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1 **Biotechnological applications of the sepiolite interactions with**
2 **bacteria: bacterial transformation and DNA extraction**

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24 **Highlights:**

25

26 • Bacteria/sepiolite interaction can filtrate water.

27 • DNA/sepiolite biohybrids can be used to improve the plasmid DNA
28 transformation efficiency into bacteria

29 • Sepiolite binding capacity towards DNA can be used to purify plasmids from
30 bacteria

31

32 **Abstract:**

33 Among the various clay minerals, sepiolite, which is a natural nanofibrous silicate
34 that exhibit a poor cell toxicity, is a potential promising nanocarrier for the non-viral
35 and stable transfer of plasmid DNA into bacteria, mammalian and human cells. We
36 first show here that sepiolite binds to bacteria, which can be useful in decontamination
37 protocols. In a previous research we have shown that is possible to modulate the
38 efficiency of the absorption of different types of DNA molecules onto sepiolite, and
39 that the DNA previously adsorbed could be recovered preserving the DNA structure
40 and biological activity. Taking advantage of both, the sepiolite/bacteria and
41 sepiolite/DNA interactions, we show that pre-assembly of DNA with sepiolite and
42 incubation of bacteria with this obtained biohybrid strongly improve the
43 transformation efficiency, in a rapid, convenient and inexpensive method that doesn't
44 require competent cell preparation. In addition, we also show that the controlled
45 sepiolite and DNA binding capacities can be used to purify plasmids from bacteria,
46 representing an advantageous alternative to onerous commercial kits. All of these
47 results open the way to the use of sepiolite-based bionanohybrids for the development
48 of novel biological models of interest for academic and applied sciences.

49

50

51 **Keywords:** sepiolite, bionanohybrids, nanomaterial, DNA, bacterial transformation,
52 plasmid extraction

53 **1. Introduction**

54 Clay minerals represents one of the most abundant groups of inorganic solids in
55 interaction with the biosphere (Bergaya and Lagaly, 2006). They have been implicated
56 in the prebiotic synthesis of biomolecules at origins of life (Fripiat, 1984). Moreover,
57 because of their functional properties, bionanohybrids materials resulting from the
58 combination of biopolymers with nanoparticles such as clay minerals and other related
59 solids (Avérous and Pollet, 2012; Chivrac et al., 2010; Darder et al., 2007; Mittal,
60 2011; Ruiz-Hitzky et al., 2000), represent alluring prospects for a wide variety of
61 application ranging from decontamination absorbent (oil pollution, pet litter) to
62 biomedical applications (Ruiz-Hitzky *et al.*, 2010, 2013) such as biosensors, scaffolds
63 for tissue engineering, effective drug-delivery nano-vehicles, vaccination, wound
64 dressings and DNA delivery into mammalian cells (Wicklein *et al.*, 2010, 2011, 2012;
65 Kam *et al.*, 2006; Lacerda *et al.*, 2012; Dutta & Donaldson, 2012; Kim *et al.*, 2013;
66 Park *et al.*, 2013; Castro-Smirnov *et al.*, 2016; Piétrement *et al.*, 2018). In this context,
67 the study of the interaction of nucleic acids with sepiolite (Castro-Smirnov et al., 2016,
68 2017; Piétrement et al., 2018) as well as other clay minerals showing one-dimensional
69 aspect, such as imogolite (Jiravanichanun et al., 2012; Ma et al., 2012) and halloysite
70 nanotubes (Long et al., 2017; Santos et al., 2018), is attracting growing interest in view
71 to applications dealing with uses as vector for DNA transfection, and other biomedical
72 applications. Moreover, the role of clays in their interaction with living organisms, as
73 for instance bacteria, is also a relevant point of research of study as they can be
74 involved in the origin of certain clays, such as sepiolite and palygorskite (Leguey et
75 al., 2014; del Buey et al., 2018), may influence the growth of microorganisms or may
76 show antimicrobial activity *per se* or after modifications (Abhinayaa et al., 2019;
77 Gaálová et al., 2019; Ito et al., 2018; Li et al., 2019; Williams et al., 2011).

78 Among the various clay minerals, sepiolite, which is a natural nanofibrous
79 silicate, presents many advantages for various applications: it is abundant, inexpensive
80 and biocompatible. At concentrations below 10 ng/ μ l, sepiolite exhibit a poor cell
81 toxicity in mammalian cells (Castro-Smirnov *et al.*, 2017) and, in addition,
82 epidemiologic studies concluded so far that, unlike carbon nanotubes (Kobayashi *et*
83 *al.*, 2017), sepiolite does not constitute a health risk particularly those with fiber
84 lengths below 5 μ m (Denizeau *et al.*, 1985; Maisanaba *et al.*, 2015). Therefore,
85 International Agency of Research on Cancer (IARC, affiliated to World Health
86 Organization: WHO) does not classified sepiolite as hazardous or carcinogenic
87 (Wilbourn *et al.*, 1997). Thanks to its nanofibrous nature, sepiolite facilitates the
88 transfer of DNA into bacteria through a process called the Yoshida effect, which was
89 first described with asbestos (Rodríguez-Beltran *et al.*, 2013; Rodríguez-Beltrán *et al.*,
90 2012; Tan *et al.*, 2010; Wilharm *et al.*, 2010; Yoshida, 2007; Yoshida and Sato, 2009).
91 The Yoshida effect could be described as following: when a colloidal solution
92 containing nano-sized acicular material and bacterial cells is stimulated by sliding
93 friction at the interface between hydrogel and an interface-forming material, the
94 frictional coefficient increases rapidly and the nano-sized acicular material and
95 bacterial cells form a chestnut bur-shaped complex. This complex increases in size and
96 penetrates the bacterial cells, thereby forming a penetration-intermediate, due to the
97 driving force derived from the sliding friction (Yoshida, 2007). A hydrogel shear stress
98 greater than or equal to 2.1 N is essential for the Yoshida effect to occur and has been
99 observed with agarose, gellan gum, and χ -carrageenan. In addition, polymers such as
100 polystyrene, polyethylene, acrylonitrile-butadiene rubber, and latex rubber, as well as
101 silicate minerals such as quartz and jadeite, are all suitable interface-forming materials.
102 With regard to nanosized acicular materials, the Yoshida effect has also been

103 confirmed with multi-walled carbon nanotubes, maghemite ($\gamma\text{-Fe}_2\text{O}_3$), chrysotile, and
104 sepiolite, having diameters of 10–50 nm (Yoshida and Sato, 2009). It must be noted
105 that Yoshida effect does not involve the pre-assembly of DNA with the fibres.

106 Because of its high specific surface area, surface activity and high porosity, as
107 previously described, sepiolite has received considerable attention its ability to adsorb
108 a huge number of different molecules on the surface. In a detailed analysis of the
109 interaction between sepiolite and DNA, we have previously shown that sepiolite
110 reversibly binds different types of DNA molecules (genomic, plasmid, single strand
111 and double strand oligonucleotides) (Castro-Smirnov et al., 2016). Therefore, we
112 addressed here whether the DNA binding capacities of sepiolite can be used i) to
113 improve the efficiency of plasmid DNA transformation into bacteria and ii) to extract
114 plasmid DNA from bacteria.

115 Plasmids are small circular DNA molecules that are naturally maintained in
116 bacteria. To describe non-viral DNA transfer in bacteria and non-animal eukaryotic
117 cells (like yeast and plant cells) is used the term “transformation” (or bacterial
118 transformation). Such a mechanism is thought to have been involved in gene transfers
119 during evolution and particularly in transfers among unrelated organisms such as
120 plants and bacteria (Demanèche et al., 2001). Plasmids represent now essential tools
121 in molecular biology and biotechnology, from bacteria to mammalian cells. Indeed,
122 they are the basal vector/backbone carrying DNA for gene transfer technologies, but
123 they need first to be amplified and produced into bacteria. Therefore, the ability of
124 introducing individual molecules of plasmid DNA into cells by transformation has
125 been of central importance to the recent rapid advancement of plasmid biology and to
126 the development of DNA cloning methods. Transformation protocols include
127 techniques that are generally tedious and time-consuming because they required first

128 to generate competent bacteria, *i.e.* bacteria able to take up extracellular DNA.
129 Alternatively, commercial competent bacteria are available but are very expensive.
130 The advantage of transforming bacterial cells based on the Yoshida effect is that
131 competent cell preparation is not required. Using sepiolite should circumvent the
132 toxicity associated with asbestos.

133 Here we described protocols that strongly improve the efficiency of sepiolite-
134 mediated bacterial transformation. Indeed, first we show that sepiolite binds to bacteria
135 (which can be useful in water decontamination protocols). Taking advantage of i) the
136 sepiolite/bacteria interaction and ii) of sepiolite/DNA interaction, we show that pre-
137 assembly of DNA with sepiolite (Sep/DNA) and incubation of bacteria with Sep/DNA
138 strongly improve the transformation efficiency, in a rapid, convenient and inexpensive
139 method. In addition, we also show that sepiolite DNA binding capacities can be used
140 to purify plasmids from bacteria, representing an advantageous alternative to onerous
141 commercial kits.

142

143 **2. Experimental Section**

144 Sepiolite from Vallecas-Vicalvaro (Madrid, Spain) was provided by TOLSA
145 S.A. as Pangel S9, rheological grade commercial product of very high sepiolite content
146 (>95%) and cation exchange capacity (CEC) value close to 15 mEqiv/100 g. The total
147 specific surface area of this sepiolite was determined by N₂ adsorption-desorption
148 isotherms and found to be 330 m²/g from Brunauer-Emmett-Teller (BET) calculations,
149 whereof the external specific surface area is 160 m²/g (Perez-Carvajal *et al.*, 2019).
150 The mean fibers width of the sonicated sepiolite was 15 nm; the fibres length is: 80%
151 of fibers between 200 and 400 nm long, (maximal length of 800 nm) (Castro-Smirnov
152 *et al.*, 2016).

153 A sepiolite dispersion of 2 mg/ml was prepared in 10 mM TrisHCl buffer,
154 pH=7.5, under a vigorous vortexing at maximum speed during a minimum of 10min
155 in order to properly disperse the clay.

156 Bacteria used are the *Escherichia coli* (gram-negative) XL-2 Blue strain.

157 Circular DNA plasmid (5.7 kbp long, pCMV) was obtained by amplification
158 of bacterial culture and purified using the commercial kit from Macherey-Nagel. DNA
159 pUC19 plasmid was supplied by New England BioLabs at 1 mg/ml (pUC19 Vector,
160 2686 bp, #N3041S, lot# 0361204).

161 The adsorption of DNA molecules onto sepiolite was firstly studied carrying
162 out experiments of adsorption in isothermal conditions at 25 °C to determine the
163 influence of cations in DNA adsorption. 50 µl of sepiolite/DNA (Sep/DNA) mixtures
164 were prepared containing in this order: 25 µl of the stock solution of the sepiolite
165 dispersion (2 mg/ml); 5 µl of 10 mM TrisHCl for the case of Sep/DNA alone, and 5µl
166 of 10 times concentrated solutions of 5 mM MgCl₂, or 5 mM CaCl₂. The final sepiolite
167 concentration was fixed to 1 mg/ml, and the final DNA concentration of 800 ng/µl.

168 Then the Sep/DNA mixtures were stirred for 24 h at 25° C at 700 rpm using a
169 Thermomixer (Eppendorf) and then centrifuged for 5 min at 5000 rpm. Finally, the
170 DNA concentration in the supernatants was measured using a Nanodrop ND1000
171 spectrophotometer (UV-vis spectroscopy). Experiments were carried out 3 times for
172 each experiment. The amount of DNA bounds on sepiolite was estimated by
173 adsorption isotherms method, as described (Castro-Smirnov et al., 2016).

174 In this work a NanoDrop 1000 spectrophotometer from Thermo Scientific was
175 used to determine the concentration and purity of double and single – stranded DNA
176 in aqueous solution. The purity of DNA is obtained by the “260/280” ratio (ratio of
177 sample absorbance measured at 260 nm and 280 nm respectively). A ratio of ~1.8 is
178 generally accepted as “pure” for DNA. The 260/230 ratio is a secondary measure of
179 nucleic acid purity, the values for “pure” nucleic acid being often higher than the
180 respective 260/280 values. They are commonly in the range of 1.8 - 2.2. If the ratio is
181 appreciably lower, this may indicate the presence of contaminants.

182 TEM imaging was carried out with a ZEISS 912 AB microscope working in
183 either filtered crystallographic dark-field mode and bright-field modes. Electron
184 micrographs were recorded with a ProScan 1024 HSC digital camera and iTEM
185 software (Olympus, Soft Imaging Solutions). Samples were prepared from 5µl of DNA
186 or sepiolite-bacteria solution deposited for 1 min. on a 600-mesh copper grid covered
187 with a thin layer of carbon activated by glow-discharge of amylamine solution (Sigma-
188 Aldrich, France) (Dupaigne et al., 2018). Grids were washed with few droplets of
189 uranyl acetate solution 2% (w/v) and dried with ashless filter paper. The sample for
190 TEM imaging was prepared from a logarithmic-phase culture of XL2 bacteria (OD₆₀₀
191 0.5-1) in 10ml of LB. In this experiment, 500µl of the culture were centrifuged at
192 3000rpm for 5min, and then the pellet was re-suspended in 200µl of a sepiolite

193 dispersion containing 1 mg of clay per ml. Finally, 5 μ l of sample were placed in a
194 carbon grid for TEM analyses, as described above.

195 The final protocol for plasmid extraction from bacteria, using sepiolite:

196 • Overnight saturated culture of bacteria at 37 °C in 20ml of LB (Luria-Bertani)
197 medium, with the adequate antibiotic and at constant shaking (200–250rpm).

198 • Centrifugation of bacteria culture at 4,500g for 15min at 4 °C.

199 • Carefully re- dispersion of the pellet of bacterial cells in 4ml of Re- dispersion
200 Buffer (S1).

201 • Addition of 4ml of Lysis Buffer to the dispersion (S2).

202 • Gently mixing by inverting the tube 6–8 times.

203 • Incubation of the mixture at room temperature (18–25 °C) for 2– 3min.

204 • Addition of pre-cooled (4°C) Neutralization Buffer (S3) to the suspension.

205 Immediately gently mixing of the lysate by inverting the flask 6–8 times until a
206 homogeneous dispersion containing an off-white flocculate is formed. Incubation
207 of the dispersion on ice for 5min.

208 • Filtration of the dispersion using Wattman paper.

209 • Addition of concentrated MgCl₂ to the filtered dispersion in order to obtain a final
210 MgCl₂ concentration of 20mM.

211 • Addition of binding solution in order to have a final sepiolite concentration of 1
212 mg/ml. At this step the DNA is adsorbed onto sepiolite.

213 • Stirring of the dispersion for 90 min.

214 • Centrifugation at 4,500 g for 5 min (discard supernatant).

215 • Re- dispersion with 12 ml of washing solution and replacing the tube.

216 • Centrifugation at 4,500 g for 5 min (discard supernatant).

217 • Re-dispersion in 1 ml of Elution Buffer.

- 218 • Ethanol precipitation into 1 ml of 10 mM TrisHCl pH = 7.5.
- 219
- 220 Buffer S1: 50mM TrisHCl, 10 mM EDTA, 100µg / ml RNase A, pH 8.0.
- 221 Lysis Buffer S2: 200mM NaOH, 1% SDS.
- 222 Neutralization Buffer S3: 2.8M KAc, pH 5.1.
- 223 Binding solution: 10mM TrisHCl, 20mM MgCl₂, 4mg/ml sepiolite suspension, pH =
- 224 7.5.
- 225 Washing solution: 10 mM TrisHCl, 20 mM MgCl₂, pH 7.5.
- 226 Elution Buffer: 10 mM TrisHCl, 20 mM EDTA, pH 7.5.

227 **3. Results**

228 *3.1. Bacteria-sepiolite interaction*

229 In order to know the dose response and the kinetics response of sepiolite for
230 potential binding to bacteria, different amounts of sepiolite powder were added to
231 overnight saturated bacterial cultures or tubes containing only LB growth medium
232 without bacteria, as control. Pictures were taken every 10 min to visualize bacteria
233 putative sedimentation fostered by sepiolite (Figure 1A). The use of 40 mg and higher
234 amount of sepiolite allows clarifying 2 ml of the saturated bacterial culture, which
235 corresponds to a concentration of 20mg/ml (Figure 1).

236 In order to quantify this phenomenon, optical density using a
237 spectrophotometer (at 600 nm) were monitored (Figure 1 B and C). Almost all
238 bacterial dispersion sediments in 20 minutes with 10 or 20 mg/ml of sepiolite (Figure
239 1B). Moreover, between 10 and 15 mg/ml of sepiolite were sufficient to settle almost
240 all bacteria in 1 hour (Figure 1C). These data support a physical interaction between
241 sepiolite and bacteria. In order to visualize and confirm this physical interaction
242 between sepiolite and bacteria, we performed an analysis by transmission electron
243 microscopy (TEM) (Figure 2). This analysis confirms the interaction between sepiolite
244 fibers and the bacterial membrane. Note that almost all bacteria were found with
245 sepiolite nanofibers in contact with their cell membrane (Figure 2A-D). Zooms in
246 contact regions (Figure 3), show that nanofibers interact mostly with the edge of the
247 bacteria and occasionally pierce its lateral surface.

248

249 *3.2. Bacterial transformation*

250 Because of these data, and because sepiolite interact with DNA (Castro-Smirnov et al.,
251 2016), we hypothesized that it might be possible to improve bacterial transformation,
252 which was previously based on the Yoshida Effect, by sonication of sepiolite (sSep)

253 that avoid sepiolite aggregation (Castro-Smirnov *et al.*, 2016). Thus pre-incubation of
254 DNA sonicated sepiolite (sSep), was first carried out, forming the sSep/DNA
255 bionanohybrid then sSep/DNA with bacteria (XL-2 Blue *Escherichia coli* strain).
256 Using the Yoshida Effect protocol (Wilharm *et al.*, 2010), we obtained from 3×10^4 to
257 6×10^4 transformants per μg of pUC19 plasmid. In this referred work (Wilharm *et al.*,
258 2010), the bacterial pellet was resuspended in a solution of 100 μl of 5 mM HEPES
259 pH 7.4 and 200 mM KCl, containing 10 μg of sepiolite, and then adding 50 ng of
260 pUC19 plasmid and immediately spread on agar plate containing ampicillin (Wilharm
261 *et al.*, 2010). With our protocol we will need to use a different buffer for the pre-
262 assembly of DNA on sepiolite, *i.e.* 10 mM TrisHCl pH = 7.5 and in the presence of a
263 divalent cation at lower concentration (5 and 10 mM of MgCl_2 instead of 200 mM
264 KCl). Therefore, we first compared the transformation efficiencies with the two
265 buffers, without Sep/DNA pre-assembly. Therefore, in this method DNA is not
266 previously assembled to sepiolite, prior spreading of the bacteria. In our case, we
267 follow the same protocol but using the current buffer for Sep/DNA employed along in
268 this work (*i.e.* 10 mM TrisHCl pH=7.5), and in the presence of a divalent cation at
269 lower concentration (5 and 10 mM of MgCl_2 instead of 200 mM KCl as used elsewhere
270 (Wilharm *et al.*, 2010). Thus, 50 ng of pUC19 plasmid was previously adsorbed in 10
271 μg of sepiolite dispersed in a solution of 100 μl of 10 mM TrisHCl pH 7.5 in the
272 presence of 5 mM MgCl_2 . The bacterial pellet was then re-suspended in the pre-
273 assembled bionanocomposite dispersion and immediately spread on agar plate.
274 Following this protocol we obtained transfection efficiencies similar to that reported
275 by Wilharm and co-workers (Wilharm *et al.*, 2010)

276 We then tested as whether incubation of the Sep/DNA complex with bacteria,
277 prior spreading, might improve transformation efficiency. To probe this hypothesis,

278 the two experiments above mentioned were repeated, but in this case following by 1
279 hour of incubation at 37 °C of the re-suspended bacterial pellet (from growing phase
280 cells), in LB medium. The incubation bacteria with Sep/DNA prior spreading resulted
281 in a 6- and 10-fold increase of the transformation efficiency, with the HEPES buffer
282 (Wilharm *et al.*, 2010) and the Tris buffer (our method), respectively.

283 We also addressed the influence of the diverse polyvalent cations for Sep/DNA
284 assembly, on bacterial transformation efficiency. Thus, various Sep/DNA
285 bionanocomposites were prepared in the presence of 5 mM MgCl₂, 5 mM CaCl₂, 0.5
286 mM spermidine and 0.5 mM spermine. The amount of adsorbed DNA varies
287 depending on the nature of the cation present in the following sequence: MgCl₂ <
288 CaCl₂ < spermidine < spermine (Castro-Smirnov *et al.*, 2016). However, the bacterial
289 transformation efficiency decreased with higher valences (Table 1). This may be due
290 to the stronger interaction between sepiolite and DNA cations of higher valence,
291 resulting in lower efficiency of DNA delivery into cells. Note that incubation of
292 sep/DNA with bacteria (in suspension) without using the spreader, fail to transfer DNA
293 into bacteria. This suggests that the sliding friction on solid agar plates is necessary,
294 suggesting that DNA internalize into bacteria in a Yoshida effect manner.

295 Finally, since sepiolite spontaneously forms aggregates, which can be
296 dissociated by sonication (Castro-Smirnov *et al.*, 2016), we assayed the influence of
297 sonication of the sepiolite and co-workers prior to assembly with DNA. Using the
298 same experimental conditions above mentioned, two bionanocomposites were
299 prepared, but one with non-sonicated sepiolite and the other with sSep. Remarkably,
300 sonication of sepiolite prior assembly with DNA, more than 2-fold increases the
301 bacterial transformation efficiency (Table 2).

302

303 3.3. DNA extraction and purification from bacteria

304 Extracting and purifying nucleic acids are critical steps and it is important to
305 have methods that allow separating nucleic acids from biological medium with a high
306 efficiency, and obtaining nucleic acids having a high degree of purity. Such methods
307 are generally based on the absorption of plasmid DNA on affinity resin, which can be
308 expensive. Because of the interaction of DNA with sepiolite and that DNA can be
309 desorbed with EDTA (Castro-Smirnov *et al.*, 2016), we address the question as
310 whether sepiolite can be used for plasmid extraction from bacteria.

311 The plasmid pUC19 was amplified in bacteria (XL2). After cells lysis and
312 denaturation step using classical methods (see Experimental Section), the extract was
313 then incubated with sepiolite and, after centrifugation, DNA was recovered by washing
314 with EDTA (Figure 4). Interestingly, efficient DNA recovery from bacteria was
315 observed with sepiolite. The efficiency of DNA extraction was 78.9 µg for 20 ml of
316 cultured bacteria at saturation. The index of purity was calculated by the ratio A_{260}/A_{280}
317 (absorbance at 260 nm for DNA, on absorbance at 280nm for proteins), which should
318 be near 1.8. Here, the A_{260}/A_{280} of DNA plasmid extracted with sepiolite was 1.7,
319 indicating a good purity index. Moreover, the ratio of the different isoforms of the
320 plasmid DNA, *i.e.* supercoiled (high quality plasmid) *versus* open circle and linear
321 (altered plasmid) was not modified, compared to that of the commercial initial plasmid
322 DNA, and, importantly almost only supercoiled plasmids (high-quality) were
323 recovered (Figure 4). This shows that the extraction with sepiolite did not altered the
324 plasmid structure.

325 The quality of the plasmid extracted from bacteria was confirmed by digestion
326 with a restriction enzyme (Figure 4 lane D) and by TEM (Figure 5). The fact that the

327 restriction enzyme efficiently cleaved the DNA indicates that the DNA extracted with
328 sepiolite is of sufficient quality for further molecular biology experiments. TEM image
329 confirms the supercoiled structure of the sepiolite-extracted plasmid (Figure 5).

330 **4- Discussion**

331 Bacterial transformation is a pivotal process for plasmid preparation in most
332 molecular biology approaches, in academic as well as applied research. Because
333 bacteria do not efficiently spontaneously uptake DNA, methods have been designed to
334 render bacteria competent to plasmid transformation. However, these methods are
335 tedious and time consuming. Alternatively, competent bacteria are commercialized,
336 but are expensive. The methods based on Yoshida effects, and optimized here, are
337 rapid, convenient. Indeed, bacteria in their growing phase can be directly transformed.
338 The use of sepiolite for such purposes present several advantages. First, sepiolite is not
339 onerous. Second, sepiolite-mediated bacterial transformation can be optimized by
340 using sonicated sepiolite, specificities of sepiolite, which i) sepiolite spontaneously
341 interacts with DNA generating the Sep/DNA bionanohybrid (Castro-Smirnov *et al.*,
342 2016); ii) sepiolite interacts with bacteria and concentrate them. Therefore, pre-
343 incubation sepiolite with DNA (Sep/DNA) followed by incubation of Sep/DNA with
344 bacteria in their growing phase, prior spreading, should increase transformation
345 efficiency. The combination of the different improvements led to transformation
346 efficiency close to 10^6 transformants/ μg of DNA, *i.e.* a 30-fold increase. We
347 summarize our proposed method in Figure 6. The bacteria used here are gram-negative
348 (*Escherichia coli*). Since Yoshida effect has been shown to work with gram-positive
349 bacteria (Yoshida and Sato, 2009), our improvement should likely also work with such
350 kind of bacteria. This is discussed in the revised version line

351

352 In addition, the capacity of sepiolite to interact with DNA allowed us to design
353 a protocol for plasmid extraction from bacteria (see Figure 7 and Experimental
354 Section).

355 Moreover, it is worth to note that the interaction between sepiolite and bacteria
356 might constitute the basis of promising applications aiming at filtrating and
357 decontaminating water.

358 The low toxicity of sepiolite compared to asbestos, could also be an advantage.
359 However, the potential toxicity of sepiolite is a very important debate, both with regard
360 to environmental concerns (sepiolite is present as natural deposits) as well as for the
361 numerous uses of sepiolite including biomedical applications. Because , like asbestos,
362 sepiolite can generate a Yoshida effect and DNA breaks into the bacterial genome,
363 potential carcinogenic risks have been addressed by extrapolation from bacteria to
364 mammals (González-Tortuero *et al.*, 2018). However, one can object that these
365 extrapolations could correspond to over-interpretations: First, the mean sizes of
366 bacteria is 50-fold smaller than the mean size of mammalian cells; thus the size-ratio
367 sepiolite/cells is very different; this is very important to reaching any cell
368 compartment, notably the nucleus that contains the DNA. Second, their DNA
369 organisation is completely different, originating the Latin/Greek etymology,
370 prokaryote *versus* eukaryotes: i) bacteria are prokaryotes meaning that they do not
371 contain a nucleus, the DNA being in the cytoplasm, frequently bound to the membrane;
372 therefore it is directly accessible and threaten by the friction with fibres; ii) mammals
373 are eukaryotes meaning that they contain a nucleus in which the genome is embedded
374 into chromosomes; therefore to directly assault DNA sepiolite should penetrate into
375 the nucleus, which would not immediate because of the sizes differences. Moreover,

376 mammals have developed protection mechanisms both at the cell and the organism
377 levels (see below). Fourth, at the doses used for DNA transfection, sepiolite (without
378 friction) do not (or barely) affect human cells viability (Castro-Smirnov et al., 2017;
379 Piétrement et al., 2018). Fifth, if sepiolite can be spontaneously internalized, cells are
380 also able to expel it (Castro-Smirnov et al., 2017). Sixth, inhalation exposure of rats to
381 sepiolite dust produced neither fibrosis nor increase incidence of tumours (Wagner et
382 al., 1987). Seventh, *in vitro* and *in vivo* toxicological assays, as well as epidemiological
383 studies, conclude that sepiolite from Taxus Basin (Spain) does not present asbestos-
384 like effect and health risks (Denizeau et al., 1985; Maisanaba et al., 2015; Santarén
385 and Alvarez, 1994). Therefore, the International Agency of Research on Cancer
386 (IARC, a World Health Organization agency) does not classified sepiolite as hazardous
387 or carcinogenic (Wilbourn et al., 1997).

388

389 **5- Conclusion**

390 This study confirms that incubation of bacteria with biohybrid materials resulting from
391 the pre-assembling of DNA with sepiolite, strongly improves the plasmid DNA
392 transformation efficiency into bacteria in a rapid, convenient and inexpensive method
393 that does not require competent cell preparation. Therefore there is no need to prepare
394 or buy competent bacteria, thus saving time consuming and being less expensive.
395 Although, the final transformation efficiency is lower than with commercial competent
396 bacteria, it is largely sufficient for most applications in molecular biology, allowing
397 thus to save time and money. Hence, sepiolite binding capacities can be used to purify
398 plasmids from bacteria, since it reversibly absorbs DNA, with controlled efficiency,
399 representing an advantageous alternative to onerous commercial kits. Moreover, the

400 physical interaction between sepiolite and bacterial membrane might constitute the
401 basis of promising applications such as filtration and decontamination of water.
402 Anyway, sepiolite, at least that from Taxus Basin in Spain, does not present asbestos-
403 like effect and health risk as potential risks of DNA breaks into the bacterial genome
404 cannot be extrapolated from bacteria to mammalian and human cells.

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411

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- 543
- 544

545 **Figures legends**

546

547 **Figure 1.** Sepiolite-mediated bacterial sedimentation. **A.** Pictures sepiolite-mediated
548 bacterial sedimentation. Left panel: overnight saturated bacterial cultures. Right panel:
549 LB medium without bacteria. The sepiolite concentrations and time of contact are
550 indicated on the picture. **B.** Time course sedimentation measured by optical density,
551 with 3 different concentration of sepiolite (indicated in the picture). **C.** Dose response
552 of sepiolite-mediated bacterial sedimentation after 1 hour of contact.

553

554 **Figure 2.** TEM images of sepiolite nanofibers in contact with XL2 bacteria. Arrows:
555 contact points between sepiolite fibers and bacteria.

556

557 **Figure 3.** TEM images of a complete XL2 bacteria (A) with their respective zooms in
558 contact regions (B and C). I and II are two different examples.

559

560 **Figure 4.** Gel shift assay of extracted PUC19 plasmid from bacteria using sepiolite.
561 **A:** ladder. **B:** Commercial pUC19 plasmid. **C:** sepiolite-extracted pUC 19 plasmid. **D:**
562 sepiolite-extracted plasmid digested (linearized) with EcoRI.

563

564 **Figure 5.** TEM image of extracted pUC19 using sepiolite.

565

566 **Figure 6.** Optimized protocol for sepiolite-mediated bacterial transformation. First, the
567 sSep/DNA bionanohybrid were prepared, in which 50 ng of pUC19 plasmid was

568 previously adsorbed in 10 µg of sonicated sepiolite dispersed in a solution of 100µl of
569 10m M TrisHCl pH 7.5 in the presence of 5 mM MgCl₂. Then, the bacterial pellet
570 (from growing phase cells) is resuspended in 100 ml of sSep/DNA in 10 mM TrisHCl
571 and 5 mM MgCl₂ pH 7.5, following by 1 hour of incubation at 37 °C in LB medium.
572 Then spreading in agar plate containing the selection antibiotic.

573

574 **Figure 7.** Scheme of sepiolite-mediated extraction of plasmid DNA from bacteria.

575 See detailed protocol in Experimental Section.