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Sandrine Ragu, Olivier Piétrement, Bernard Lopez

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# Clays and Clay Minerals

## Binding of DNA to Natural Sepiolite: Applications in Biotechnology and Perspectives

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<b>Abstract:</b>	<p>DNA manipulation is crucial for many biotechnological prospects and for medical applications such as gene therapy. This requires the amplification and extraction of DNA from bacteria and the transfer of these DNA molecules into cells, including bacterial and mammalian cells. The capacity of the natural magnesium silicate clay mineral sepiolite to bind to DNA makes it a potential useful tool for biotechnological/medical strategies. In addition, sepiolite is inexpensive and classified as non-toxic and non-carcinogenic. This review will first describe the physico-chemical interactions between sepiolite and DNA. Then, the leverage of sepiolite/DNA interactions for DNA extraction from bacteria, to optimize DNA transfer into bacteria and DNA transfection into mammalian cells are presented. Finally, the putative toxicity of sepiolite and its advantages and perspectives for future prospects, such as the improvement of immunotherapy are also discussed.</p>	
<b>Corresponding Author:</b>	Bernard Lopez Institut Cochin FRANCE	
<b>Corresponding Author Secondary Information:</b>		
<b>Corresponding Author's Institution:</b>	Institut Cochin	
<b>Corresponding Author's Secondary Institution:</b>		
<b>First Author:</b>	Sandrine Ragu	
<b>First Author Secondary Information:</b>		
<b>Order of Authors:</b>	Sandrine Ragu	
	Olivier Piétrement	
	Bernard Lopez	
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**Clays & Clay Minerals**

1 Binding of DNA to Natural Sepiolite: Applications in Biotechnology and Perspectives

2 Sandrine Ragu<sup>#,1</sup>, Olivier Piétrement<sup>#,2</sup>, and <sup>1</sup>Bernard S. Lopez<sup>\*,1</sup>

3 <sup>1</sup> Université de Paris, INSERM U1016, UMR 8104 CNRS, Institut Cochin, Equipe Labellisée

4 Ligue Contre le Cancer, Paris, France

5 <sup>2</sup> Laboratoire Interdisciplinaire Carnot de Bourgogne, CNRS UMR 6303, Université de

6 Bourgogne, 9 Avenue Alain Savary, 21078 Dijon Cedex, France

7 #These authors contributed equally to this work

8 \*Corresponding author: [bernard.lopez@inserm.fr](mailto:bernard.lopez@inserm.fr), Tel: +33 1 5373 2740, Institut Cochin, 24

9 rue du Faubourg St Jacques, 75014, Paris, France.

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ABSTRACT

DNA manipulation is crucial for many biotechnological prospects and for medical applications such as gene therapy. This requires the amplification and extraction of DNA from bacteria and the transfer of these DNA molecules into cells, including bacterial and mammalian cells. The capacity of the natural magnesium silicate clay mineral sepiolite to bind to DNA makes it a potentially useful tool for biotechnological/medical strategies. In addition, sepiolite is inexpensive and classified as non-toxic and non-carcinogenic. This review will first describe the physico-chemical interactions between sepiolite and DNA. Then, the leverage of sepiolite/DNA interactions for DNA extraction from bacteria, to optimize DNA transfer into bacteria and DNA transfection into mammalian cells, are presented. Finally, the putative toxicity of sepiolite and its advantages and perspectives for future prospects, such as the improvement of immunotherapy, are also discussed.

Keywords: Sepiolite; dna interaction; bacterial transformation ; DNA plasmid extraction; DNA transfection in mammalian cells

27 INTRODUCTION

28 Genome engineering is a major strategy for the development of new biological models of  
29 interest in academia and the applied sciences, such as biotechnological, biomedical, and  
30 agronomic research. Remarkably, this constitutes the basis of gene therapy aiming to correct  
31 an endogenous, mutated, defective gene and to restore normal physiological functions. DNA  
32 is the central biomolecule bearing the genetic information that is transmitted from one  
33 generation to another. Hence, methods aimed at extracting and purifying DNA and/or  
34 transferring DNA into living cells represent central issues in biotechnology and biomedical  
35 applications.

36 Notably, the development of vectors based on biohybrid materials for DNA transfer, which  
37 allows the avoidance of virus-based vectors, represents an appealing approach for the  
38 treatment of different genetic disorders (Choy et al., 2000; Lin et al., 2006; Shi et al., 2011;  
39 Wu et al., 2014; Choi et al., 2014). Among the different micro/nanoparticles, sepiolite  
40 represents an alluring solution.

41 Sepiolite is a natural magnesium silicate clay mineral with a micro-fibrous morphology. The  
42 size of sepiolite fibres varies according to geographical origin. For example, the length of  
43 sepiolite fibres from Taxus Basin deposits in Spain ranges from 0.2 to 0.8  $\mu\text{m}$  (Castro-  
44 Smirnov et al., 2016; Piétrement et al., 2018). Interestingly, sepiolite is able to bind different  
45 kinds of biological molecules, including polysaccharides (Alcântara et al., 2014), lipids  
46 (Wicklein et al., 2010), proteins (Alcântara et al., 2012), and virus particles (Ruiz-Hitzky et al.,  
47 2012). These capacities make sepiolite a promising micro/nanovector for the non-viral  
48 transfer of biomolecules (Piétrement et al., 2018). Particularly, combining analyses from  
49 physics, chemistry, and biology, sepiolite has been shown to bind to DNA (Castro-Smirnov et

50 al., 2016). These capabilities can be leveraged for biotechnology applications for future  
51 promising biomedical strategies.  
52 This aim of this review was to discuss the potential biotechnological and biomedical uses of  
53 sepiolite, based on the physico-chemical characterisation of sepiolite/DNA interactions. Such  
54 interactions allow consideration of sepiolite as a promising substrate for DNA extraction  
55 from bacteria, for improving sepiolite-mediated bacterial transformation, and for  
56 mammalian cell DNA transfection. A further objective was to envision the putative toxicity of  
57 sepiolite *versus* its advantages and future perspectives.

#### 58 SEPIOLITE AND ITS INTERACTION WITH DNA

##### 59 *Sepiolite Structure.*

60 DNA/sepiolite interactions depend mainly on the sepiolite nanostructure and surface  
61 properties. Sepiolite belongs to a family of clays called pseudo-layered hydrated fibrous  
62 magnesium phyllosilicates. Its chemical formula is  $\text{Si}_{12}\text{Mg}_8\text{O}_{30}(\text{OH})_4(\text{H}_2\text{O})_4(\text{H}_2\text{O})_8$  and it has a  
63 trioctahedral structure: two layers of tetrahedral silica sandwiching a central octahedral  
64 magnesium oxide-hydroxide layer (TOT family). Unlike other phyllosilicates, sepiolite, like  
65 palygorskite, exhibits a three-dimensional crystalline organization with surface channels and  
66 internal tunnels. The crystal structure of sepiolite was studied by Brauner and Preisinger  
67 (Brauner & Preisinger, 1956) and is shown in Fig. 1. The internal channels have dimensions of  
68 close to  $0.37 \times 1.06\text{-nm}^2$ .

69 Many active sites on the sepiolite surface consist of both silanol groups (SiOH) located at the  
70 periphery of the fibers and magnesium hydroxyl type ( $\text{Mg}-(\text{H}_2\text{O})_2$ ) groups located on either  
71 side of the channels (see Fig. 1). These sites play an essential role in DNA adsorption (Castro-  
72 Smirnov et al., 2016) and allow the grafting of numerous chemical groups (García et al.,  
73 2011; Moreira et al., 2017; Undabeytia et al., 2019) and are responsible for the remarkable

74 physico-chemical properties of sepiolite. Sepiolite also has a sorption capacity mainly due to  
75 the edge surfaces: the tunnels can accept molecules and retain them in a three-dimensional  
76 space, whereas the surface channels interact with the outside. Both participate in very  
77 different sorption mechanisms.

78 Like all clays, sepiolite has the property of retaining and exchanging cations with its  
79 environment, which is called the cation exchange capacity (CEC). However, the structure of  
80 sepiolite makes the internal faces of the channels difficult to access, and its three-  
81 dimensional structure prevents swelling, unlike many of the two-dimensional phyllosilicate  
82 clays. Sorption is, therefore, limited to the capacity of binding to the faces of the edges of  
83 the sheets that emerge at the periphery of the fibres. For sepiolite, the CEC is quite low (20  
84 to 30 meq/100 g of mineral) compared to other clays, but nevertheless plays an essential  
85 role in the ability of sepiolite to adsorb various biomolecules, such as polysaccharides  
86 (Darder et al., 2006), lipids (Wicklein et al., 2012), proteins (Alcântara et al., 2012), and  
87 viruses (Ruiz-Hitzky et al., 2009), giving rise to a great diversity of bio-nanocomposites.  
88 Finally, as a consequence of its structure, sepiolite has a large interaction surface that can be  
89 split into two contributions; the internal surface is close to 300 m<sup>2</sup>/g, and the external  
90 surface ranges from 200 to 300 m<sup>2</sup>/g (mainly depending on the fineness of the fibres). This  
91 large surface of interaction (both internal and external) partly gives an understanding of how  
92 sepiolite can interact strongly with biomolecules.

93 This ability to adsorb cations has helped to develop the theory that clays could be the cradle  
94 of life on Earth, or, more specifically, of complex biochemical compounds that made life  
95 possible (Yang et al., 2013). The hypothesis is that the clay hydrogel, thanks in part to its  
96 structure organized in microcavities, plays a containment role for basic chemical elements  
97 that could have carried out the complex reactions at the origin of the formation of proteins,

98 DNA, and ultimately all of the machinery involved in the functions of a living cell. The clay  
99 hydrogels could also then have confined and protected these chemical processes until the  
100 formation of the membrane surrounding the living cells.

101 In addition, the adsorption of DNA on mineral surfaces might favor horizontal gene transfer  
102 into living organisms, which may play a significant role in the evolution of living species. All  
103 these studies suggest a specific interaction between clays and nucleic acids.

#### 104 *Sepiolite and DNA*

105 With the aim of using sepiolite as a new DNA vector, a study have thus shown that DNA  
106 could efficiently bind to sepiolite, notably highlighting the role of multivalent cations as  
107 enhancers of the DNA binding process (Castro-Smirnov et al., 2016). However, while  
108 monovalent cations reduce the adsorption of DNA molecules to mica (Pastre et al., 2003),  
109 surprisingly, these cations promote the adsorption to sepiolite fibres. While monovalent  
110 cations are less effective than multivalent cations, they unequivocally contribute to the  
111 adsorption process (see Fig. 2). Interestingly, the presence of polyvalent cations ( $Mg^{2+}$ ,  $Ca^{2+}$ ,  
112 spermidine, or spermine) strongly stimulates the adsorption of DNA depending on the  
113 valence of the cation (see Fig. 2). These results suggest an adsorption mechanism mediated  
114 by an electrostatic bridge through hydrogen bonds between the DNA phosphate groups and  
115 the silanol groups located on the outer surface of the sepiolite, which is unlike the mica  
116 surface, where counterion correlations occur (Pastre et al., 2003; Pastré et al., 2006).  
117 Fourier-transform infrared (FTIR) spectroscopy analysis thus showed that, indeed, the  
118 interaction of DNA with sepiolite was mediated by only the external silanol groups, whereas  
119 the hydroxyl groups of the octahedral sheets of sepiolite play no role (Castro-Smirnov et al.,  
120 2016).



121 If the DNA is adsorbed onto the sepiolite fibres, the question of its desorption also arises in  
122 the context of a project to create a nano-cargo for cell transfection. In cellulose, the  
123 mechanisms that could induce this desorption remain unknown, but the possibility of DNA  
124 desorption *in vitro* has been tested through the desorption of DNA from the  
125 bionanocomposite by incubating it with EDTA, a well-known metal ion chelating agent. By  
126 using different DNA plasmids, the quality of the DNA recovered after its attachment to  
127 sepiolite was examined and the different DNA isoforms (super-coiled, open circle, or linear)  
128 were unmodified after their binding to sepiolite. This shows that sepiolite does not alter the  
129 quality of DNA and that this method can be considered a new method of DNA purification  
130 (Castro-Smirnov et al., 2016).

131 All these results confirmed the potential of sepiolite as an efficient cargo for DNA transfer  
132 into cells. Moreover, the capacity of desorption suggests the possibility of using sepiolite for  
133 DNA extraction.

#### 134 EXTRACTION OF PLASMID DNA FROM BACTERIA

135 DNA extraction and purification, especially of plasmids, is the basis of molecular biology.  
136 Plasmids are circular DNA molecules that autonomously replicate in bacteria and are  
137 essential tools in molecular biology and biotechnology, from bacteria to mammalian cells.  
138 Indeed, they are used as DNA vectors/backbones in gene transfer technology. Hence, fusion  
139 of an exogenous DNA (for example, a piece of a human DNA) allows replication and  
140 amplification into bacteria. After this replication and amplification process, their extraction is  
141 necessary for subsequent experiments.  
142 Several methods of plasmid extraction are available commercially. These methods are more  
143 or less expensive and time-consuming. The DNA binding capability of sepiolite, associated  
144 with the possibility of DNA release, makes sepiolite a simple and inexpensive alternative

145 method (Castro-Smirnov et al., 2020). Indeed, the binding of DNA onto sepiolite requires  
146 cations, notably divalent cations such as  $Mg^{2+}$  or  $Ca^{2+}$  (Castro-Smirnov et al., 2016). The  
147 chelation of cations with EDTA, therefore, enables the release of DNA from sepiolite, which  
148 is not covalently bound (Castro-Smirnov et al., 2020). The general protocol (Castro-Smirnov  
149 et al., 2020) can be summarized as follows:

- 150 a- Bacteria bearing plasmids are grown in the appropriate medium with the suitable  
151 selection of antibiotics.
- 152 b- At the mid-saturation state (as monitored by spectrophotometry), bacteria are  
153 pelleted by centrifugation and then re-suspended in specific buffers. Bacteria are  
154 lysed, and the DNA is denatured (the two complementary strands are separated) in  
155 alkaline buffers.
- 156 c- The addition of neutralizing buffer allows plasmid DNA (which are small DNA  
157 molecules) to renature (return to the bicatenary DNA structure), remaining soluble.  
158 In contrast, bacterial genomic DNA (which are long DNA molecules) cannot properly  
159 renature, and so they precipitate.
- 160 d- Centrifugation precipitates genomic DNA pellets and bacterial debris.
- 161 e- The supernatant (containing the plasmid DNA) is recovered. Sepiolite plus  $Mg^{2+}$  or  
162  $Ca^{2+}$  is added, allowing plasmid DNA to bind to sepiolite, allowing the subsequent  
163 washing steps to eliminate contaminants.
- 164 f- Sepiolite-bearing DNA is centrifuged and washed with Tris buffer.
- 165 g- Then, EDTA is added to the sepiolite/DNA pellets, releasing the plasmid DNA from  
166 the sepiolite.
- 167 h- Plasmid DNA and sepiolite are then separated by centrifugation (sepiolite in the  
168 pellet and plasmid DNA in the supernatant).

169 Steps (a) to (d) are common to almost all methods of plasmid extraction.  
170 Importantly, the structures of plasmid DNA molecules are not altered by this procedure, as  
171 shown by electrophoresis and transmission electron microscopy analyses. Moreover,  
172 plasmid DNA can be processed by enzymes such as restriction enzymes (Castro-Smirnov et  
173 al., 2020). These facts attest to the good quality of the plasmid DNA extracted by the  
174 sepiolite-based method.

#### 175 IMPROVEMENT OF THE YOSHIDA EFFECT ON BACTERIAL TRANSFORMATION

176 In bacteria and non-animal eukaryotic cells, the non-viral transfer of DNA is called  
177 transformation. In animal cells, it is called transfection.  
178 Efficient transformation requires first rendering the bacteria to be competent for DNA  
179 transfer. However, the protocols to prepare competent bacteria are generally laborious and  
180 time consuming. One alternative is to purchase commercially competent bacteria, but this  
181 method is expensive. One advantage of the Yoshida effect protocol for bacterial  
182 transformation is that it does not require the use of competent bacteria.  
183 In the Yoshida effect protocol, the solution containing the bacteria and the acicular material  
184 forms a colloidal solution. Upon spreading on solid agar medium in Petri dishes using a solid  
185 spreader, the sliding friction stimulates bacterial transformation (Yoshida et al., 2001;  
186 Yoshida, 2007; Yoshida & Sato, 2009). However, the Yoshida effect was first described with  
187 asbestos, thus raising health and toxicity concerns for its use. Sepiolite, which is considered  
188 non-toxic and non-carcinogenic (see below), can circumvent this health worry. Indeed, the  
189 fibrous nature of sepiolite favors bacterial transformation through a Yoshida-like effect  
190 (Yoshida, 2007; Yoshida & Sato, 2009; Tan et al., 2010; Wilharm et al., 2010; Rodríguez-  
191 Beltrán et al., 2012; Rodríguez-Beltrán et al., 2013).

192 The Yoshida effect does not absolutely require the pre-assembly of DNA with the fibers.  
193 However, combining different capacities of sepiolite (i.e. the use of sonicated sepiolite, the  
194 capacity to bind DNA and bacteria), a 100-fold improvement in transformation efficiency has  
195 been observed (Castro-Smirnov et al., 2020). The general protocol can be summarized as  
196 follows.

197 a- The use of sonicated sepiolite (sSep). Indeed, sepiolite spontaneously aggregates and  
198 can be dissociated by sonication, increasing its spreading efficiency.

199 b- Pre-assembly of DNA and sSep, using the characteristics (incubation in the presence of  
200 divalent cations) then centrifugation) described above, before adding the bacteria.

201 c- Pre-incubation of the sSep/DNA biohybrid material with bacteria (at the mid-phase of  
202 growth) before spreading. Indeed, sepiolite can spontaneously bind to bacteria (Castro-  
203 Smirnov et al., 2016, 2020) and it can thus be used for liquid decontamination. In  
204 addition, this procedure favors bacterial transformation (Castro-Smirnov et al., 2020).  
205 However, the Yoshida effect is still necessary because sepiolite-bacteria assembly alone is  
206 not sufficient for efficient internalization.

207 Adapting these 3 conditions to the Yoshida effect, transformation efficiencies reached close  
208 to  $10^6$  transformants per microgram of DNA (Castro-Smirnov et al., 2020). Such  
209 transformation efficiency is lower than that of the commercially available competent  
210 bacteria but is largely sufficient for most molecular biology applications at a lower cost. This  
211 method is also much less time-consuming than the classical protocols for preparing  
212 competent bacteria.

### 213 TRANSFECTION OF MAMMALIAN CELLS

214 Transfer of DNA into mammalian cells is an essential issue in biotechnology and biomedical  
215 applications. Indeed, this constitutes the basis of strategies aimed at designing new model

216 organisms for academic, biomedical, or agronomical research and gene therapy. However,  
217 mammalian cells do not efficiently incorporate exogenous DNA.  
218 The Yoshida effect cannot work with mammalian cells because frictional forces kill them.  
219 However, sepiolite can be spontaneously internalized into mammalian cells without  
220 requiring friction, mainly through endocytosis and macropinocytosis pathways that involve  
221 the invagination of the cell membrane around the sepiolite fibers, resulting in its  
222 incorporation in the endosomes (intracellular [organelles](#) embedded into membranes) of the  
223 cells (Castro-Smirnov et al., 2017). Interestingly, this process has a low effect on viability  
224 (Castro-Smirnov et al., 2017; Ragu, Dardillac, et al., 2020). Given that sepiolite can bind DNA,  
225 it can serve as a vector for stable DNA transfection into mammalian cells (Castro-Smirnov et  
226 al., 2016). This application relies on the binding of DNA to sSep prior to the addition of the  
227 sSep/DNA bio-hybrid material to the cell media. The uptake of the sSep/DNA bio-hybrid also  
228 acts through the endocytosis pathway, and the intracellular sSep/DNA bio-hybrid is then  
229 embedded in endosomes. The addition of drugs that destabilize endosome membranes,  
230 such as chloroquine, results in a 2-fold increase in the transfection efficiency (Castro-  
231 Smirnov et al., 2016).

232 These methods of cell transfection are somewhat less efficient than the use of commercial  
233 reagents but are much less expensive. Different strategies can be envisioned to increase the  
234 efficiency of intake of the nanoparticles into the cell. These strategies might be based on the  
235 structure of the cell membrane, which is constituted of a bi-layer of lipids. Moreover,  
236 techniques that could favor the delivery from the endosomes, such as favoring endosome  
237 collapse into the cells, are now address; this might favor DNA delivery into cells. Moreover,  
238 the pH of the endosome is very acid, and thus might lead to hydrolysis of the DNA. Methods  
239 controlling this issue are, therefore, under investigation. The injection of sepiolite in vivo

240 should be precisely analyzed. Indeed, it could be injected into the blood micro-vessel and/or  
241 accumulated in the liver. Moreover, the production of ROS (reactive oxygen species) and of  
242 inflammatory cytokines might generate unwanted inflammation and/or premature ageing.  
243 Therefore, the behavior and the fate of sepiolite, as well as the consequences of sepiolite  
244 injection (through different procedures such as in blood versus intra-peritoneal), should be  
245 addressed and analyzed in depth and in vivo in a mouse model before attempting this  
246 transfer in humans.

#### 247 TOXICITY OF SEPIOLITE IN HUMAN CELLS

248 Because of the multiple potential uses of sepiolite, including its biomedical applications, the  
249 question of its toxicity has become an important issue. Its fibrous nature has led to concerns  
250 of asbestos-like effects. This can be supported by the fact that, like asbestos, sepiolite can  
251 generate a Yoshida effect and, in addition, generate DNA damage in bacteria (González-  
252 Tortuero et al., 2018). However, translating these conclusions to mammalian cells would  
253 represent an over-interpretation. Indeed, bacteria are much smaller than mammalian cells  
254 (approximately 1000-fold). Therefore, while the length of a sepiolite fibre is similar to that of  
255 a bacterial cell, the sepiolite fiber is much smaller than that of a human cell (Castro-Smirnov  
256 et al., 2017; Ragu, Dardillac, et al., 2020). In addition, the genome of eukaryotes (including  
257 human cells) is embedded into a nuclear compartment, while in prokaryotes (bacteria) no  
258 nucleus exists, and DNA is contained directly in the cytoplasm. Therefore, a sepiolite fiber  
259 that penetrates bacteria can directly interact with its genomic DNA to alter it. In contrast, in  
260 mammalian cells, the sepiolite fibre that enters the cell will be in the cytoplasm and not in  
261 the nucleus. Hence, the sepiolite is not in contact with the genomic DNA (Castro-Smirnov et  
262 al., 2017; Ragu, Dardillac, et al., 2020). For DNA interaction, the sepiolite fiber should be  
263 transported into the nucleus, which appears to be an infrequent process (Castro-Smirnov et

264 al., 2017; Ragu, Dardillac, et al., 2020). In agreement with this observation, the interaction of  
265 human cells with sepiolite does not trigger the DNA damage response (Ragu, Dardillac, et al.,  
266 2020), supporting the fact that sepiolite does not attack genomic DNA in mammalian cells.  
267 Coherently, sepiolite appears to be weakly toxic in cultured mammalian cells (Castro-  
268 Smirnov et al., 2017; Ragu, Dardillac, et al., 2020), especially at the doses used for DNA  
269 transfection, which are much lower than other classical transfection methods.  
270 Sepiolite also induces the production of reactive oxygen species (ROS) in cells (Ragu,  
271 Dardillac, et al., 2020). Because ROS can potentially alter any biological component,  
272 including DNA, and lead to mutagenesis, this has raised some concerns about the potential  
273 toxicity of sepiolite. The respiratory chains that synthesize ATP and provide the energy  
274 required for various metabolic pathways generate ROS as by-products. However, cells can  
275 also control the production of ROS that act as secondary messengers in physiological  
276 processes (Ameziane-El-Hassani et al., 2016). In this situation, ROS are beneficial to the  
277 organism. Notably, while antioxidants have been proposed to protect against tumorigenesis,  
278 in contrast, treatment with antioxidants favors the development of lung carcinomas and  
279 metastasis (Breau et al., 2019; Le Gal et al., 2015; Wiel et al., 2019), thus underlying the  
280 potential benefit of ROS production. In addition, ROS can also trigger the induction of  
281 apoptosis, eliminating stressed cells, to the benefit of the whole organism. Hence, an  
282 intracellular increase in ROS can result from the detection of sepiolite by cells and their  
283 response but does not automatically imply that the fate of the cell or organism will be  
284 jeopardized.

285 Finally, ROS can oxidize lipid cell membranes, leading to lipid peroxidation (LP). However,  
286 sepiolite represses LP from the supernatants of rat brain homogenates, suggesting a putative  
287 antioxidant role for sepiolite (Cervini-Silva et al., 2015). One hypothesis is that cells respond

288 to sepiolite interaction through the production of controlled ROS but, in parallel, sepiolite  
289 could also scavenge ROS, thus protecting against potential noxiousness.

290 One important component of the potential toxicity of fibres (such as asbestos) relies on the  
291 fact that they remain stuck in tissues, generating chronic inflammation, which can become  
292 pathogenic with time. These concerns connect two issues: the size of the fibres and the  
293 exclusion capacity of the cells to the fibres. Indeed, small fibres should more easily be  
294 excluded, thereby avoiding a potential asbestos-like effect. The large majority of the  
295 sepiolite fibres from Vallecas-Vicalvaro deposits in Spain range in length from 200 to 800 nm  
296 (Castro-Smirnov et al., 2016), values that are lower than those reported in the literature  
297 (Bellmann et al., 1997). Such sizes are much smaller than those of mammalian cells;  
298 therefore, the capacity of the cells themselves to exclude such sepiolite fibres thus becomes  
299 prominent for its harmlessness. Interestingly, the stable natural intrinsic fluorescence of the  
300 sepiolite used in the present study( from Vallecas-Vicalvaro deposit) enabled examination of  
301 its fate in cells (Castro-Smirnov et al., 2017; Ragu, Dardillac, et al., 2020). In particular, time-  
302 lapse fluorescent video microscopy analysis revealed the spontaneous exclusion of these  
303 sepiolite fibers from mammalian cells, likely through an exocytosis process that involved the  
304 membranes of endosomes (Castro-Smirnov et al., 2017). These data can account for the low  
305 cellular toxicity of sepiolite (Denizeau et al., 1985; Castro-Smirnov et al., 2016; Ragu,  
306 Dardillac, et al., 2020). More generally, and in agreement with the size effect hypothesis,  
307 both epidemiological studies and in vitro and in vivo analyses have led to the conclusion that  
308 sepiolite, especially with fiber lengths less than 5  $\mu\text{m}$ , does not present a health risk  
309 (Maisanaba et al., 2015). Moreover, previous biological and epidemiological analyses have  
310 led the International Agency for Research on Cancer (IARC) to classify sepiolite as non-



311 hazardous and non-carcinogenic (McConnochie et al., 1993; Santarén & Alvarez, 1994;  
312 Wagner et al., 1987).

313 PERSPECTIVES FOR IMPROVEMENT OF IMMUNOTHERAPY

314 Additional bio-medical applications can be derived from the interaction of sepiolite with  
315 cells, such as immunotherapy. This strategy takes advantage of the capacity of the immune  
316 system to eliminate stressed cells. It has been applied to cancer therapy (Esfahani et al.,  
317 2020) and was recognized by the Nobel Academy (Nobel prize of Physiology or Medicine to  
318 James Allison and Tasuku Honjo, in 2018). This process is triggered by the production and  
319 excretion of inflammatory cytokines that modify the microenvironment of the cell. This leads  
320 to activation of the innate immune response, which recruits immune system cells (for  
321 instance, natural killer cells, macrophages, and lymphocytes). Tumors are classified as hot or  
322 cold, depending on whether they respond to immunotherapy. Unfortunately, the majority of  
323 tumors are cold. Therefore, strategies that are able to change the status of cold tumors to  
324 hot tumors should represent important progress in immunotherapy (Duan et al., 2020).  
325 Sepiolite is internalized into cells and recognized, leading to the production of inflammatory  
326 cytokines (Ragu, Dardillac, et al., 2020). Hence, this particularity opens a hopeful perspective  
327 to turn cold tumors into hot tumors for immunotherapy.

328 Additionally, grafting DNA onto sepiolite can be expected to optimize the inflammatory  
329 response. Indeed, cytoplasmic DNA is recognized by cells that induce inflammatory cytokines  
330 (Ragu, Matos-Rodrigues, et al., 2020). Thus, one hope would be that the bio-hybrid  
331 sepiolite/DNA material that delivers DNA into the cytoplasm of the cell might synergize the  
332 efficiency of cytokine production and thus the immune response.

333 CONCLUSIONS

334 DNA is negatively charged, and the first obstacle is the cell membrane, which is made of lipid  
335 bilayers that have charges incompatible with the spontaneous DNA uptake by cells. Non-  
336 viral methods use physical and chemical strategies to bypass the membrane barrier but are  
337 generally toxic to the cell. One limitation of both viral and physicochemical methods is the  
338 fact that they can transfer DNA only. One ideal strategy would be based on transport  
339 systems that can co-convey different kinds of molecules. The development of novel  
340 nanocarriers using biohybrid materials for non-viral gene transfer constitutes thus a  
341 promising approach. In this context, sepiolite is an enticing candidate as a nanocarrier for  
342 the non-viral vectorization of DNA, because it is biocompatible and is spontaneously  
343 internalized and excreted by mammalian cells. Moreover, thanks to its spontaneous  
344 fluorescence, sepiolite can be detected in cells (and maybe hopefully in animals) without  
345 requirement of fluorescent chemical grafting and can be followed in cells. Importantly,  
346 sepiolite is weakly toxic to mammalian cells and is classified as non-hazardous and non-  
347 carcinogenic by the IARC (WHO). Strategies based on natural internalization/externalization  
348 capacities of the mammalian cells are preferable to limit cell toxicity. These strategies  
349 implied that DNA should first bind to sepiolite to form a sepiolite/DNA (Sep/DNA) biohybrid.  
350 Indeed, sepiolite can adsorb different molecules very efficiently, thus allowing the  
351 development of many elaborate strategies, and, as a natural clay, sepiolite represents an  
352 answer to societal concerns regarding challenging nanotechnologies.

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357 DECLARATIONS

358 *To be used for non-life science articles*

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366 Availability of data and material: not applicable

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368 Authors' contributions: SR, OP, and BSL wrote and corrected the manuscript.

369 *To be used for articles with biological applications*

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***Clays & Clay Minerals***

381 Code availability: Not applicable

382 Authors' contributions: SR, OP and BSL wrote and corrected the manuscript.

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546 Fig. 1. Sepiolite structure (adapted from (Brauner & Preisinger, 1956)).

547

548 Fig. 2. (A) The effects of the presence of cations with various valences on DNA adsorption.

549 Reaction conditions: 10 mM Tris-HCl pH 7.5, salmon sperm DNA and sepiolite concentrations

550 of 615 ng/ $\mu$ l and 1 mg/mL, respectively (from (Castro-Smirnov et al., 2016)). TEM images of

551 DNA condensed with (B) spermidine and (C) the interaction with sepiolite fibers.

552 Transmission electron microscopy (TEM) imaging: DNA molecules (5 nM) were condensed in

553 10 mM Tris-HCl pH 8, 50 mM NaCl, and 1 mM spermidine. 5  $\mu$ L of reaction mixture was

554 deposited onto a 600 mesh copper grid coated with a thin carbon film previously activated

555 by glow discharge in the presence of 1-aminopentane (Merck, Saint Quentin Fallavier,

556 France) (Dupaigne et al., 2018). After 1 min, the grids were washed with aqueous 2 wt.%

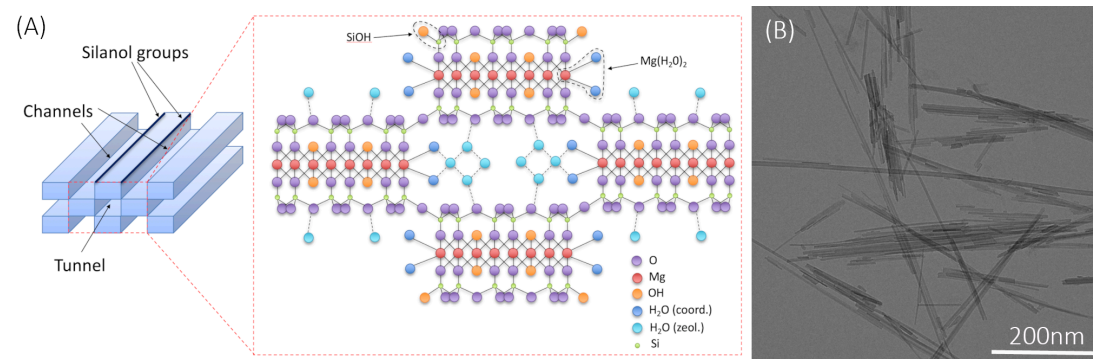
557 uranyl acetate (Merck, Saint Quentin Fallavier, France) and then dried with ashless filter

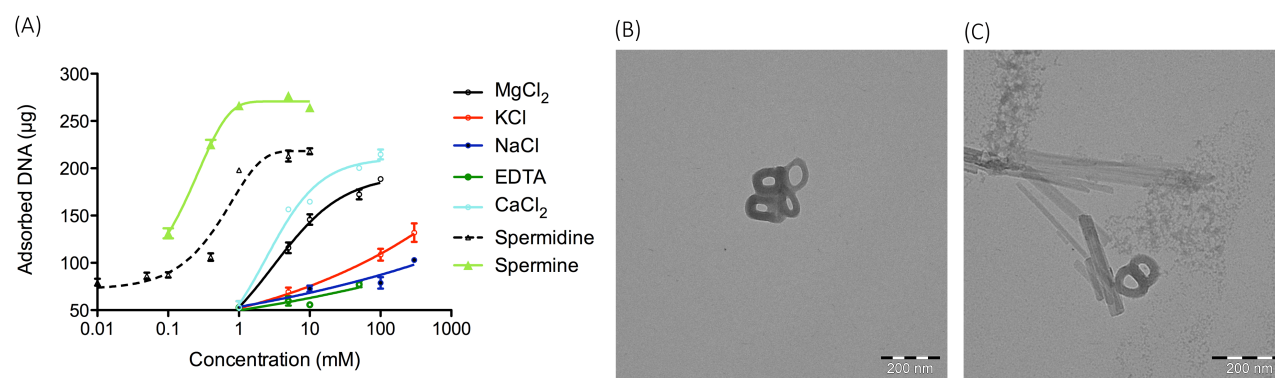
558 paper (VWR, Rosny sous bois, France). TEM observations were carried out on a Zeiss 912AB

559 transmission electron microscope in bright field mode. Electron micrographs were obtained

560 using a ProScan 1024 HSC digital camera and iTEM software (Olympus, Soft Imaging

561 Solutions, Munster, Germany).







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**Supplementary Material**

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