presence of the CTEAB or the non-access to the DNA double
452 strand to the enzyme because of its compaction. It was also previously observed that
453 after 10 min irradiation, the T4 treatment revealed again the DNA photodamage.¹¹ In
454 these experiments and for a lower time of irradiation (1 min), the photooxidative
455 damage of the plasmid induced by the CTEAB alone are limited (lane 5*). That's what
456 the SSB ratio observed should be due, only to abasic lesions already present in native
457 DNA, and to the CPD formed under irradiation. After T4 treatment of the sample (lane
458 11*), the SSB ratio becomes again equivalent to the SSB ratio for DNA alone (lane
459 10*) and the native DNA abasic site level is recovered and no increase of the SSB ratio
460 is observed in presence of CTEAB and one minute of irradiation, compared to DNA
461 alone (Fig. 8B). No CPD are formed and the T4 enzyme works again if the sample is
462 irradiated. The small amount of damage formed by the irradiation seems enough to
463 induce some modification on the DNA compaction and to allow recovering the
464 enzymatic activity of the T4 endonuclease V.



465
 466 **Fig. 8.** DNA cleavage for 1 min of irradiation at $\lambda \geq 335\text{nm}$ and 20°C with CTEAB (5×10^{-4}
 467 $\text{mol}\cdot\text{L}^{-1}$) or {CTEAB@VitE} after or not phage T4 endonuclease V treatment (T4) (*
 468 indicates the irradiated samples). A: Electrophoresis agarose gel of plasmid pBR322
 469 (9×10^{-5} $\text{mol}\cdot\text{L}^{-1}$) in 20 mM TRIS/HCl buffer (pH = 7.4). Lanes 1 and 4*: DNA alone;
 470 lanes 2 and 5*: DNA + CTEAB; lanes 3 and 6*: DNA + {CTEAB@VitE}; lanes 7 and
 471 10*: DNA alone + T4; lanes 8 and 11*: DNA + CTEAB + T4; lanes 9 and 12*: DNA +
 472 {CTEAB@VitE} + T4. B: Single strand break (SSB) ratio of DNA, CTEAB-DNA and
 473 {CTEAB@VitE}-DNA lipoplexes \pm T4 with and without irradiation.

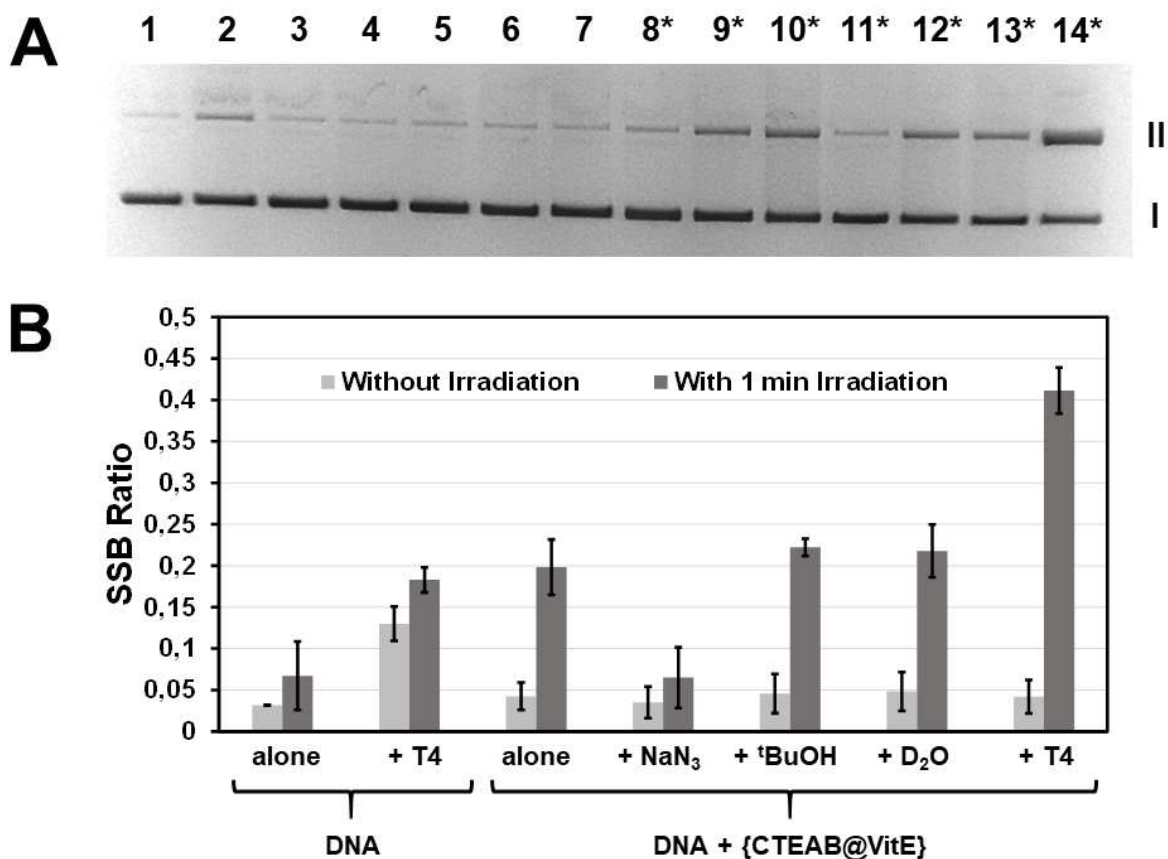
474
 475 The same behavior is observed for the {CTEAB@VitE}-DNA lipoplex. Without
 476 irradiation and after T4 treatment, no CPD are revealed (lane 9) and with irradiation
 477 and T4 treatment, the SSB ratio corresponds to addition of native abasic site level and

478 photooxydative damage due to the irradiation (lane 12*). Thus, no significant CPD
479 formation is shown in our experimental conditions in presence of the {CTEAB@VitE}
480 nanoemulsion and under UVA irradiation.

481 Three specific scavengers were also used to understand the mechanism of the DNA
482 photodamage by the {CTEAB@VitE} nanoemulsion. The sodium azide (NaN_3) reveals
483 the role of the singlet oxygen in the DNA photodamage by quenching it, D_2O also by
484 increasing the singlet oxygen lifetime and the *tert*-Butanol ($t\text{BuOH}$) shows the
485 implication of the hydroxyl radical species by quenching them (Fig. 9). In a previous
486 paper,¹¹ it was shown that singlet oxygen was responsible for the oxidative damage in
487 CTEAB-DNA complex. For the {CTEAB@VitE}-DNA lipoplex under UVA irradiation
488 and with NaN_3 (lane 11*), the SSB ratio observed decreases dramatically, compared
489 to {CTEAB@VitE}-DNA lipoplex alone (lane 10*) and until to be equal to the one for
490 DNA alone (lane 8*). It means that the singlet oxygen is again involved on the
491 photooxydation of the plasmid DNA by the {CTEAB@VitE} nanoemulsion. D_2O was
492 also used to bear out the formation of singlet oxygen and its involvement on the
493 photodegradation process. On lane 13* and Fig. 9B, a slight increase of the SSB ratio
494 is observed for the sample on D_2O . By means of *tert*-Butanol, the involvement of
495 hydroxyl radical species can be shown. In presence of *tert*-Butanol, the SSB ratio stays
496 equivalent to the SSB ratio for the {CTEAB@VitE}-DNA lipoplex alone. The hydroxyl
497 radical species are not involved on the oxidative damage of the plasmid DNA.

498 Consequently, the singlet oxygen is the major responsible for the direct UVA
499 photooxydation of DNA on the {CTEAB@VitE}-DNA lipoplex. The mechanism of this
500 process is a type II mechanism by triplet triplet energy transfer from the
501 {CTEAB@VitE}-DNA lipoplex to the ground state of the molecular oxygen leading to
502 the formation of the reactive singlet oxygen.

503 This study highlights the role of the vitamin E as a pro-oxidant species on the
 504 {CTEAB@VitE}-DNA lipoplex by favoring the direct UVA photooxydation of the DNA
 505 duplex.



506
 507 **Fig. 9.** DNA cleavage for 1 min of irradiation at $\lambda \geq 335\text{nm}$ and 20°C with
 508 {CTEAB@VitE} and NaN_3 ($200 \times 10^{-3} \text{ mol}\cdot\text{L}^{-1}$), $^t\text{BuOH}$ (4% v/v), in D_2O (80% v/v) or
 509 phage T4 endonuclease V treatment (T4) (* indicates the irradiated samples). A:
 510 Electrophoresis agarose gel of plasmid pBR322 ($9 \times 10^{-5} \text{ mol}\cdot\text{L}^{-1}$) in 20 mM TRIS/HCl
 511 buffer (pH = 7.4). Lanes 1 and 8*: DNA alone; lanes 2 and 9*: DNA + T4; lanes 3 and
 512 10*: DNA + {CTEAB@VitE}; lanes 4 and 11*: DNA + {CTEAB@VitE} + NaN_3 ; lanes 5
 513 and 12*: DNA + {CTEAB@VitE} + $^t\text{BuOH}$; lanes 6 and 13*: DNA + {CTEAB@VitE} in
 514 D_2O ; lanes 7 and 14*: DNA + {CTEAB@VitE} + T4. B: Single strand break (SSB) ratio

515 of + {CTEAB@VitE}-DNA lipoplex \pm NaN₃, ^tBuOH, D₂O or T4 with and without
516 irradiation.

517 **4. Conclusion**

518 Various studies raise the question of the stability of Vitamin E in nanoemulsion-type
519 systems under UVA radiation. In this work, the photosensitization of singlet oxygen by
520 Vitamin E into self-assembled systems under UVA was studied. The cationic surfactant
521 CTEAB has shown to be an efficient complexing agent to DNA. CTEAB surfactant was
522 then used to form a Vitamin E-containing nanoemulsion that could interact with DNA.

523 The self-organization properties of the CTEAB and vitamin E were studied by different
524 physico-chemical characterizations (tensiometry, pyrene fluorimetry, and scattering
525 experiments). The overall description of the system leads a complex fluid composed
526 of different aggregates ranging from CTEAB micelles to a fractal network of large oil
527 Vitamin E droplets. The study of the interactions between CTEAB-Vitamin E and
528 plasmid DNA highlighted the gradual DNA compaction followed by gradual DNA
529 decompaction for increasing CTEAB-Vitamin E amounts. Vitamin E seems to promote
530 DNA decompaction.

531 Formulated CTEAB-vitaminE-DNA lipoplex were irradiated under UVA light. Presence
532 of vitamin E generates a significant enhancement of the DNA photodamage. The
533 mechanistic interpretation of the results has been highlighted by the presence of
534 peculiar scavengers. It reveals that the singlet oxygen is responsible of the direct UVA
535 photooxydation of DNA on the CTEAB-Vitamin E-DNA lipoplex. This study underlines
536 the role of vitamin E as a pro-oxydant species by favoring the UVA DNA
537 photooxydation. From a safety point of view, a warning is necessary regarding the
538 topical pharmaceutical or cosmetic applications of such a formulation.

539 **Acknowledgements**

540 This work benefited from the use of the SasView application, originally developed
541 under NSF award DMR-0520547. SasView contains code developed with funding from
542 the European Union's Horizon 2020 research and innovation program under the
543 SINE2020 project, grant agreement No 654000.

544 The research federation FERMAT (Université de Toulouse, France) is acknowledged
545 for providing access to the Xeuss 2.0 SAXS instrument (Xenocs) through the CPER I-
546 MATECBIO 2015-2020 grant funded by the Région Occitanie and Toulouse Métropole,
547 and Dr. P. Roblin from the Laboratoire de Génie Chimique de Toulouse is thanked for
548 technical support in SAXS measurements.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Supporting information for:

Formulation induces Direct DNA UV-A photooxidation. Part II. Pro-oxidant effect of formulated Vitamin E *via* generation of singlet oxygen

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1. Multi-angle dynamic light scattering (MADLS)

In MADLS the measured autocorrelation function $g^{(2)}(q, \tau)$ of the scattered intensity $I(q, t)$ normalized over time is expressed as (1):

$$g^{(2)}(q, \tau) = \frac{\langle I(q, t)I(q, t + \tau) \rangle}{\langle I(q, t) \rangle^2} \quad (1)$$

It affords the correlation function of the electric field $g^{(1)}(q, \tau)$ using the Siegert relationship (2):

$$g^{(2)}(q, \tau) = 1 + \beta |g^{(1)}(q, \tau)|^2 \quad \beta \leq 1 \quad (2)$$

In the ideal case of monodisperse spherical particles under Brownian motion, the autocorrelation function decays exponentially over time (3):

$$g^{(1)}(q, \tau) = e^{-\Gamma\tau} \quad (3)$$

where $\Gamma = D_0 q^2$ is the decay rate and D_0 is the translational diffusion coefficient of the particles. Real situations are more complex and different algorithms can afford the decay rate from $g^{(1)}(q, \tau)$, among them Cumulant, non-negatively constrained least squares (NNLS), and CONTIN analysis. For this purpose, we used a home-made software (namely MULTI-STORMS, written and compiled with MATLAB® by Dr. C. Mingotaud). It offers the choice in the methods used to analyze the correlograms mathematical adjustments are performed using different algorithms based on the shape and the scattering properties of the particles to be analyzed.

The hydrodynamic radius of equivalent sphere (R_h) is then obtained by the Stokes-Einstein relationship (4):

$$R_h = \frac{kT}{6\pi\eta D_0} \quad (4)$$

where k is the Boltzmann constant, T the absolute temperature, and η the solvent viscosity.

Experimentally, multiple scattering was observed when measuring the {CTEAB@VitE} system (slightly opalescent) and its dilutions. The 3D cross-correlation configuration was then used to eliminate the multiple scattering contribution.¹ The

experimental autocorrelation functions measured on {CTEAB@VitE} are shown in Figure S1. The curves were well fitted with a 2nd order Cumulant analysis² (5):

$$g^2(\tau) = B + \beta \cdot e^{-2\bar{\Gamma}\tau} \cdot \left(1 + \frac{\mu_2}{2!}\tau^2 - \frac{\mu_3}{3!}\tau^3 + \dots\right)^2 \quad (5)$$

where B is the background value, β the amplitude of the autocorrelation function, $\bar{\Gamma}$ the mean value of the decay rates and μ_n the n^{th} moment of the distribution function of decay rates, $f(\Gamma)$. This analysis provided the intensity-weighted decay rates Γ , that did not vary linearly with respect to q^2 . It indicated several contributions in the overall apparent diffusion coefficient D_{app} (translations, internal motions such as rotations, etc.).

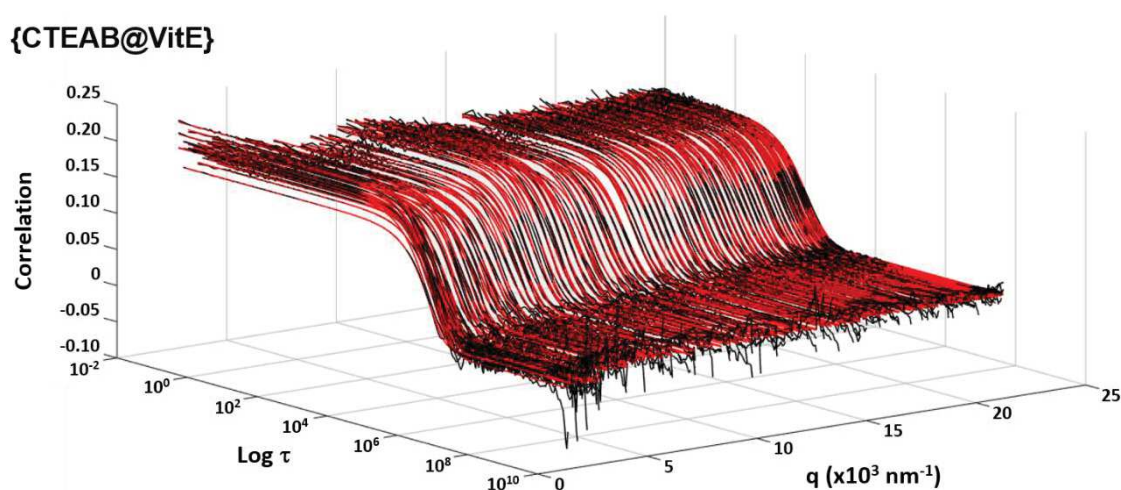


Figure S1. Auto-correlation functions obtained from MADLS measurements of the {CTEAB@VitE} system (black curves) and the corresponding fits using a second-order Cumulant analysis (red curves).

Similar measurements were performed in the same conditions on {CTEAB@VitE} samples after filtrations at 450 nm and 200 nm. Correlograms were also well adjusted with a 2nd order Cumulant analysis. Measurements and analysis are shown in Figures S2 and S3 (filtration at 450 nm) and in Figures S4 and S5 (filtration at 200 nm).

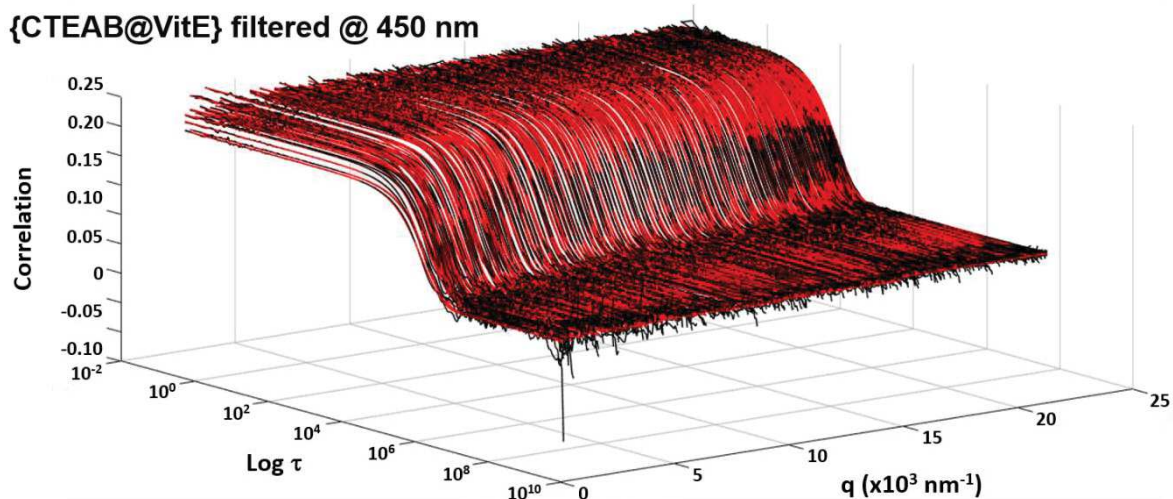


Figure S2. Auto-correlation functions obtained from MADLS measurements of {CTEAB@VitE} filtered at 450 nm (black curves) and the corresponding fits using a second-order Cumulant analysis (red curves).

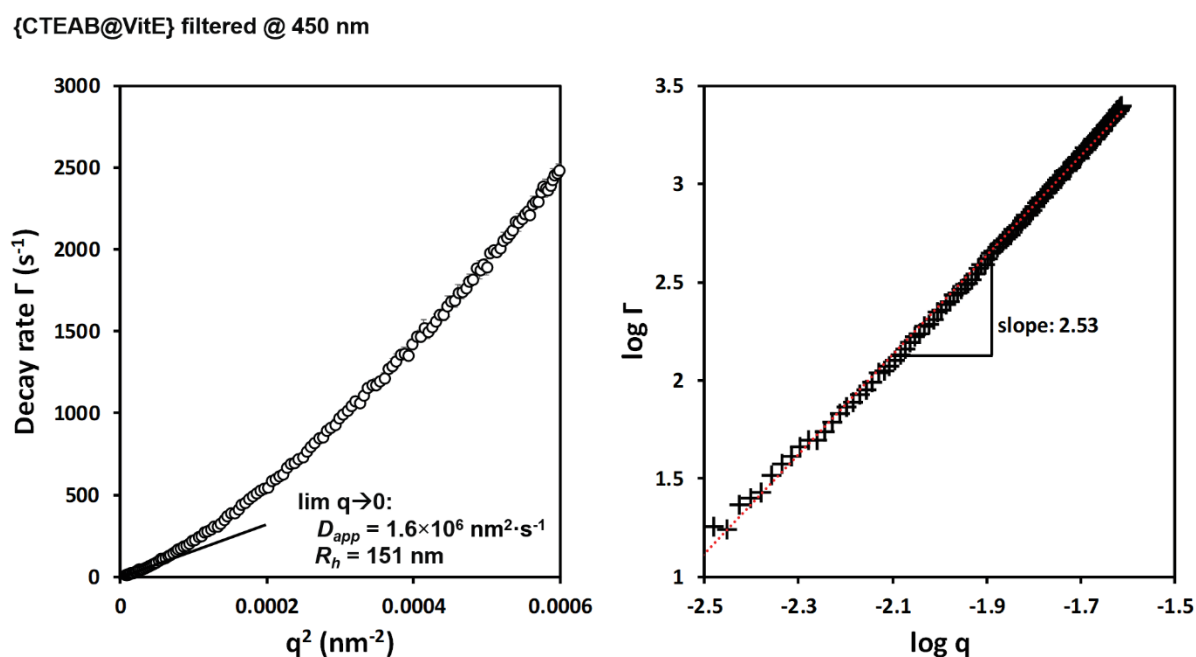


Figure S3. (left): decay rate Γ (s^{-1}) as determined from a 2nd order Cumulant analysis, plotted as a function of q^2 from MADLS measurements of {CTEAB@VitE} filtered at 450 nm; open symbols are the experimental data and the line is the linear fit at very low q values. (right): plot of $\log \Gamma$ as a function of $\log q$ showing a linear dependence.

{CTEAB@VitE} filtered @ 200 nm

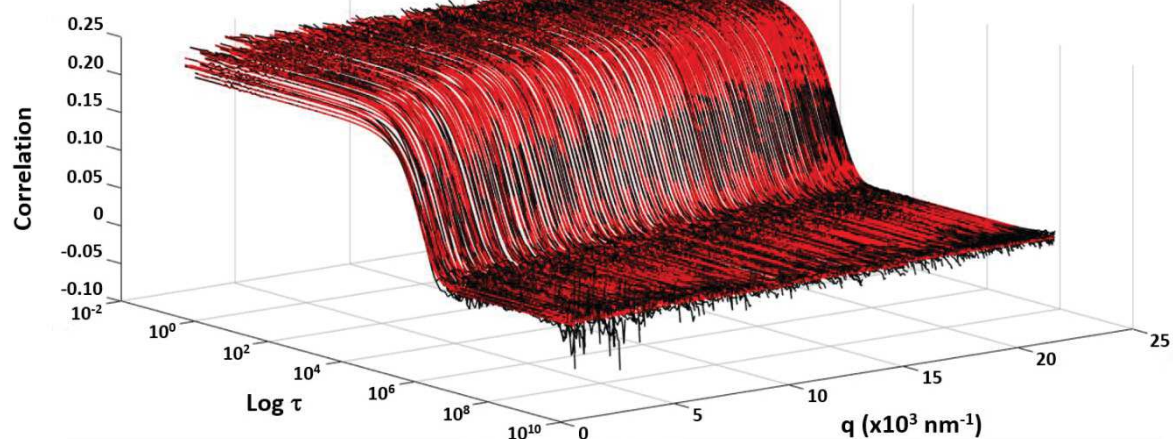


Figure S4. Auto-correlation functions obtained from MADLS measurements of {CTEAB@VitE} filtered at 200 nm (black curves) and the corresponding fits using a second-order Cumulant analysis (red curves).

{CTEAB@VitE} filtered @ 200 nm

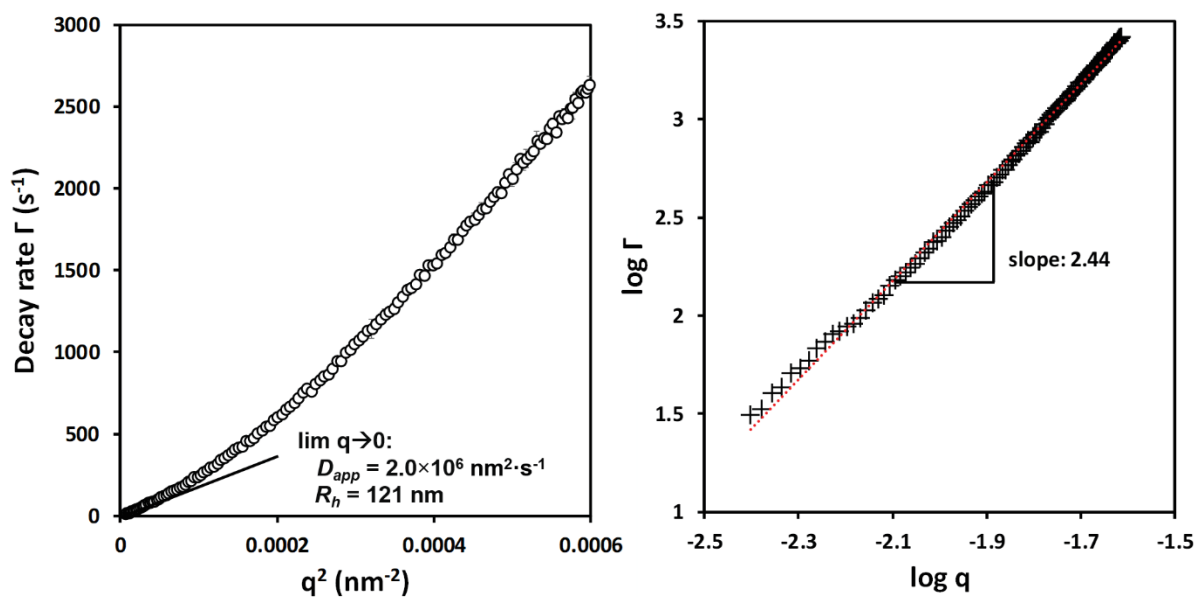


Figure S5. (left): decay rate Γ (s^{-1}) as determined from a 2nd order Cumulant analysis, plotted as a function of q^2 from MADLS measurements of {CTEAB@VitE} filtered at 200 nm; open symbols are the experimental data and the line is the linear fit at very low q values. (right): plot of $\log \Gamma$ as a function of $\log q$ showing a linear dependence.

2. Multi-angle static light scattering (MASLS)

In MASLS experiments, the excess of scattered intensity with respect to the solvent is converted into absolute intensity (Rayleigh ratio R_θ , in cm^{-1}) using toluene as a reference, for which the excess Rayleigh ratio is known ($R_{\theta,\text{toluene}} = 1.19 \times 10^{-5} \text{ cm}^{-1}$ at 660 nm)³ as described in equation (6):

$$R_{\theta,\text{sample}} = \frac{I_{\text{sample}} - I_{\text{solvent}}}{I_{\text{toluene}}} \cdot \left(\frac{n_{\text{solvent}}}{n_{\text{toluene}}} \right)^2 \cdot R_{\theta,\text{toluene}} \quad (6)$$

where I_{sample} , I_{solvent} , and I_{toluene} are the scattering intensities of the sample solution, the solvent, and the toluene respectively, and where n_{solvent} and n_{toluene} are the refractive indexes of the solvent and the toluene at 660 nm, respectively ($n_{\text{water}} = 1.33$ and $n_{\text{toluene}} = 1.49$ at 25 °C).

MASLS profiles were then fitted using the SasView software (version 4.2.2.) applying different models:

- (i) a general, empirical Guinier-Porod fit to estimate the size and the dimensionality of scattering aggregates and defined as (7-9):⁴

$$I(q) = \begin{cases} \frac{G}{q^s} \cdot \exp\left\{\frac{-q^2 R_g^2}{3-s}\right\} & q \leq q_1 \\ \frac{D}{q^d} & q \geq q_1 \end{cases} \quad (7)$$

$$q_1 = \frac{1}{R_g} \cdot \left[\frac{(d-s)(3-s)}{2} \right]^{1/2} \quad (8)$$

$$D = G \cdot \exp\left\{\frac{-q_1^2 R_g^2}{3-s}\right\} \cdot q_1^{(d-s)} \quad (9)$$

where G and D are the Guinier and Porod scale factors respectively, R_g the radius of gyration, d the Porod exponent, and s a parameter depending on the geometry ($s=0$ for spheres, $s=1$ for rods, $s=2$ for lamellae or platelets).

- (ii) A mass fractal model using the form factor $P(q)_{sphere}$ of a sphere, and described in equations (10-12):⁵

$$I(q) = scale \cdot P(q) \cdot S(q) + background \quad (10)$$

$$P(q)_{sphere} = 9 \cdot \left[\frac{\sin(qR) - qR \cos(qR)}{(qR)^3} \right]^2 \quad (11)$$

$$S(q) = \frac{\Gamma \cdot (D_m - 1) \cdot \zeta^{D_m - 1} \cdot \sin[(D_m - 1) \tan^{-1}(q\zeta)]}{q \cdot [1 + (q\zeta)^2]^{(D_m - 1)/2}} \quad (12)$$

where R is the radius of the primary particle, D_m the mass fractal dimension, and ζ the cut-off length.

- (iii) The sticky hard sphere adjustment was realized combining the form factor of a sphere $P(q)_{sphere}$ (11) and a interparticle structure factor $S(q)_{stickyHS}$ for a hard sphere fluid with a narrow attractive well and using a perturbative solution of the Percus-Yevick closure. The full description of $S(q)_{stickyHS}$ can be found in the literature.^{6,7}

3. Light scattering of concentrated samples

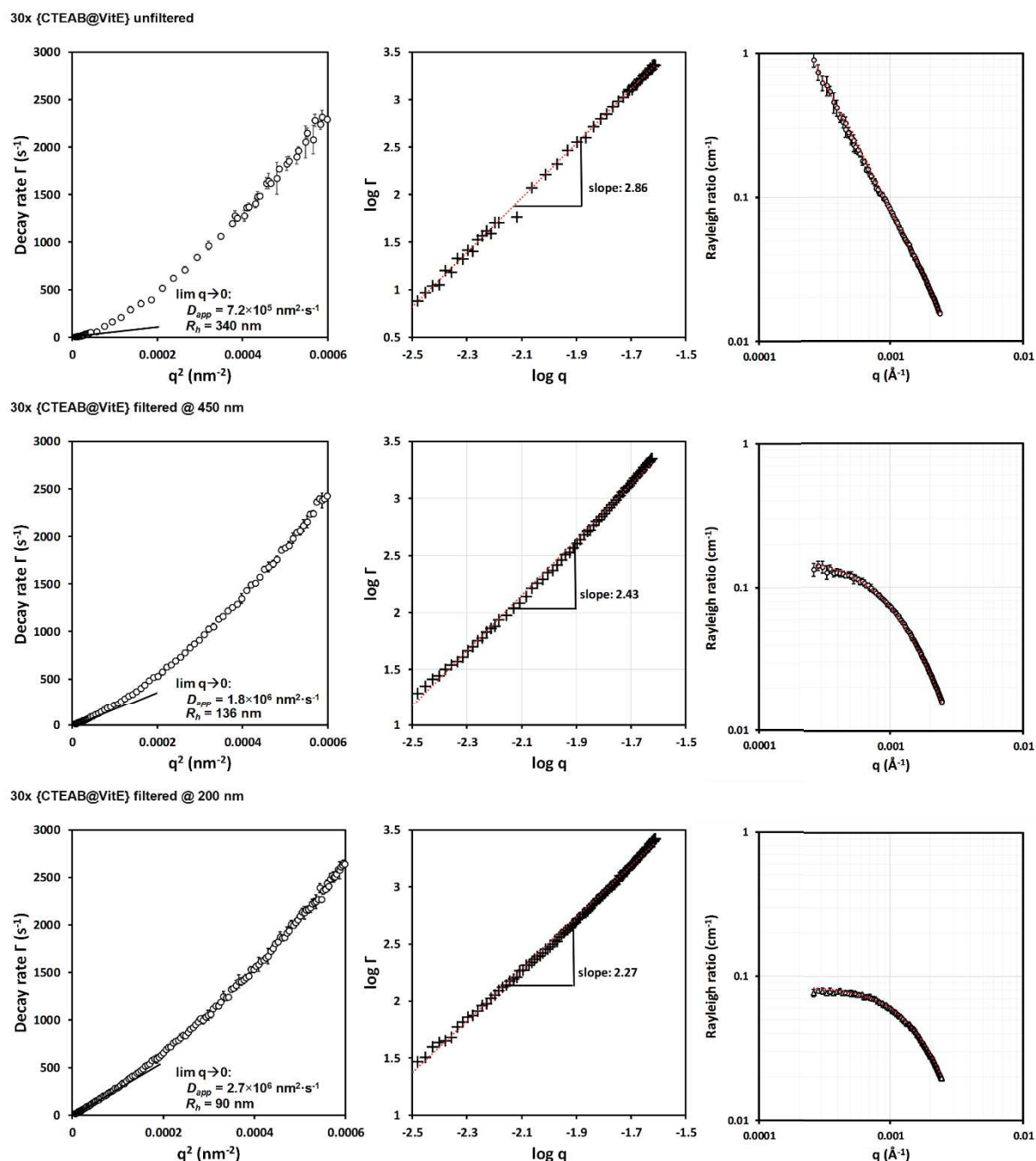


Figure S6. (left): decay rate Γ (s^{-1}) as determined from a 2nd order Cumulant analysis, plotted vs q^2 from MADLS measurements. (center): plot of $\log \Gamma$ as a function of $\log q$ showing a linear dependence. (right): absolute scattered light intensities as obtained by MASLS. From top to bottom: 30x {CTEAB@VitE} unfiltered, filtered at 450 nm, filtered at 200 nm.

4. Analysis of small angle X-ray scattering data (SAXS)

The general expression of the scattered intensity $I(q)$ is given by equation (13):

$$I(q) = \Phi \cdot V \cdot (\Delta\rho)^2 \cdot P(q) \cdot S(q) \quad (13)$$

where $q = [4\pi \sin(\theta/2)]/\lambda$ is the scattering vector (θ is the scattering angle), Φ is the volume fraction of the scatterers, V is their volume and $\Delta\rho$ is their difference in scattering length density (SLD) with respect to the solvent. $P(q)$ is the scatterers' form factor and $S(q)$ is the structure factor accounting for inter-particle interactions, for which various analytical models are available.

Combining SLS and SAXS curves, the overall scattered intensity could be adjusted by a combination (14) of a fractal model and two additional sticky hard sphere models (as described above in the MASLS section):

$$I(q)_{total} = I(q)_{fractal} + I(q)_{stickyHS,1} + I(q)_{stickyHS,2} \quad (14)$$

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