REPORT

New biohybrid materials as nanocarriers of nucleic acids, and their biotechnological applications

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ABSTRACT

This work focused on the synthesis, physicochemical characterization and biotechnological applications of new nanomaterials composed of sepiolite for gene transfer. Biohybrids were obtained of sepiolite nanofibers covered by nucleic acids (DNA and RNA molecules). They were characterized by novel techniques and procedures, identifying that the DNA gets adsorbed onto the surface of sepiolite nanofibers that were previously subjected to disaggregation procedures for a maximum fractionation. Such interaction is mediated by silanol groups evenly spaced on the outer surface of silicate particles, through electrostatic interactions, hydrogen bonds, cation bridges and Van der Waals forces. It was shown that sepiolite can be spontaneously internalized by eukaryotic cells in culture, and the mechanisms mediating such internalization were characterized, both endocytic and non-endocytic. Similarly, it was found that sepiolite nanofibers can be transferred between neighboring cells spontaneously, these particles displaying intrinsic fluorescence that facilitates its detection within the cell as gene transfer vector. The biohybrids were assayed with satisfactory results for the transfer of a plasmid DNA vector into cancer cell lines, and strategies were also established to significantly increase their transfection efficiency. These studies demonstrated for the first time the use of sepiolite nanofibers as a platform for transferring nucleic acids and potentially other types of molecules into mammalian cells, those molecules administered for functional or therapeutic purposes, among other applications. This work granted the Annual Award of the National Academy of Sciences of Cuba for the year 2016.

Keywords: Nanomaterials, nanofibers, clays, sepiolite, gene transfer, transfection, nanocarriers, endocytosis

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RESUMEN

Nuevos materiales biohíbridos como nano-portadores de ácidos nucleicos, y sus aplicaciones biotecnológicas. Este trabajo se centró en la síntesis, caracterización físicoquímica-biológica y aplicaciones biotecnológicas de nuevos nanomateriales basados en el uso de la sepiolita como vector de transferencia génica. Se obtuvieron biohíbridos de nanofibras de sepiolita con ácidos nucleicos (ADN y ARN) adsorbidos, caracterizados mediante técnicas y metodologías novedosas. Se identificó que el ADN se adsorbe de manera reversible y eficiente en las nanofibras de sepiolita previamente desagregadas e individualizadas al máximo. Dicha interacción es mediada por los grupos silanoles regularmente localizados en la superficie externa del silicato, por interacciones que pueden implicar fuerzas electrostáticas, puentes de hidrógeno, puentes a través de cationes, y fuerzas de Van der Waals. Se observó que las nanofibras de sepiolita pueden ser internalizadas de manera espontánea por células eucariotas en cultivo, y se identificaron los mecanismos endocíticos y no endocíticos predominantes de internalización. De igual manera, pueden ser transferidas entre células vecinas de forma espontánea, además de poseer fluorescencia intrínseca, lo cual facilita la detección de su localización como vehículo de transferencia génica. Los biohíbridos se ensayaron de forma satisfactoria para la transferencia de un vector génico plasmídico en líneas celulares cancerígenas, y se ensayaron estrategias que incrementaron significativamente la eficiencia de transfección. Estos estudios demostraron por primera vez el uso de las nanofibras de sepiolita como una plataforma para la transferencia de ácidos nucleicos y potencialmente otras moléculas a células de mamíferos, con posible acción terapéutica o funcional, u otras aplicaciones. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2016.

Palabras clave: Nanomateriales, nanofibras, arcillas, sepiolita, transferencia génica, transfección, nanoportadores, endocitosis

*I*ntroduction

DNA transfer into mammalian cells is a state-of-the-art technique in nanobiotechnology, playing a pivotal role between the design of novel and promising strategies for gene therapy and the development of new

experimental models for academic and applied research in medicine, biotechnology and agronomy.

However, its effectiveness depends on the availability of efficient nanocarriers for the delivery of new gene vectors for non-viral gene transfer. One alternative to cope with this challenge comprises the use of biohybrid nanomaterials, and particularly bionanocomposites, with attractive properties for this purpose [1]. This work is focused on new approaches with potential impact on the treatment of genetic diseases, cardiovascular diseases, AIDS, Alzheimer's disease, and several types of cancer, by using gene transfer. In this paper we report the synthesis and physicochemical-biological characterization of new biohybrid nanomaterials, in which the DNA is adsorbed on composites of clay mineral of fibrous morphology: sepiolite. In addition, novel protocols for the use of these composites as gene vectors were developed for the first time, to transfer DNA and RNA into mammalian cells.

Results and discussion

Spontaneous internalization of sepiolite in mammalian cells

Sepiolite is a clay mineral of high purity (> 95 %) obtained by a wet grinding process. It was supplied by TOLSA SA (Spain) as Pangel S9, previously obtained from the Vallecas-Vicalvaro deposits near Madrid. Sepiolite is a natural hydrated magnesium silicate with micro-nanofibrous morphology, with a theoretical cell formula $Si_{12}O_{30}Mg_8(OH,F)_4(H_2O)_4 \cdot 8H_2O$ [2]. We took advantage of the nature of the sepiolite surface, which shows extensive negatively charged area, and its nanofibrous morphology, as potential nanoplatform for the co-transfection of different types of active molecules. In fact, we formerly found that sepiolite was capable of interacting with polysaccharides, lipids, proteins and viruses, resulting in a wide variety of biohybrid and bionanocomposite materials for diverse applications, which could find applications in biotechnology and biomedicine [3].

A first point on this study was to analyze the aspect and size distribution of these clay fibers by transmission electron microscopy (TEM). For this, clay was submitted to ultrasonication to conveniently detangle the fibers. This treatment may have shorten them but also generated an relatively uniform particle size range and reactivity, with an average width of 15 nm and about 80 % of fibers 200-400 nm in length [4].

The sub-micrometric size combined with the low toxicity described for sepiolite fibers, makes it potentially suitable for delivery of molecules into mammalian cells. Considering this, working concentrations of sepiolite suspensions in the range of 1-10 ng/μL were prepared and assessed for toxicity. They were found to be non-toxic in different mammalian cell lines. Moreover, it was observed that sepiolite has a high natural stable fluorescence (green excitation at 488 nm and emission between 498 nm and 530 nm, red excitation at 532 nm and emission between 542 nm and 685 nm), which allows to directly follow up the uptake process of sepiolite nanofibers by cells without adding any other compound as required for other gene transfer vectors [5]. Taking advantage of this property, the internalization of sepiolite fibers was analyzed by laser confocal microscopy and the spontaneous cellular uptake was confirmed in V79 cells. Remarkably, time-lapse video-fluorescent microscopy revealed that mammalian cells were able to spontaneously internalized sepiolite fibers but were also able to eject them, and by this way, sepiolite nanofibers can be transported between adjacent cells. Additionally, the incorporation kinetics was followed by Flow Activated Cell Sorting (FACS), with 45 % of cells becoming fluorescent after 6 h, up to 55 % after 24 h [5]. Using TEM we found that in the cytoplasm the sepiolite fibers were surrounded by membranes or endosomes, suggesting the internalization by endocytosis (Figure 1).

Specific endocytosis and macropinocytosis structures were observed at the membrane/sepiolite junction (Figure 2). It was also found that some sepiolite fibers were not surrounded by endosomal membranes, suggesting an alternative pathway for endocytosis in the internalization of sepiolite (direct cytoplasmic membrane insertion). We used FACS analysis to quantitatively determine the involvement of macropinocytosis and clathrin-mediated endocytosis following incubation with sepiolite in the presence of endocytosis inhibitors including chloroquin (which blocks the clathrin-mediated endocytosis) and amiloride (which inhibits macropinocytosis). While chloroquine reduced only 20 % of the sepiolite cellular

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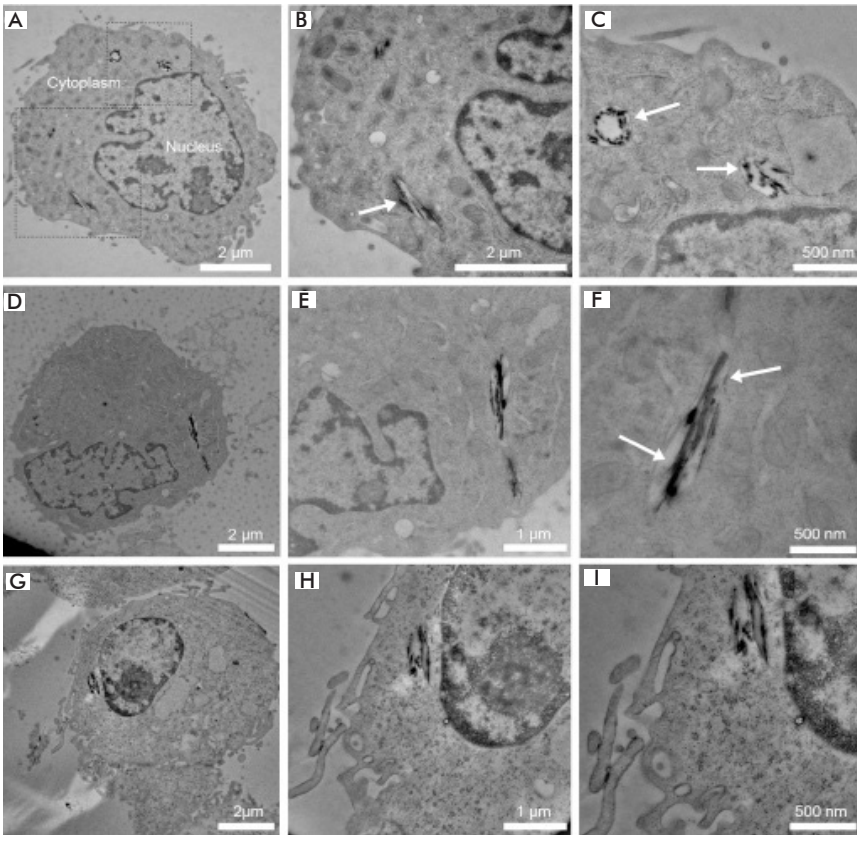

Figure 1. TEM images of a set of three different V79 cells incubated with sepiolite nanofibers. (A, D and G) with progressive 2× zooming in respective internal sepiolite fiber localizations (10 ng/μL of sepiolite in 5 × 10^6 cells). The arrows point to sepiolite nanofibers embedded into membranes.

internalization, amiloride inhibited it by 50 %, showing that one of the main mechanisms of cellular internalization of sepiolite is macropinocitosis, in agreement with previous observations using TEM [5].

Synthesis of biohybrids based on sepiolite and DNA

In order to demonstrate the use of sepiolite as DNA transfer vector, sepiolite fibers were incubated with plasmid DNA. It was shown that DNA could actually be adsorbed onto sepiolite spontaneously up to 80 µg of DNA per mg of sepiolite, as deduced from the analysis of adsorption isotherms (determined using UV-vis spectrometry to calculate DNA concentration in solution before and after adsorption). It was shown that polyvalent cations such as Mg^{2+}, Ca^{2+}, spermidine and spermine strongly stimulate DNA adsorption in a way directly correlated to the valence of the cations. Up to nearly 300 µg of DNA could be adsorbed per mg of sepiolite in the presence of the tetravalent cation (spermine). Most DNA molecules were absorbed instantaneously, suggesting a predominance of electrostatic and hydrogen bridging interactions between sepiolite and DNA. Subsequently, the changes in the surface electric charge of biohybrids due to the presence of multivalent cations and DNA were studied by determining zeta potential through electrokinetic analysis. Different DNA conformations were characterized: linear genomic DNA (300 bp in average) containing complex sequences (salmon sperm DNA), covalently closed circular plasmid DNA (5.7 kb), double-stranded linear DNA (dsDNA, 15 bp) and single-stranded linear DNA (non-translated single stranded DNA, 15 bp). Analysis using Fourier transform infrared spectroscopy (FTIR) confirmed that the interaction of DNA with sepiolite was occurred through the external silanol groups of sepiolite [4]. Moreover, analyzes using TEM and atomic force microscopy (AFM) showed that sepiolite fibers assembled DNA biomolecules on their outer surface (Figure 3).

Additionally, the reversibility of the DNA adsorption process onto sepiolite was confirmed, i.e. the possibility of recovering DNA previously adsorbed on sepiolite. For this, the biohybrid was re-suspended in ethylenediaminetetraacetic acid (EDTA), which is a standard metal ion chelating agent. The displacement and sequestration of poly-cations favoring DNA binding to sepiolite, allowed the recovery of DNA previously adsorbed on the clay surface by cation bridges. The quality of the DNA plasmids recovered by EDTA re-suspension was estimated by electrophoresis: the distribution of different DNA isoforms (supercoiled, open circle, linear) was unchanged during the sepiolite adsorption-desorption processes. This demonstrated that interaction with sepiolite does not affect the biological quality of DNA and even more, that the method of incubation of DNA with sepiolite nanoparticles and a chelating agent can be established as a new methodology for the extraction and purification of DNA, much cheaper than some available commercial systems [4].

Transfer of nucleic acids into mammalian cells

Since it was determined that sepiolite is able to efficiently transfer small interfering RNA (FTIC labeled siRNA) into human cancer cells (A673 sarcoma cells) [4], its ability as biohybrid nanoplataform to transfer DNA into the nucleus of mammalian cells was evaluated by synthesizing a sepiolite/DNA (Sep/DNA) bionanohybrid. It was prepared by incubating the pCMV plasmid vector, which harbors a gene conferring resistance to G418 antibiotic cells, with sepiolite nanofibers. The efficiency of DNA transfer was then measured in cells through selection of G418-resistant cell colonies after the exposure of cells to Sep/DNA. Numerous resistant colonies were observed in V79 hamster cancer cells and U2OS human osteosarcoma cells after 10 days of incubation with the G418 antibiotic, demonstrating the ability of sepiolite to stably transfer an exogenous DNA into mammalian cells [4].

Subsequently, another strategy was tested to increase the efficiency of sepiolite-mediated DNA transfection. It consisted on disaggregating the

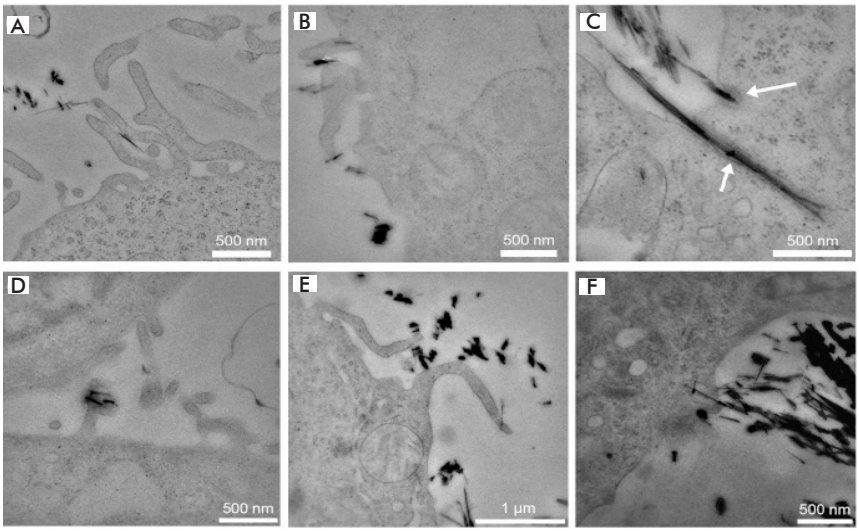

Figure 2. TEM images of V79 cells showing different cellular uptake mechanisms of sepiolite fibers after incubation for 6 h. A, B, D and E): Images showing the macropinocytosis mechanism for sepiolite uptake in V79 cells. C) Endocytosis with membrane invagination (up arrow) and direct nanofiber insertion (down arrow). F) Direct nanofiber insertion.

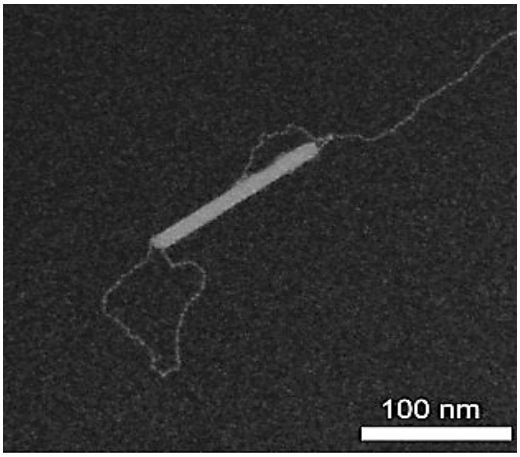

Figure 3. Image of sepiolite/DNA bionanohybrid using TEM, showing a sepiolite nanofiber with a plasmid DNA molecule adsorbed onto its surface.

sepiolite fibers more efficiently by ultrasonication of the sepiolite suspension (sonicated sepiolite; SSEP). Then, new biohybrids were synthesized with the above mentioned plasmid and SSEP, and mammalian cells were then incubated with the resulting SSEP/DNA complexes (Figure 4).

Surprisingly, ultrasonicated sepiolite complexes increased transfection efficiency in two orders of magnitude when applied to human cells in culture. In fact, the number of transfected colonies reached 350 per µg of DNA [4]. The efficiency of SSEP/DNA transfection was further measured by prior incubation of the cells with chloroquine or amiloride, to try to favor the endosomal escape of nanofibers. While amiloride does not produce a significant effect, prior incubation of the cells with chloroquine stimulated transfection efficiency by a factor of 3 in human cells. These data indicate that, in human cells and using SSEP/DNA biohybrids, chloroquine favors endosomal escape more effectively than the inhibition of the internalization of the bionanohybrid, thus increasing the transfection efficiency to reach 900 resistant colonies per microgram of DNA. Furthermore, a transfection efficiency similar and only slightly lower than that obtained by using conventional transfection methods as Jet PEI was achieved by combining the two protocols, chloroquine pretreatment plus SSEP/DNA [5]. This strategy is further complemented by the sepiolite advantage of having a relatively lower cost than existing transfection agents and being simultaneously non-toxic to cells. Overall, our results indicate that sepiolite could provide an efficient transfection nanohybrid system for the delivery of nucleic acids into cells.

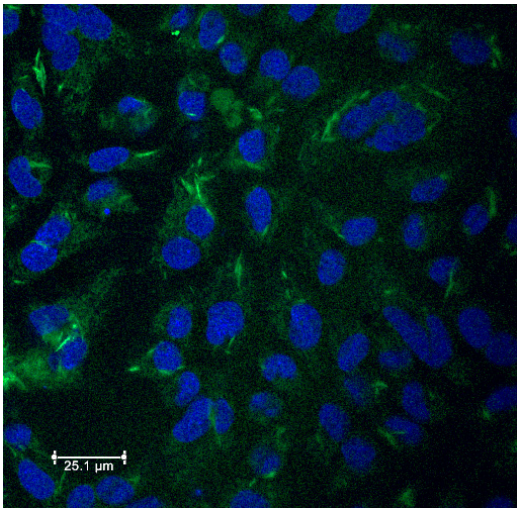

Figure 4. Laser confocal microscopy images of V79 cells previously incubated with sonicated sepiolite at 10 ng/µL.

Scientific relevance

Due to its relatively low cost, simplicity and feasibility of preparation, and convenience of the synthesis methods described in this work, sepiolite-based biohybrids offer a new and attractive platform for the transfer of nucleic acids in mammalian cells, and particularly humans, with potentially promising future developments with application in Nanomedicine and Nanobiotechnology. Higher co-transfection efficiencies could be also possible with sepiolite nanofibers than with typical small spherical nanoparticles due to the higher surface area of the nanofibers for non-viral gene transfer. This is also attractive for the co-transfection of DNA molecules together proteins and targeting or therapeutic, for instance, monoclonal antibodies, in order to achieved synergic effects. They can also be applied as delivery vectors for gene editing purposes, for cellular engineering to produce recombinant proteins of biomedical use, to generate new transgenic models in plants and animals, in gene and cell therapies, and to develop new treatments against diseases of genetic origin. Our results supported 3 international patent applications, one of them already granted (EP3009514A1).

This work also granted Castro Smirnov FA with a National Award to a Young Researcher from the Cuban Ministry of Science, Technology and the Environment (CITMA). It also contributed to a multidisciplinary Ph.D. thesis in Biological Sciences at the Université Paris XI and in the Institute of Cancerology Gustave-Roussy in Paris (discipline Molecular Biophysics, Life Sciences and Health), then homologated by the Cuban National Commission of Scientific Degrees in Physical Sciences in 2014. Six international articles were published in high prestige journals, the two most recent in Scientific Reports, from the Nature Publishing Group. Oral presentations and keynotes were delivered at 10 international scientific conferences, including the 251th National Meeting of the American Chemical Society in USA, Nanotech France 2016, the 4th International Conference on Multifunctional, Hybrid and Nanomaterial 2015 and the International Clay Conference 2017, and 20 technical lectures were imparted in universities and research centers in Cuba and abroad, including the Imperial College of London, Johns Hopkins University in Baltimore, New York University, Tel Aviv University, Federal University of Roraima, and the Istituto Italiano di Tecnologia, Politecnico di Torino, Italy.

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