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

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Highlights:

- Bacteria/sepiolite interaction can filtrate water.
- DNA/sepiolite biohybrids can be used to improve the plasmid DNA transformation efficiency into bacteria
- Sepiolite binding capacity towards DNA can be used to purify plasmids from bacteria

Abstract:

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

Keywords: sepiolite, bionanohybrids, nanomaterial, DNA, bacterial transformation, plasmid extraction

1. Introduction

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

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

confirmed with multi-walled carbon nanotubes, maghemite (γ-Fe_2O_3), chrysotile, and sepiolite, having diameters of 10–50 nm (Yoshida and Sato, 2009). It must be noted that Yoshida effect does not involve the pre-assembly of DNA with the fibres.

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

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

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

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

2. Experimental Section

Sepiolite from Vallecas-Vicalvaro (Madrid, Spain) was provided by TOLSA S.A. as Pangel S9, rheological grade commercial product of very high sepiolite content (>95%) and cation exchange capacity (CEC) value close to 15 mEquiv/100 g. The total specific surface area of this sepiolite was determined by N_2 adsorption-desorption isotherms and found to be 330 m^2/g from Brunauer-Emmett-Teller (BET) calculations, whereof the external specific surface area is 160 m^2/g (Perez-Carvajal *et al.*, 2019). The mean fibers width of the sonicated sepiolite was 15 nm; the fibres length is: 80% of fibers between 200 and 400 nm long, (maximal length of 800 nm) (Castro-Smirnov et al., 2016).

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

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

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

The adsorption of DNA molecules onto sepiolite was firstly studied carrying out experiments of adsorption in isothermal conditions at 25 ºC to determine the influence of cations in DNA adsorption. 50 µl of sepiolite/DNA (Sep/DNA) mixtures were prepared containing in this order: 25 µl of the stock solution of the sepiolite dispersion (2 mg/ml); 5 µl of 10 mM TrisHCl for the case of Sep/DNA alone, and 5µl of 10 times concentrated solutions of 5 mM $MgCl_2$, or 5 mM $CaCl_2$. The final sepiolite 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^{o} 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 (OD_{600}
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, 5µl 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 $MgCl_2$ to the filtered dispersion in order to obtain a final
210 $MgCl_2$ 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.

- Ethanol precipitation into 1 ml of 10 mM TrisHCl pH = 7.5.

Buffer S1: 50mM TrisHCl, 10 mM EDTA, 100µg / ml RNase A, pH 8.0.

Lysis Buffer S2: 200mM NaOH, 1% SDS.

Neutralization Buffer S3: 2.8M KAc, pH 5.1.

Binding solution: 10mM TrisHCl, 20mM $MgCl_2$, 4mg/ml sepiolite suspension, pH = 7.5.

Washing solution: 10 mM TrisHCl, 20 mM $MgCl_2$, pH 7.5.

Elution Buffer: 10 mM TrisHCl, 20 mM EDTA, pH 7.5.

3. Results

3.1. Bacteria-sepiolite interaction

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

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

3.2. Bacterial transformation

Because of these data, and because sepiolite interact with DNA (Castro-Smirnov et al., 2016), we hypothesized that it might be possible to improve bacterial transformation, 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 $3x10^4$ to
257 $6x10^4$ 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,

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

We also addressed the influence of the diverse polyvalent cations for Sep/DNA assembly, on bacterial transformation efficiency. Thus, various Sep/DNA bionanocomposites were prepared in the presence of 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5 mM spermidine and 0.5 mM spermine. The amount of adsorbed DNA varies depending on the nature of the cation present in the following sequence: $MgCl_2$ < $CaCl_2$ < spermidine < spermine (Castro-Smirnov *et al.*, 2016). However, the bacterial transformation efficiency decreased with higher valences (Table 1). This may be due to the stronger interaction between sepiolite and DNA cations of higher valence, resulting in lower efficiency of DNA delivery into cells. Note that incubation of sep/DNA with bacteria (in suspension) without using the spreader, fail to transfer DNA into bacteria. This suggests that the sliding friction on solid agar plates is necessary, suggesting that DNA internalize into bacteria in a Yoshida effect manner.

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

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

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

4- Discussion

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

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

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

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

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

5- Conclusion

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

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

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

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

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

Figure 5. TEM image of extracted pUC19 using sepiolite.

Figure 6. Optimized protocol for sepiolite-mediated bactial transformation. First, the 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 $MgCl_2$. 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 ^{o}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.